Role of Nitric Oxide-responsive Repressor NsrR in Global Transcriptional Control

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ABSTRACT

Role of Nitric Oxide-responsive Repressor NsrR in Global Transcriptional Control

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Doctor of Philosophy

Division of Environmental and Biomolecular Systems within

the Institute of Environmental Health

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Our long-term research goal is to understand how *Bacillus subtilis* senses and responds to oxygen limitation. *B. subtilis* can survive anaerobically by opting for nitrate respiration or fermentation. The ResDE signal transduction system has a key role in the transcriptional activation of genes required to support nitrate respiration such as *nasDEF* (nitrite reductase genes) and *hmp* (flavohemoglobin gene). However, the full induction of *nasDEF* and *hmp* transcription by the ResD response regulator requires NO despite oxygen limitation. Previous study showed that NsrR is responsible for the upregulation of *nasD* and *hmp* when cells were treated with exogenous NO. An intriguing question remains as to how NsrR-dependent transcriptional regulation works in response to NO.

The overall aim of my research is to understand the mechanism by which NsrR repressor activity is modulated by NO and uncover the realm of NsrR regulation in *B. subtilis*. NsrR has a [4Fe-4S] cluster and exposure of the purified protein to NO results in iron dinitrosylation in the cluster. Electrophoretic mobility shift assays and *in vitro* transcription experiments using apo- and holo-NsrR demonstrated that the [4Fe-4S] cluster is essential for its NO-sensitive high-affinity interaction with the *nasD* promoter. NsrR represses transcription initiation at the *nasD* promoter by dissociating a preformed *nasD*-ResD-RNA polymerase complex. This study led to a new finding that two different NsrR-binding sites exist in the *nasD* promoter, namely class I and class II binding sites. Mutational and deletion analysis of the NsrR-binding regions showed that holo-NsrR recognizes a partial dyad symmetry in the class I site, whereas holo- and apo- equally bind to the A+T-rich class II site with a relaxed sequence specificity. Genome-wide transcriptome analysis revealed many candidate genes for the class II NsrR regulon, which include genes in the Fur and the AbrB/Rok regulons. *In vivo* transcription assays showed that the NsrR regulon is under complex control exerted by multiple regulators including AbrB, Rok, Fur, and ResD.

We addressed NO-sensitive direct interaction of NsrR with class I and II genes *in vivo* by using ChAP-qPCR (chromatin affinity precipitation followed by qPCR). Both ResD and NsrR do not interact with the promoter of *sdpA* (encoding sporulation-delaying factor) in the AbrB/Rok regulon, indicating that ResD and NsrR play indirect roles in *sdpA* transcription. In contrast, NsrR binds to *nasD* and *ykuN* (flavodoxin gene) in the

Fur regulon in a NO-sensitive manner. ResD also associates with the *nasD* and *ykuN* promoters and NsrR inhibits ResD binding to *nasD*, whereas either NsrR or Fur enhances ResD binding to *ykuN*. The study presented in this thesis laid the foundation for the further investigation of the detailed mechanism of the interplay among these regulators.

CHAPTER 1

INTRODUCTION

For prokaryotes, adaptation to constant changes in their environment is the key to their survival. Challenges like changes in the physical and chemical composition of their habitats, limited nutrient supply, oxygen limitation or depletion are some of the common changes they encounter. Moreover, in their natural habitat, bacteria have also evolved to compete with other microorganisms that produce toxic biomolecules (like antibiotics or free radicals) for the available resources. Survival under these conditions is achieved primarily by the combination of an economical use of the limited nutrient sources and intricate gene or cell regulation mechanisms.

Bacillus subtilis is a low G+C content, heterotrophic, spore-forming, gram-positive soil bacterium that has developed sophisticated mechanisms to sense, adapt, and survive the constant fluctuations in its habitat. One such survival mechanism that is being studied in our laboratory is oxygen limitation conditions which *B. subtilis* can encounter naturally through fluctuating water content in the soil. Under oxygen limiting conditions, facultative anaerobes like *B. subtilis* and *Escherichia coli* have evolved mechanisms to make use of terminal electron acceptors other than oxygen. For example, when oxygen becomes limited, *E. coli* opt for anaerobic respiration or mixed acid fermentation. *E. coli* utilizes several alternative electron acceptors that include nitrate, nitrite, fumarate, and DMSO (Unden *et al.* 1997). Alternatively, fermentation which is the less energy-generating pathway, is preferred only during the absence of preferred electron acceptors. Hence, the reactions and the components required for the shift in metabolism involve many sensory proteins, signals, signal transducers, together with

network of proteins that activate genes, which are necessary for selecting appropriate energy generating pathway for their survival in different anoxic conditions. *B. subtilis* senses oxygen limitation and chooses to switch to anaerobic respiration using nitrate as a terminal electron acceptor or by fermentation (Nakano *et al.* 1998).

1.1 ANAEROBIOSIS IN *BACILLUS SUBTILIS*

Genome-wide comparison studies of gene expression profiles in aerobically and anaerobically cultured *B. subtilis* revealed many differences in the expression patterns throughout the genome, some of which include changes in carbon metabolism, electron transport, iron uptake, antibiotic production and stress response (Ye *et al.* 2000). The overall goal of the research conducted in our laboratory is to elucidate the role and mechanism of transcriptional regulators in controlling differential gene expression upon shift to anaerobic conditions. To date, *B. subtilis* is known to use nitrate as an alternative terminal electron acceptor, and in the absence of nitrate, it can survive by mixed acid fermentation (Nakano *et al.* 1997; Cruz Ramos *et al.* 2000). In this section, I will briefly summarize the genes that are required for driving nitrate respiration and I will provide an outline of the network of regulation that is known.

1.1.1 Nitrate Respiration

Figure 1.1 shows genes coding for enzymes that function in nitrate respiration of *B. subtilis* and the regulatory pathways that control transcription of these genes. There are two nitrate reductase gene clusters in *B. subtilis*, each having different physiological functions (Glaser *et al.* 1995; Ogawa *et al.* 1995). The *nasBC* operon that codes for a

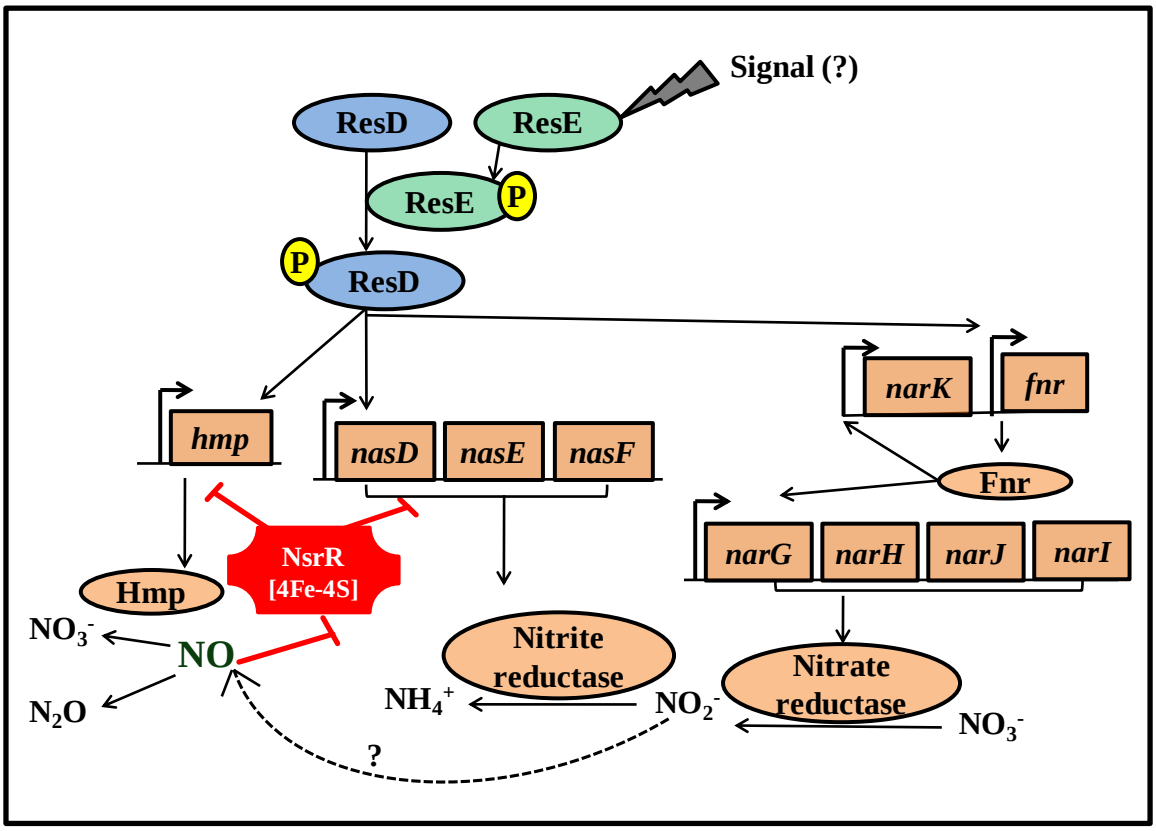

Fig. 1.1. The pathway of anaerobic nitrate respiration in *B. subtilis*. Positive regulation is labeled with an arrow, while repression is labeled with a ⊥. The regulation that is marked in red is the focus of this thesis.

cytoplasmic nitrate reductase is required for nitrate assimilation during aerobic growth (Ogawa *et al.* 1995). The *narGHJI* operon encodes a membrane-integrated nitrate reductase and functions in nitrate respiration during anaerobic growth (Glaser *et al.* 1995; Nakano *et al.* 1995). The transcription of *narGHJI* is strongly induced under anaerobiosis and the induction is dependent on the Fnr transcriptional regulator (Cruz Ramos *et al.* 1995).

Unlike nitrate reduction that is catalyzed through the aerobic assimilatory enzyme (NasBC) or anaerobic respiratory enzyme (NarGHJI), the NasDE nitrite reductase plays a dual role in assimilatory and respiratory function (Nakano *et al.* 1998). Amongst the genes of the *nasDEF* operon, *nasDE* codes for the nitrite reductase subunits and the *nasF* gene product is an enzyme required for the synthesis of siroheme cofactor formation (Nakano *et al.* 1998). The *nas* operon that constitutes *nasBC* and *nasDEF* is transcriptionally induced upon nitrogen limitation during aerobic growth and the induction is dependent on nitrogen regulator, TnrA (Nakano *et al.* 1998). However, under anaerobic conditions, only the transcription of *nasDEF* is highly induced. The transcriptional activation of *nasDEF* requires the ResDE two-component regulatory system (Hoffmann *et al.* 1998; Nakano *et al.* 1998). Later studies on the ResDE-dependent activation of the *nasDEF* operon identified an additional regulatory mechanism involving the nitric oxide (NO)-responsive transcriptional repressor NsrR (Nakano *et al.* 2006). Elucidating the role of NsrR in regulating ResDE-dependent *nasDEF* expression is one of the major aims of the thesis research described herein and will be discussed in detail in the next chapter.

Another *B. subtilis* gene that is highly induced during nitrate respiration is *hmp*. The *hmp* gene codes for a two-domain flavohemoglobin that is found in various bacteria and fungi. Biochemical studies showed that Hmp in *E. coli* has NO dioxygenase activity to convert NO to nitrate in the presence of oxygen (Gardner *et al.* 2000; Hausladen *et al.* 2001; Gardner *et al.* 2002; Hernandez-Urzua *et al.* 2003). Apart from the NO dioxygenation function, Hmp can also execute NO reduction under anaerobic conditions albeit at a slower rate (Kim *et al.* 1999). Therefore, Hmp has emerged as an important enzyme in NO detoxification (Gardner 2005). The role of Hmp in NO detoxification in various bacteria will be further discussed in later sections.

Expression of the genes that encode the above-mentioned enzymes during anaerobic growth is tightly controlled by two transcriptional regulators and a two-component signal transduction system [(Nakano *et al.* 2006); reviewed in (Nakano *et al.* 1998)]. First, for the transition from aerobic to anaerobic metabolism, the ResDE two-component signal transduction system is required. The ResDE regulatory system will be described in more detail in the next sections. Second, Fnr, a member of the Crp-Fnr regulatory protein family, is responsible for the anaerobic induction of the *narGHJI* and *narK* operons through interaction with a conserved DNA binding site at their promoter regions (Cruz Ramos *et al.* 1995; Reents *et al.* 2006). *fnr* transcription is induced from the Fnr-dependent *narK* operon promoter (Cruz Ramos *et al.* 1995) and the ResDE-dependent *fnr* promoter (Nakano *et al.* 1996). Third, the NO-sensitive NsrR repressor controls *nasD* and *hmp* transcription (Nakano *et al.* 2006). Detailed discussion of NsrR and its role in regulating ResDE-dependent gene expression is presented in later chapters (Chapter 2).

1.1.2 Fermentation

In the absence of an alternative electron acceptor (nitrate), *B. subtilis* can opt to survive through fermentation process. Fermentation in general is the process of anaerobic degradation of glucose or other organic nutrients to generate energy as ATP (Scott 1945). ATP is generated through substrate-level phosphorylation and NADH produced through glycolysis is reoxidized by internal electron acceptors generated during pyruvate metabolism (Scott 1945). Unlike *E. coli* that ferments glucose under anaerobic conditions, *B. subtilis* undergoes fermentation only in the presence of either glucose and pyruvate or glucose with a mixture of 20 amino acids (Nakano *et al.* 1997; Cruz Ramos *et al.* 2000). The exact reason for the dependence on pyruvate for the efficient glucose fermentation is not known (Nakano *et al.* 1997). The fermentative growth on glucose is poor and the growth can be recovered by the addition of pyruvate (Nakano *et al.* 1997). The transcriptome analysis collected in the inefficient fermentative growth on glucose showed a reduction in *pdhAB* (encoding pyruvate dehydrogenase E1 alpha and beta subunits) expression and a dramatic increase in *lctPE* (L-lactate permease and L-lactate dehydrogenase respectively) expression (Ye *et al.* 2000). The addition of pyruvate to the slow fermentative growth on glucose relieved the repression on *pdhAB* (Ye *et al.* 2000). It might be possible that pyruvate is required for the transcription of the *pdhAB* operon, which is needed to support efficient fermentative growth. Although the ResDE two-component signal transduction system and anaerobic gene regulator Fnr are essential for

nitrate respiration, mutations in these genes show moderate (in case of *resD* and *resDE)* or no effect (in case of *fnr*) on pyruvate-dependent fermentative growth (Nakano *et al.* 1997). These studies showed that different regulatory pathways direct nitrate respiration and fermentation in *B. subtilis*.

1.2 RESD-RESE TWO-COMPONENT SIGNAL TRANSDUCTION SYSTEM

1.2.1 Two-component signal transduction system in bacteria

Two-component signal transduction systems can trigger diverse responses to environmental changes by serving as a basic sensing system coupled to an immediate response mechanism resulting in the adaptation process. As two-component signal transduction systems are predominant signal pathways in bacteria, they have been considered as potential targets for development of new therapeutic drugs (Barrett *et al.* 1998). The simplest system consists of a histidine protein kinase (HK) and a response regulator protein (RR) [reviewed in (Stock *et al.* 2000)]. HK responds to a stimulus that is generated endogenously or exogenously by undergoing autophosphorylation at a conserved histidine residue using ATP as a substrate. Then HK transfers its high-energy phosphoryl group to a conserved aspartate residue of its cognate RR. Usually, only the phosphorylated RR can elicit the response, often by binding to the promoter DNA and regulating gene expression, thus resulting in adaptation to the new environment [reviewed in (Stock *et al.* 2000)]. Some of the well characterized systems are known to function in bacterial chemotaxis (Kirby 2009), aerobic/anaerobic transition (Iuchi *et al.* 1996), sporulation (Hoch 1993; Perego 1998), osmolarity changes (Forst *et al.* 1989), and cell differentiation (Jenal 2000).

1.2.2 Role of ResD in low GC gram-positive bacteria

resD and *resE* were first identified by *B. subtilis* genome sequencing project (Sorokin *et al.* 1993) and later studies showed their essential roles in aerobic respiration and anaerobic nitrate respiration in *B. subtilis* (Sun *et al.* 1996). ResE is a HK and ResD is a RR that belong to the OmpR-EnvZ subfamily of two-component regulatory proteins (Sun *et al.* 1996). *resD* and *resE* constitute an operon with the upstream *resABC* genes, which code for proteins involved in cytochrome *c* biogenesis (Sun *et al.* 1996). ResA is a thiol-disulfide oxidoreductase and functions in redox state-dependent cytochrome *c* maturation (Erlendsson *et al.* 2003; Crow *et al.* 2005; Colbert *et al.* 2006). ResB and ResC are essential for cytochrome *c* synthesis (Le Brun *et al.* 2000). *resD* and *resE* transcription is controlled from an upstream *resA* promoter and an intergenic *resD* promoter (Sun *et al.* 1996). During anaerobic growth or at the end of aerobic exponential growth, the transcription of *resDE* is activated by phosphorylated ResD (ResD~P) from the *resA* promoter, while the transcription from the promoter upstream of *resD* is ResD-independent (Sun *et al.* 1996).

ResDE orthologs are present in low GC gram-positive bacteria such as *Bacilli*, *Listeria* and *Staphylococci* and were found to be essential for anaerobiosis and/or virulence gene expression. In contrast to *Bacilli*, *Listeria* and *Staphylococci* have a dicistronic *resDE* operon that lacks the upstream *resABC* genes (Larsen *et al.* 2006; Pragman *et al.* 2007). In *L. monocytogenes, resE* is transcribed from the *resE* promoter in addition to the *resD* promoter. In *B. cereus* the ResDE system plays an important role in both fermentative growth and enterotoxin production (Duport *et al.* 2006; Esbelin *et al.* 2009). Initial studies showed that ResDE controls the transcription of the anthrax toxin

gene, *pagA*, and that of the master regulator for toxin production, *atxA* in *B. anthracis* (Vetter *et al.* 2007). However, later studies provided a contradictory report on the role of *resDE* as having no affect on the virulence gene expression (Wilson *et al.* 2008). The latter study suggested that the contradictory result was likely due to the possible compensatory mutations generated by the *resD* mutation (Wilson *et al.* 2008). ResD plays a role in virulence gene expression in response to carbohydrates and controls sugar uptake in *L. monocytogenes* (Larsen *et al.* 2006). SrrAB, the ResDE orthologs of *S. aureus*, also regulates virulence factor expression (Yarwood *et al.* 2001; Pragman *et al.* 2004). SrrAB is a major activator for *icaADBC* operon that codes for polysaccharide intercellular adhesin (PIA) under anaerobic conditions, thus contributing towards protection against non-oxidative defense mechanisms by neutrophils (Ulrich *et al.* 2007). In addition, the SrrAB two-component system was found to regulate the expression of NO-induced genes confirming its role in NO resistance mechanism in *S. aureus* (Richardson *et al.* 2006).

1.2.3 The ResDE two-component signal transduction system is required for aerobic and anaerobic respiration in *B. subtilis*

ResD exists as a monomer regardless of its phosphorylation state (Zhang *et al.* 2000). The signal ligand that the ResE senses remains unknown (Nakano *et al.* 1996; Sun *et al.* 1996; Nakano *et al.* 1997). ResD is required for the transcriptional activation of *ctaABCDEFG* (required for heme A synthesis and cytochrome caa_3 oxidase), the *petCBD* operon (encoding subunits of the cytochrome *bf* complex), *cydABCD* (coding for subunits of cytochrome *bd* oxidase and associated ABC transporter) (Puri-Tanejaet al.,

2007), and *resABC* (Sun *et al.* 1996) under both aerobic and anaerobic conditions. Therefore, *resD* mutants (and to a lesser extent, *resE* mutants) exhibit pleiotropic phenotypes relating to respiration such as streptomycin resistance and dependency on 6-carbon sugars for growth (Sun *et al.* 1996). Due to its pleiotropic phenotype, the *resDE* mutant has the propensity to generate suppressor mutations that can bypass the phenotypic changes. For example, suppressor mutants were identified to be the loss of function alleles of *ydiH* (Schau *et al.* 2004). YdiH (renamed as Rex) is a negative regulator of *cydABCD* (Schau *et al.* 2004; Puri-Taneja *et al.* 2007), as the *rex* mutation causes the derepression of *cydABCD* (Schau *et al.* 2004; Puri-Taneja *et al.* 2007). The resultant increase in the cytochrome *bd* terminal oxidase compensates for the loss of cytochrome aa_3 in the *resDE* mutant (Schau *et al.* 2004). As described above, these defects are not confined to aerobic growth. *resDE* mutants are unable to grow under nitrate respiration conditions, as they are required for the expression of *fnr* and the *nasDEF* operon. Mutations in *resD, resE* and *resDE* completely abolished the transcription of *fnr,* indicating that ResD phosphorylated through ResE is required for initiating the transcriptional activation of *fnr* upon transition from an aerobic to anaerobic environment (Nakano *et al.* 2000). This initial induction of *fnr* transcription from the *fnr*-specific promoter is needed to further elevate the transcription of *fnr* through the upstream *narK* operon promoter as well as the induction of *narGHIJ* that is indispensable for nitrate respiration (Geng *et al.* 2007).

The ResDE system is also required for *phoPR* induction upon phosphate starvation (Hulett 1996; Sun *et al.* 1996), though the detailed mechanism is unknown. PhoPR system is a two-component signal transduction system that functions in inorganic

phosphate metabolism (Hulett 1996). Moreover under phosphate-limited conditions, ResD protein level is in turn controlled by the PhoPR system by its dual role in *resD* transcription (Birkey *et al.* 1998). Phosphorylated PhoP directly interacts with the *resA* promoter and activates transcription of *resABCDE*, while repressing *resDE* transcription from the *resD*-specific promoter (Birkey *et al.* 1998). Hence, the factors constitute a positive feedback loop that is co-dependent on *phoPR* and *resDE* induction (Birkey *et al.* 1998). The Pho response is dependent on the expression of one of the terminal oxidases (which are in turn dependent on ResD) (Schau *et al.* 2004). Terminal oxidases are required for maintaining the redox status of the quinone pool, and the reduced quinones resulting from *resDE* mutation inhibit PhoR autophosphorylation (Schau *et al.* 2004). Thus expressing at least one terminal oxidase in the *resDE* mutant was shown to bypass the requirement of ResD for Pho induction (Schau *et al.* 2004).

1.2.4 Characterization of ResDE-dependent transcriptional activation

In vitro binding experiments and *in vitro* transcription experiments showed that ResD directly binds to the promoter regions to activate transcription of *ctaA* (Zhang *et al.* 2000), *nasD* (Nakano *et al.* 2000; Geng *et al.* 2004), *hmp* (Nakano *et al.* 2000; Geng *et al.* 2004), *fnr* (Nakano *et al.* 2000; Geng *et al.* 2004), and *yclJK* (Hartig *et al.* 2004). Hydroxyl radical footprinting analysis revealed a phosphorylation enhanced tandem binding of ResD to the *nasD* and *hmp* promoters (Geng *et al.* 2004). Mutational analysis of ResD binding regions led to the identification of a consensus binding sequence as $TTGTGAAN_3TTTN_4A$ in ResD-controlled promoters (Geng *et al.* 2007). Consensus sequence alignment in various ResD-interacting promoters, led to the speculation that

ResD might bind to the promoter DNA in different orientations (Geng *et al.* 2007). Phosphorylation of ResD by ResE also enhanced the transcription of target genes (Zhang *et al.* 2000; Geng *et al.* 2004). However, several questions remain to be answered such as (1) what kind of signal does ResE perceive to undergo autophosphorylation? (2) what is the exact mechanism of regulation that differentiates ResD-dependent transcriptional activation between aerobically and anaerobically expressed genes? Studies have proposed that the ResE kinase possesses a dual function as a kinase and as a phosphatase towards the response regulator ResD based on the availability of oxygen, hence controlling the level of phosphorylated ResD (Nakano *et al.* 1999; Nakano *et al.* 2001). It was speculated that an increase in phosphorylated ResD in the cell could be a reason for the dramatic increase in the transcription of ResDE-controlled genes under anaerobic conditions (Nakano *et al.* 1999). A mutant ResE, which retains autophosphorylation and ResD phosphorylation activities but is defective in phosphatase activity, allowed partial aerobic derepression of the ResDE-controlled *nasD, fnr, sbo* and *hmp* transcription (Nakano *et al.* 2001). These studies suggested that anaerobic induction of the ResDE regulon is at least partly due to a reduction of the ResE phosphatase activity.

Later studies showed that NO is required for the full induction of ResD-dependent gene expression (Nakano 2002) and that inactivation of NsrR repression via NO production (Nakano *et al.* 2006) during nitrate respiration leads to the up-regulation of ResDE-dependent activation of *nasD* and *hmp*. This NO-sensitive repressor activity of NsrR partly answered the question about higher levels of anaerobic expression of ResDE-controlled *nasD* and *hmp* expression. One of the aims of this thesis research is to

understand how the inactivation of NsrR repression enhances expression of the *nasD* operon during nitrate respiration.

1.3 NITRIC OXIDE AND ITS RESPONSIVE REGULATORS IN BACTERIA

1.3.1 The source of NO and its toxic effects on bacteria

NO is a very reactive and toxic free-radical gas and is known to perturb the activity of many regulatory proteins (Figure 1.2) (Culotta *et al.* 1992; Stamler *et al.* 1992). Besides its toxicity at high concentrations, its ubiquitous presence in low concentrations plays an essential role in biogeochemical nitrogen cycling in water and soil ecosystems (Ducluzeau *et al.* 2009). It functions as an important constituent of the atmospheric chemistry and plays an important role in regulating many physiological functions in living systems ranging from microorganisms (Bogdan 2001; Gusarov *et al.* 2005) to multicellular organisms including humans [reviewed in (Gross *et al.* 1995)]. NO is well studied in higher eukaryotes as a signaling molecule and for its role in mammalian innate immunity (Gross *et al.* 1995).

Bacteria encounter NO as an effector molecule of the innate immune system in the mammalian host, through the interferon γ stimulated macrophages (Hibbs *et al.* 1988). As one of the first line of defense, stimulated macrophages use their inducible NO-synthases (iNOS) to generate NO. Inside the phagocytosed cell, NO can damage the bacteria cell by protein modifications such as 3-nitrotyrosine adduct formation (Kooy *et al.* 1997), nitrosylation of iron-sulfur centers of the enzymes like aconitase (Drapier 1997), inactivation of ribonucleotide reductase (Lepoivre *et al.* 1991). NO also has the potential to cause cytotoxic DNA damage (Kwon *et al.* 1991). Interaction of NO with

reactive oxygen species produced by phagocytes results in cytotoxic effects due to the formation of peroxynitrite ($ONNO^-$). Thus, NO can be further oxidized into potent reactive nitrogen species (RNS). During nitrosative stress high concentrations of NO can lead to peroxidation of lipids (O'Donnell *et al.* 2001) and deamination of DNA (Dong *et al.* 2003; Glaser *et al.* 2005; Dong *et al.* 2006). NO can also indirectly damage DNA and was shown to be a potent mutagen in *E. coli* during nitrate metabolism (Weiss 2006). It is speculated that, during nitrosative stress conditions, an unidentified endogenous DNA-methylating species, most likely a nitrosamine, is produced, leading to DNA damage (Taverna *et al.* 1996). The toxic chemistry resulting from the NO attack is listed in Figure 1.2.

The effector molecule NO is also produced by NO synthases (NOSs) in bacteria. Some gram-positive bacteria such as *Actinobacter*, *Deinococcus*, and *Firmicutes*, carry NOS that catalyzes the conversion of L-arginine to L-citrulline and NO in the presence of NADPH and oxygen [reviewed in (Sudhamsu *et al.* 2009; Crane *et al.* 2010). The major difference between bacterial and mammalian NOS is that the former enzyme lacks the reductase domain (Adak *et al.* 2002; Pant *et al.* 2002). The *B. subtilis* flavodoxin, YkuN, was shown to donate electrons to NOS (Wang *et al.* 2007) and, when *ykuN* is deleted, other cellular reductases support NOS-dependent production of NO (Gusarov *et al.* 2008). Another major difference is that some bacteria lack the biosynthesis pathway for tetrahydrobiopterin, the mammalian NOS cofactor and utilize tetrahydrofolate instead (Reece *et al.* 2009). Given its anti-microbial properties in eukaryotes, it was surprising when researchers discovered NOS in bacteria (Sudhamsu *et al.* 2009). In *B. subtilis* NO was shown to reduce the Fenton reaction by inhibiting the

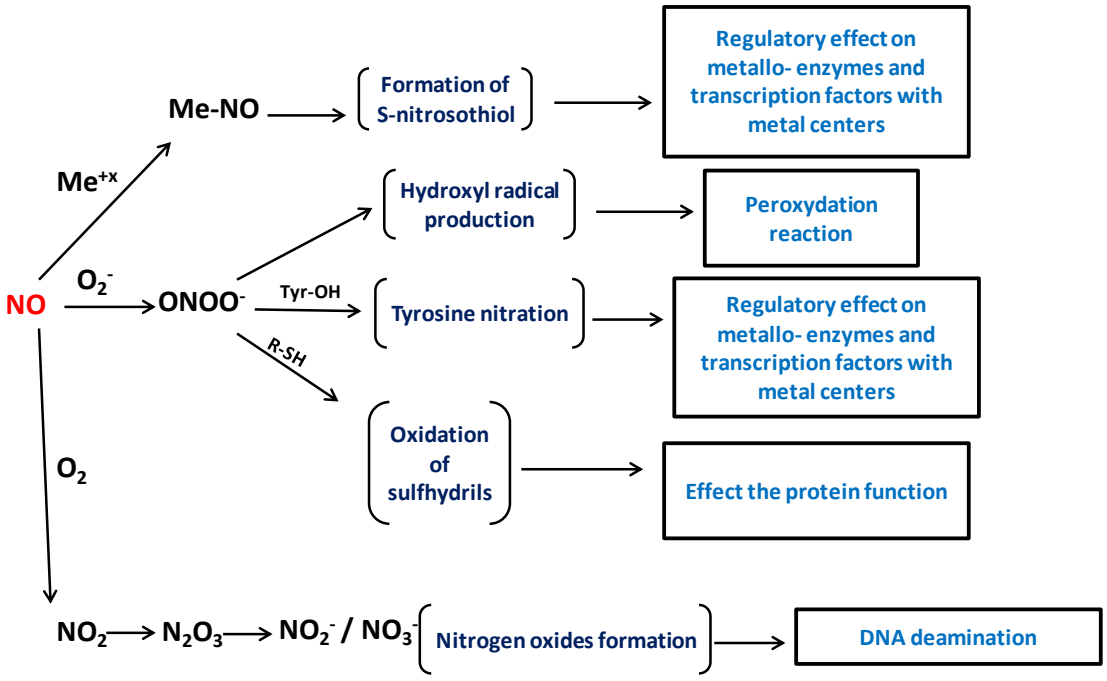

Fig. 1.2. Chemistry of nitric oxide (NO) radicals in a living cell. In the presence of oxygen, NO is oxidized to NO_2. Further reaction with NO gives rise to N_2O_3 . N_2O_3 results in deamination of DNA and chromosome fragmentation. In the presence of superoxide (O_2^-), NO is converted to peroxynitrite ($ONOO^-$). $ONOO^-$ causes tyrosine nitration, oxidation of thiol residues to sulfenic and sulfonic acids, and forms NO_2 and hydroxyl radical. NO_2 gives rise to nitrite (NO_2^-) and nitrate (NO_3^-) depending on the oxygen concentration. NO can nitrosylate thiol groups of cysteines in proteins, which results in the formation of intermolecular disulfide bonds (-S-S-), mixed disulfide bonds (-S-S-R), cysteine sulfenic acids (R-S-OH), sulfinic acids ($R\text{-}SO_2H$) or sulfonic acids ($R\text{-}SO_3H$). NO can also interact with the transition metals (Me) such as Fe, Mn or Cu and results in metal-nitrosyl complexes, thus affecting their activities and/or regulatory properties [reviewed in (Bogdan 2001)].

cysteine reduction during oxidative damage (Gusarov *et al.* 2005). During oxidative stress, *B. subtilis* can utilize NOS-generated NO for the activation of catalases (KatA) that detoxify H_2O_2 (Gusarov *et al.* 2005). NO activates KatA by reacting with the iron-heme center via S-nitrosylation mechanism (Gusarov *et al.* 2005). Therefore, the role of NOS in some pathogenic bacteria could be to protect against oxidative stress, increase antibiotic resistance, and promote virulence (Shatalin *et al.* 2008; Gusarov *et al.* 2009). The possible reason behind the NO mediated resistance and beneficial effects against antibiotics is believed to be through NO reaction with the toxic compounds, thereby alleviating their effects (Gusarov *et al.* 2005; Shatalin *et al.* 2008; Gusarov *et al.* 2009).

NO is also an obligatory intermediate in denitrification (Hochstein *et al.* 1988). Denitrification, which is the reduction of nitrate and nitrite to NO, nitrous oxide (N_2O) and di-nitrogen (N_2), is a conserved respiratory pathway utilized by denitrifying bacteria [reviewed in (Richardson *et al.* 2001)]. Though *B. subtilis* is not a denitrifier, it can still encounter NO in natural environments where denitrifiers cohabit. In addition, NO is endogenously produced as a byproduct of nitrate respiration (Ji *et al.* 1988).

NO produced exogenously or endogenously targets iron-sulfur clusters or mononuclear iron centers of many cellular proteins, leading to the formation of dinitrosyl iron complexes that could hamper the function of metalloproteins (Stamler *et al.* 1992). NO also affects metabolic pathways by targeting enzyme functions. *Salmonella. typhmurium* exhibits methionine (M) and lysine (K) auxotrophy during nitrosative stress (Richardson *et al.* 2011). M and K synthesis is dependent on the availability of succinyl CoA, a TCA cycle intermediate (Richardson *et al.* 2011). The reduced succinyl CoA production by α-ketoglutarate dehydrogenase under NO stress is caused due to the

inhibition of lipoamide-dependent lipoamide dehydrogenase (LpdA) activity, an essential component of the enzyme (Richardson *et al.* 2011).

1.3.2 Bacterial defense against nitrosative stress

In order to protect themselves from the harmful effects of NO and its derivatives, many bacteria have evolved sophisticated regulatory mechanisms to counteract cytotoxic effects of NO by either upregulating genes involved in NO detoxification (Poole 2005; Stephen 2007), maintaining metal homeostasis (Fleischhacker *et al.* 2011), activating genes that encode less NO-sensitive counterparts (Dunn *et al.* 2010), or activating those that function in repairing NO damage (Overton *et al.* 2008; Forrester *et al.* 2012). Depletion of thiol pools and inactivation of metal centers during nitrosative stress stimulates cysteine and homocysteine biosynthesis operons in *E. coli* and *Salmonella,* glutathione biosynthesis in *Candida albicans,*and iron repair systems and iron regulation genes (De Groote *et al.* 1996; Mukhopadhyay *et al.* 2004; Hromatka *et al.* 2005).

NO detoxification is catalyzed by flavohemoglobin and other globin-like enzymes in bacteria. There are three classes of bacterial globin that are identified so far, namely, myoglobin-like globin, truncated globin, and flavohemoglobin. Flavohemoglobin or Hmp, is one of the most extensively studied bacterial enzyme for its chemistry with NO (Poole *et al.* 2000; Angelo *et al.* 2008). Hmp was shown to provide protection to the living cell against nitrosative stress by directly consuming NO (Poole *et al.* 2000). These proteins are comprised of a globin domain and a FAD-binding domain. Aerobically, Hmp is able to convert NO to nitrate through NO dioxygenase activity (Hernandez-Urzua *et al.* 2003). Hmp is also able to reduce NO to N_2O under oxygen limited conditions at a

lower rate (Kim *et al.* 1999). *hmp* mutants of pathogenic bacteria show poor survival in the host macrophages (Stevanin *et al.* 2002; Stevanin *et al.* 2007). On the other hand, constitutive expression of *hmp* in *E. coli* and *Salmonella* in the absence of NO results in increased susceptibility to oxidative stress as oxygen reduction by the heme group in Hmp generates superoxide anion (Poole *et al.* 2000; Poole 2005; Gilberthorpe *et al.* 2007). Therefore, *hmp* transcription is tightly regulated at the transcriptional level by NO-sensing NsrR repressor, along with additional regulators such as Fnr and MetR (in *E. coli, Salmonella enterica*) (Membrillo-Hernandez *et al.* 1998; Cruz-Ramos *et al.* 2002), the transcriptional activator NorR (in *Pseudomonas aeruginosa*) (Mukhopadhyay *et al.* 2004), or the ResDE system (in *B. subtilis)* (Nakano 2002).

The second class of NO-detoxifying globin is the myoglobin-like protein consisting of a single-globin domain. VHb of *Vitreoscilla*is, an obligate aerobe, was the first bacterial globin to be crystallized (Frey *et al.* 2003; Frey *et al.* 2005). VHb displays a classic globin fold with unusual structures in both proximal and distal heme pockets, thus conferring a unique structural organization (Tarricone *et al.* 1997). VHb has been shown to possess NO dioxygenase activity (Joshi *et al.* 1998), serve as an alternate terminal oxidase (Dikshit *et al.* 1992), modulate the redox status of cells (Tsai *et al.* 1995), be involved in NO detoxification (Kaur *et al.* 2002), and provides protection from oxidative stress (Geckil *et al.* 2003). *Campylobacter jejuni*, a microaerophillic foodborne pathogen that can be exposed to nitrosative stress during host infection, is protected by a single domain globin Cgb (Monk *et al.* 2008; Pickford *et al.* 2008; Smith *et al.* 2011). Cgb is under transcriptional control of NssR, a member of the Crp-Fnr subfamily (Elvers *et al.* 2005; Monk *et al.* 2008).

The third group of globin in bacteria that confers protection against nitrosative stress is the truncated hemoglobins (trHbs). trHbs are small oxygen-binding hemeproteins, distantly related to hemoglobins and myoglobins. These globins are also widely distributed among bacteria and plants (Ascenzi *et al.* 2007). One such example is Ctb of *C. jejuni* (Monk *et al.* 2008; Pickford *et al.* 2008; Smith *et al.* 2011) that is under transcriptional control of NssR (Elvers *et al.* 2005; Monk *et al.* 2008). *Mycobacterium tuberculosis, Mycobacterium smegmatis* and *Mycobacterium bovis* trHbs scavenge NO produced by macrophages through NO dioxygenase reactions (Ouellet *et al.* 2002; Lama *et al.* 2006). *B. subtilis* possess a truncated hemoglobin (YjbI), a gene in the dicistronic operon *yjbIH* (Rogstam *et al.* 2007). YjbH is an adaptor for ClpXP-mediated proteolysis of an oxidative stress responsive protein Spx (Larsson *et al.* 2007; Engman *et al.* 2012). *yjbIH* and *yjbH* mutations make *B. subtilis* hypersensitive to sodium nitroprusside (SNP) (Rogstam *et al.* 2007) and the mutant phenotype is bypassed by the mutations in the *spx* gene (Larsson *et al.* 2007).

Mutational and transcriptomic studies also revealed many additional proteins that might have a role in protection against NO toxicity. In *E. coli*, transcriptomic studies conducted under anaerobic nitrosative stress conditions revealed that the *norVW* operon, *ytfE*, and a LysR-type regulator encoded by *yidZ* are upregulated along with *hmp* (Justino *et al.* 2005). Knockout mutations of these genes in *E. coli* lead to hypersensitivity to NO (Justino *et al.* 2005; Justino *et al.* 2006). YtfE, which is a dimeric protein with two Fe atoms per monomer, was speculated to play a role in repairing NO- or H_2O_2-damaged [Fe-S] clusters in the cell (Justino *et al.* 2006; Overton *et al.* 2008). *hcp* and *hcr* that encode an iron-sulfur-cluster-associated protein and its cognate reductase respectively,

are associated with NO tolerance mechanism in *E. coli* and *Rhodobacter capsulatus* (Wolfe *et al.* 2002; Cabello *et al.* 2004). Flavorubredoxins and associated flavoproteins encoded by the *norVW* operon were shown to possess NO reductase activity under anaerobic and microaerobic condition in *E. coli* (Gardner *et al.* 2002; Gardner *et al.* 2002; Vicente *et al.* 2008). Expression of *norVW* is regulated at the transcriptional level by NorR, a NO sensor, and its role in transcriptional regulation will be discussed in the next section.

Recent studies conducted on *Salmonella* demonstrated that the base excision repair system (BER) is involved in repairing damaged DNA caused by RNS generated in host phagocytes (Richardson *et al.* 2009). As mentioned before RNS leads to the deamination of the DNA. In order to maintain the genomic integrity, *Salmonella* uses BER components such as uracil DNA glycosylase (Ung), 3-methyladenine DNA glycosylase (AlkA), formamidopyrimidine DNA glycosylase (Fpg), exonucleases (Xth) and endonucleases (Nei, Nfo and Nth) to repair damaged or modified DNA (Richardson *et al.* 2009). Glycosylase mutants lacking Ung and Fpg displayed enhanced NO-dependent hypermutability, whereas *xth* and *nfo* mutants lacking AP (Apurinic/apyrimidinic) endonuclease activity were susceptible to increased chromosomal fragmentation during NO stress (Richardson *et al.* 2009).

In *S. aureus*, the adaptive response upon encountering nitrosative stress is provided by L-lactate dehydrogenase (encoded by *ldh1)* (Richardson *et al.* 2008). NO was shown to inhibit respiration and acetyl CoA generation by pyruvate dehydrogenase and pyruvate formate lyase through an unknown mechanism (Richardson *et al.* 2008). In order to circumvent the metabolic restrictions by NO toxicity, *S. aureus* enhances NO-

insensitive *ldh1* expression during nitrosative stress (Richardson *et al.* 2008). *ldh* mutants (*ldh1* and *ldh2)* showed inability to maintain redox homeostasis under aerobic and anaerobic conditions during NO stress (Richardson *et al.* 2008). Another mechanism of tolerance was uncovered in *Vibrio fischeri*, which produces a NO-inducible and NO-resistant alternative oxidase (Aox) (Dunn *et al.* 2010). NO can inhibit conventional respiratory oxidase function (Mason *et al.* 2009). Therefore, Aox functions in maintaining the respiration in *V. fischeri* in the presence of NO (Dunn *et al.* 2010). Transcriptome studies revealed that NO-dependent induction of *aox* gene is repressed by NO-sensitive NsrR repressor (Dunn *et al.* 2010).

1.3.3 NO stimulates ResD-dependent *nasD* and *hmp* transcription

An assumption that NO might induce expression of the ResDE regulon came from the key finding that oxygen limitation alone is not enough to fully activate transcription, as induction is dependent on nitrate or nitrite. Although *B. subtilis* grows anaerobically by generating ATP via fermentation (Nakano *et al.* 1997; Nakano *et al.* 1997), induction of ResDE-dependent genes such as *nasD* and *hmp* is much lower under these conditions compared to those of cells grown by nitrate respiration (LaCelle *et al.* 1996; Nakano *et al.* 1996; Nakano *et al.* 2000). Later studies revealed that increase in transcription during nitrate respiration is likely due to NO, which is produced during nitrate respiration (Nakano 2002). More direct evidence of the stimulatory effect of NO was obtained through introducing an NO scavenger carboxy-PTIO *in vivo* that alleviates the positive effect of NO or nitrite on *nasD* transcription (Nakano *et al.* 2006). These studies led to

the quest for a NO-sensitive transcription factor and to the discovery of NsrR, a repressor that participates in ResDE-dependent gene regulation (Nakano *et al.* 2006).

Transcriptome analysis of *B. subtilis* in response to NO revealed that under aerobic conditions the most strongly induced genes were *hmp* and members of the Fur and σ^B regulons (Moore *et al.* 2004). Whereas under anaerobic conditions the NO-dependent induction was higher with *hmp*, and members of the PerR and Fur regulons but no induction of the σ^B regulon was detected (Moore *et al.* 2004). Effect of NO on the NsrR, ResDE and the Fur regulons will be discussed in the later chapters (Chapter 3 & 4).

1.4 NITRIC OXIDE SENSORS IN BACTERIA

Molecular consequences of NO toxicity involve the inhibition of various metabolic activities as described above. In order to escape from the toxic insult, bacteria have evolved several sensory proteins that can detect NO. Based on recent reviews of NO sensors (Stephen 2007; Spiro 2008; Tucker *et al.* 2010), the regulatory proteins that sense NO can be categorized into primary and secondary sensors. Regulators for genes involved in NO detoxification are highly sensitive to NO and are classified as primary sensors. For example, primary sensors include NorR in *E. coli* (D'Autreaux *et al.* 2005), NnrR in *Rhodobacter sphaeroides* (Laratta *et al.* 2003), Dnr in *P. aeruginosa* (Giardina *et al.* 2008) and NsrR in various bacteria [reviewed in (Tucker *et al.* 2010)]. Secondary sensors are regulatory proteins that are not directly involved in NO stress response, but are targeted by NO toxicity. Some examples of secondary sensors are Fnr (Cruz-Ramos *et al.* 2002), IscR (Giel *et al.* 2006), Fur (Moore *et al.* 2004) and SoxR (Demple 1999) whose primary roles are oxygen sensing, iron sulfur cluster biogenesis, and redox stress

sensing respectively. As these secondary sensors have Fe-S clusters, they are susceptible to NO targeting (Table 1.1). This thesis focuses on the NO-sensing NsrR transcriptional repressor that has an iron sulfur cluster as a cofactor. I will discuss more examples of NO sensing regulators and their regulons (Table 1.1).

Table 1.1. Examples of some of bacterial [Fe-S]-cluster containing regulatory proteins

Regulator	Cluster type	Primary function	Reference
Fnr	[4Fe-4S]	Oxygen sensor in a wide range of bacteria	1
NreB	[4Fe-4S]	Oxygen sensor in Staphylococci	2
SoxR	[2Fe-2S]	Redox/ O_2^- stress sensor	3
NsrR	[2Fe-2S]/[4Fe-4S]	NO stress response	4
WhiB	[4Fe-4S]	Developmental processes in Actinomycetes	5
IscR	[2Fe-2S]	Sensor of cellular [Fe-S]-cluster levels	6
Aconitase	[4Fe-4S]	Aconitase and RNA binding activity	7
RirA	[2Fe-2S]	Iron response regulator in alpha-proteobacteria	8
RsmA	[2Fe-2S]	SigM anti-sigma factor	9

1. (Reents *et al.* 2006), 2. (Reinhart *et al.* 2010), 3. (Hidalgo *et al.* 1995), 4.(Tucker *et al.* 2008; Yukl *et al.* 2008; Isabella *et al.* 2009), 5. (den Hengst *et al.* 2008), 6. (Fleischhacker *et al.* 2012), 7. (Tang *et al.* 2005), 8. (Johnston *et al.* 2007), 9. (Gaskell *et al.* 2007)

1.4.1 SoxRS

The transcription factor SoxR in *E. coli* is activated upon oxidation of the redox active $[2Fe\text{-}2S]^+$ cluster to $[2Fe\text{-}2S]^{2+}$ cluster by O_2^- (Pomposiello *et al.* 2001). $[2Fe\text{-}2S]^{2+}$ SoxR in response to redox stress activates *soxS* (Demple 2002; Demple *et al.* 2002). SoxS is a transcriptional regulator that is necessary to induce ~45 genes associated with removal and repair of oxidative damage (Vasil'eva *et al.* 2001; Kiley *et al.* 2003). Studies have shown that SoxR can also be activated by NO causing the [2Fe-2S] centers to form protein-bound dinitrosyl-iron-complexes (Demple 2002; Demple *et al.* 2002). The NO effect on SoxR regulation differs from the other metal-centered regulators in

several aspects. Firstly, reaction with NO has a positive effect on the transcriptional activity of SoxR. Secondly, reaction with NO does not block the DNA binding property nor its affinity to DNA (Vasil'eva *et al.* 2001; Demple *et al.* 2002). It is believed that NO or O_2^- reaction with the [Fe-S] cluster stimulates the conformational changes in SoxR, leading to the favorable interactions with the transcription machinery (Vasil'eva *et al.* 2001).

1.4.2 Fnr-CRP family of transcriptional regulators

Fnr is the best-studied example of the Fnr-Crp (cAMP receptor protein) family of proteins that are widely distributed in bacteria. This family includes transcriptional regulators that react with a wide range of gaseous ligands (like oxygen in case of Fnr and carbon monoxide for CooA (CO-oxidizing system activator), NO for NnrR (nitrite reductase and nitric oxide reductase regulator). For example, NnrR is the first protein that was designated as a dedicated NO sensor in denitrifying strain of *R. sphaeroides* (Tosques *et al.* 1996). NnrR controls transcription of the NO reductase gene (*nor* operon) and the nitrite reductase gene (*nir*) that forms NO (Tosques *et al.* 1996). Although *in vivo* studies indicated that NO is the key molecule for activating NnrR-dependent gene expression, there is no direct evidence showing NO interaction with NnrR protein (Kwiatkowski *et al.* 1996; Kwiatkowski *et al.* 1997). Dnr (dissimilatory nitrate respiration regulator) in *P. aeruginosa*, which is a homologue of NnrR, has been studied *in vitro*. The function of Dnr which promotes the transcriptional activation of *nir, nor,* and *nos* (nitrous oxide reductase gene) in response to NO is dependent on its heme cofactor (Schreiber *et al.* 2007; Castiglione *et al.* 2009; Giardina *et al.* 2011). Another

Fnr/CRP family member that mediates a response to NO is NssR (nitrosative stress sensing regulator) of *C. jejuni* (Elvers *et al.* 2005). As described in the previous section, NssR activates the transcription of genes encoding two globin-like proteins, Cgb and Ctb. It is speculated that NssR signal-sensing mechanism might be distinct from that of other members of the Fnr-Crp family, as the NssR clusters separately from Nnr and Dnr proteins in the sequence-based phylogeny studies (Elvers *et al.* 2005). It still remains a mystery as to how NO modulates the activity of NssR, since *in vitro* studies were unable to show any effect of NO on DNA binding activity of NssR to the *cgB* promoter (Smith *et al.* 2011).

The [4Fe–4S]-cluster-bearing Fnr (regulator of fumarate and nitrate reduction) protein of *E. coli* controls transcription during anaerobic growth and it is inactivated by reaction of the [Fe-S]-cluster with oxygen under aerobic conditions (Green *et al.* 1996; Beinert *et al.* 1999). Under anaerobic conditions, purified Fnr protein is a homodimer with one oxygen- sensitive $[4Fe\text{-}4S]^{2+}$ cluster per subunit (Bates *et al.* 2000). The exposure of $[4Fe\text{-}4S]^{2+}$-Fnr to oxygen gradually converts the cluster to the $[2Fe\text{-}2S]^{2+}$ form, which leads to a loss in DNA binding activity, and finally dissociation into monomers (Khoroshilova *et al.* 1997). The monomeric apo-Fnr is proteolyzed by the ClpXP protease both *in vivo* and *in vitro* (Mettert *et al.* 2005). *E. coli* Fnr transcriptionally regulates the *hmp* gene by interaction of NO with the [Fe-S] cluster (Cruz-Ramos *et al.* 2002). Fnr binds to a region around –10 sequence of the *hmp* promoter *in vitro* and NO treatment affects its binding to target promoter regions, suggesting that NO-modified [Fe–S]-cluster lacks Fnr activity (Cruz-Ramos *et al.* 2002). Later studies demonstrated that the NO-specific upregulation of *hmp* in *E. coli* is due to

the inactivation of NsrR repression (Bodenmiller *et al.* 2006). However, as the major repression exerted by NsrR is relieved by NO at the *hmp* promoter, the exact mechanism of Fnr repression at the *hmp* promoter remains unknown.

B. subtilis Fnr recognizes a target sequence identical to the *E. coli* Crp binding site, but not to the *E. coli* Fnr binding site (Cruz Ramos *et al.* 1995). Genes (*arfM*, the *narK-fnr* and *narGHJI)* that are directly regulated by Fnr are much fewer in *B. subtilis* than in *E. coli* (Reents *et al.* 2006). *B. subtilis* Fnr functions similarly to *E. coli* Fnr with a slight difference in response to oxygen. Oxygen exposure results in the formation of the apo-form of Fnr rather than the [2Fe-2S]-form as an intermediate (Reents *et al.* 2006). The [4Fe-4S] cluster is not necessary for dimerization of *B. subtilis* Fnr, although the apo-protein is unable to bind DNA as in the case of *E. coli* Fnr (Reents *et al.* 2006). The difference in the role of the [4Fe-4S] cluster between the two orthologs could be due to their difference in the ligation of the [4Fe-4S] cluster (Reents *et al.* 2006; Gruner *et al.* 2011). The four conserved cysteines contribute to the [Fe-S] ligation for *E. coli* Fnr, whereas three cysteines and an aspartate residue are responsible for the *Bacillus* protein (Reents *et al.* 2006; Gruner *et al.* 2011).

1.4.3 Fur/PerR

In addition to iron starvation, NO stress can also induce transcription of genes in the Fur regulon in *E. coli* (Mukhopadhyay *et al.* 2004) and in *B. subtilis* (Moore *et al.* 2004). *fur* mutants are susceptible to NO stress, possibly due to the accumulation of Fe-nitrosyl complexes as shown in *E. coli* (D'Autreaux *et al.* 2002).

Intracellular iron concentration must be maintained at a delicate balance. Fur which stands for Ferric iron uptake regulator, was first identified in *E. coli* as a repressor of iron transport systems under iron-replete conditions (Hantke 1981; Hantke 1987). The iron-loaded Fur blocks the access for RNA polymerase to promoter DNA, leading to the repression of the downstream genes (Bsat *et al.* 1999). Fur, which is considered as an iron-dependent repressor, is also known to be an activator in many bacteria, although the mechanism of transcriptional activation has not been uncovererd (Isabella *et al.* 2008; Nandal *et al.* 2010; Yu *et al.* 2012).

B. subtilis has three Fur homologs with different specificities to their metal cofactors. These homologs function in peroxide stress response (PerR) (Bsat *et al.* 1998), zinc uptake (Zur) (Fuangthong *et al.* 2003), and iron uptake (Fur) regulation (Bsat *et al.* 1998). Global analysis of the *B. subtilis* Fur regulon and iron starvation stimulon identified 30 operons including those required for iron transport (Baichoo *et al.* 2002). Under normal conditions when iron is adequate in the cell, Fur is coordinated with one Fe^{2+} per monomer, allowing this dimeric protein to bind to the 15-bp Fur box (7-1-7 overlapping heptameric core motif) at the operator region (Baichoo *et al.* 2002). *B. subtilis* Fur consists of one structural Zn^{2+} per monomer along with a regulatory Fe^{2+} binding site that is required for repressor activity (Baichoo *et al.* 2002).

PerR exhibits different sensitivity to oxidation and specificity towards the target promoter based on its metallated forms (Fuangthong *et al.* 2002). PerR can use Fe^{2+} or Mn^{2+} as its metal cofactor depending on the iron availability (Fuangthong *et al.* 2002; Moore *et al.* 2004). Supporting evidence has indicated that the Fe^{2+} form of PerR is

sensitive to NO attack in iron rich conditions. On the contrary, conditions favoring incorporation of manganese make PerR insensitive to NO (Moore *et al.* 2004).

1.4.4 NorR

The flavorubredoxin-encoding *norVW* genes in *E. coli* are regulated by the NorR transcriptional regulator, the gene for which is divergently transcribed from the *norVW* operon (Hutchings *et al.* 2002). NorR is a σ^{54}-dependent enhancer binding protein that belongs to AAA+ (ATPase associated with various cellular activities) group of proteins and activates the transcription of *norVW* in response to NO (Hutchings *et al.* 2002; Gardner *et al.* 2003). NorR consists of three domains, namely the N-terminal regulatory GAF (cGMP-specific and-stimulated phosphodiesterases, *Anabaena* adenylate cylases and *E. coli* FhlA) domain ligated to a non-heme iron at its center, the central ATPase active domain, and the C-terminal HTH (helix-turn-helix) DNA-binding motif (Shingler 1996; D'Autreaux *et al.* 2005). The N-terminal GAF domain with an iron center is the sensory component that reacts with NO (Pohlmann *et al.* 2000; Gardner *et al.* 2003; D'Autreaux *et al.* 2005). Mutational studies conducted on NorR domain architecture revealed that the ATP hydrolysis activity of the central AAA+ domain is negatively regulated by the N-terminal GAF domain (Tucker *et al.* 2006; D'Autreaux *et al.* 2008). In the presence of NO, negative control exerted by the GAF domain on the central AAA+ domain is relieved via conformation changes (Tucker *et al.* 2008). This mechanism was observed through the interaction of NO with the iron center of the GAF domain, which brings about a conformational change, resulting in driving the transcriptional activation of the target genes (Tucker *et al.* 2008). The C-terminal HTH motif recognizes

conserved enhancer-like sequence (80-150bp) located upstream of the promoter and the binding of NorR to this region is facilitated by the nucleoid associated protein, IHF (integration host factor) (Tucker *et al.* 2004).

1.4.5 NsrR

NsrR belongs to the Rrf2 family of transcriptional regulators that have an N-terminal helix-turn-helix DNA binding motif and members of the NsrR subfamily have three conserved cysteines at their C-terminus (Spiro 2007) (Figure 1.3). NsrR was first identified in the NH_3-oxidizing bacterium *Nitrosomonas europaea* as a transcription repressor of the nitrite reductase gene, *nirK* (Beaumont *et al.* 2004). As the repression of *nirK* by NsrR was reversed by nitrite (NO_2^-), but not by SNP, the authors concluded that NsrR is a nitrite-sensitive transcription repressor. Comparative genomic studies proposed that NsrR is a master regulator in NO metabolism both in gram-positive and gram-negative bacteria (Rodionov *et al.* 2005). The proposed role of NsrR has been confirmed by studies conducted in various bacteria including *E. coli* (Bodenmiller *et al.* 2006; Filenko *et al.* 2007; Lin *et al.* 2007; Rankin *et al.* 2008), *N. gonorrhoeae* (Overton *et al.* 2006; Isabella *et al.* 2008; Isabella *et al.* 2009), *N. meningitidis* (Rock *et al.* 2007; Heurlier *et al.* 2008), *S. typhimurium* (Bang *et al.* 2006), and *Moraxella catarrhalis* (Wang *et al.* 2008) as well as in *B. subtilis* (Nakano *et al.* 2006). These studies indicated the direct role of NO in alleviating NsrR repressor activity. However, recent work in *M. catarrhalis* demonstrated that NsrR is a repressor of *aniA* (nitrite reductase) and *norB* (NO reductase) and that NsrR repression of *aniA* is sensitive to nitrite, whereas the

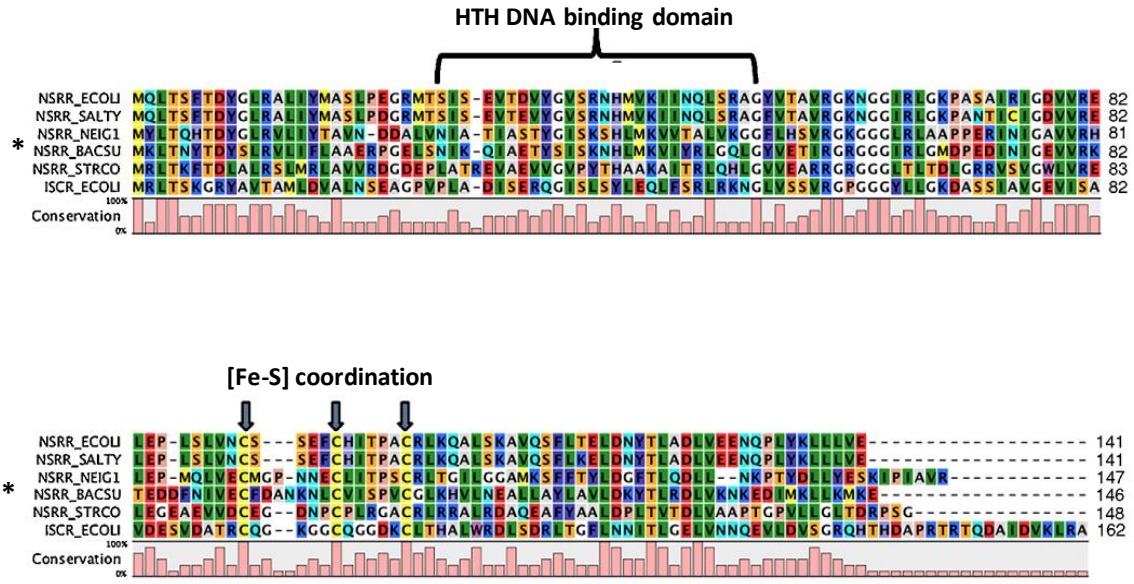

Fig. 1.3. The primary amino acid sequence alignment of NsrR with its close homologue, IscR from *E. coli*. The figure represents a ClustalW alignment of the NsrR sequences in different bacteria. ECOLI (*E. coli*), SALTY (*S. typhimurium*), NEIG1 (*N. gonorrhoeae*), and STRCO (*St. coelicolor*). Arrows indicate the three conserved cysteine residues that are required for the ligation of the [Fe-S] cluster. The N-terminal helix-turn-helix motif is required for the DNA-binding and is indicated with bracket. * represents *B. subtilis* NsrR, the focus of this thesis. The figure is modified from (Tucker *et al.* 2010).

repression of *norB* is sensitive to NO (Wang *et al.* 2008). However, the possibility remains that NsrR plays an indirect role in *aniA* transcriptional regulation.

NsrR function is also linked to the bacterial pathogenesis; for example, the survival of an *nsrR* mutant of *S.* typhimurium in IFN-γ-stimulated macrophages was reduced due to enhanced sensitivity to oxidative stress (Gilberthorpe *et al.* 2007). NsrR plays a role in enhancing the symbiotic relationship between *V. fischeri* and its squid host by regulating the *hmp* gene against the host-derived NO, thereby increasing the efficiency of colonization (Wang *et al.* 2010). *B. subtilis* NsrR is a NO-sensitive transcriptional repressor that controls the ResD-dependent transcription activation of *nasD* and *hmp* (Nakano *et al.* 2006). *B. subtilis nsrR* was first identified as the site of a null mutation that results in aerobic derepression of *hmp* in a transcription factor array analysis (Nakano *et al.* 2006). Proposed consensus NsrR-binding sites have been detected in the *nasD* and *hmp* promoter region (Rodionov *et al.* 2005; Nakano *et al.* 2006), which was later supported by DNase I footprinting analysis (Geng and Nakano, unpublished). The hypothesis that NO modulates NsrR activity is consistent with the results that cellular NsrR concentrations were unaffected by oxygen or NO (Nakano *et al.* 2006).

B. subtilis NsrR protein was purified and further characterized by our laboratory in collaboration with Moënne-Loccoz laboratory (Yukl *et al.* 2008). The use of spectroscopic techniques, including UV-vis, resonance Raman, and EPR provided evidence that anaerobically isolated *B. subtilis* NsrR (heterologously expressed in *E. coli*) contains a [4Fe-4S] cluster (Yukl *et al.* 2008). NsrR was shown to be a dimeric protein regardless of presence of the [Fe-S]-cluster co-ordination. The [4Fe-4S] cluster reacts with NO to form a dinitrosyl iron complex, which is similar to that of *E. coli* Fnr (Yukl *et*

al. 2008). As only three cysteine residues are present in NsrR, the [4Fe-4S] cluster could possibly have a non-cysteinyl ligand (Yukl *et al.* 2008). As NsrR paralogs from other bacteria (*S. coelicolor* and N. *gonorrhoeae*) were reported to bear a [2Fe-2S] cluster, my thesis work started to aim at resolving the contradictory results of the [Fe-S] cluster structure and characterizing the effect of NO on NsrR activity in transcriptional repression.

1.5 OUTLINE OF THE THESIS

The focus of my thesis research is to understand the molecular mechanism of NsrR in control of its target gene expression. Chapter 1 provides a background and literature review providing a foundation and rationale study. Chapter 2 demonstrates the molecular mechanism by which NsrR controls *nasD* expression in response to NO. In Chapter 3, I will report the identification of *cis*-regulatory regions required for NsrR-dependent *nasD* regulation and classification of the NsrR regulons into class I and class II. This chapter also highlights the new role of ResD and its coordinated regulation with NsrR in transcription of the class II regulon. In Chapter 4, I will present *in vivo* binding of NsrR and ResD on the class I and class II promoters. The results provide an evidence for a possible complex interplay involving multiple transcriptional regulators including ResD, NsrR, and Fur at the promoter DNA. Chapter 5 will summarize the research presented in this thesis and explore avenues towards future work.

CHAPTER 2

NITRIC OXIDE SENSITIVE AND INSENSITIVE INTERACTION OF *BACILLUS SUBTILIS* NSRR WITH A RESDE-CONTROLLED PROMOTER[1]

2.1 INTRODUCTION

Under anaerobic conditions, the ResDE two-component signal transduction system is required for the upregulation of genes involved in nitrate respiration (Geng *et al.* 2004). Among ResDE-controlled genes, *nasDEF* and *hmp*, which encode nitrite reductase and flavohemoglobin respectively, are the most highly induced during nitrate respiration (Ye *et al.* 2000) or by nitric oxide (NO) (Nakano 2002; Moore *et al.* 2004). However, anaerobic conditions and the ResDE system alone are not responsible for enhanced induction of these genes. Addition of exogenous NO donor (such as spermine NONOate) or endogenous NO that is produced through nitrate respiration was shown to relieve the repression on ResD-controlled *nasDEF* and *hmp* transcription *in vivo* (Nakano *et al.* 2006). Our previous studies identified the requirement of signaling molecule NO and inactivation of NO-sensitive repressor, NsrR for the transcription of *nasDEF* and *hmp* (Nakano *et al.* 2006; Yukl *et al.* 2008). NsrR, a member of Rrf2 family of transcription regulators, was proposed to be a master regulator of NO metabolism [reviewed in (Spiro 2007)].

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Kommineni S, Yukl ET, Hayashi T, Delepine J, Geng H, Moënne-Loccoz P, Nakano MM (2010) Nitric oxide-sensitive and -insensitive interaction of *Bacillus subtilis* NsrR with a ResDE-controlled promoter. *Mol Microbiol* **78:** 1280-1293

Our previous study showed that heterologously expressed and anaerobically purified *B. subtilis* NsrR (BsNsrR), bears a [4Fe-4S] cluster that, upon exposure to NO, forms dinitrosyl iron complexes (Yukl *et al.* 2008). Around the same time, two independent studies published on NsrR from *N. gonorrhoeae* (NgNsrR) (Barth *et al.* 2009) and *S. coelicolor* (ScNsrR) (Tucker *et al.* 2008), demonstrated that aerobically purified NsrR contains a [2Fe-2S] cluster and that, upon reaction with NO, diminishes NsrR DNA-binding activity *in vitro*, causing some uncertainty as to the form of Fe-S cluster that is physiologically relevant. Here, we report that BsNsrR either from aerobic or anaerobic cultures, when purified under anaerobic conditions, contains a [4Fe-4S] cluster. We also demonstrated that NsrR binds around the -35 element of the *nasD* promoter and to a further upstream region that overlaps with the ResD-binding sites. The binding of NsrR to the -35 region is greatly enhanced by the presence of the [4Fe-4S] cluster, whereas the binding to the upstream region is independent of Fe-S cluster formation.

2.2 MATERIALS AND METHODS

Bacterial strains and culture conditions

The strains and plasmids used in this study are listed in Table 2.1. *E. coli* DH5α was used for cloning of the recombinant plasmids. *E. coli* BL21 (DE3)/pLysS was used to produce NsrR proteins and ER2566 (New England Biolabs) was used for production of ResD and ResE. All *B. subtilis* strains are isogenic derivatives of JH642. ORB6559 was constructed by transforming pCm::Sp (Steinmetz *et al.* 1994) into ORB6179 (Nakano *et al.* 2006), which replaces the chloramphenicol-resistance marker in *nsrR* with

Table 2.1: *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::Spreplacement of chloramphenicol resistance with spectinomycin resistance	
	(Steinmetz *et al.* 1994)	
pDG646	erythromycin-resistance cassette	(Guerout-Fleury *et al.* 1995)
pDG795	*thrC* integration vector	(Guerout-Fleury *et al.* 1996)
pDG1515	tetracycline-resistance cassette	(Guerout-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*.

spectinomycin resistance by Michiko M. Nakano. Antibiotic concentrations used in growth media were ampicillin (50 μg ml^{-1}), chloramphenicol (5 μg ml^{-1}), spectinomycin (75 μg ml^{-1}), and erythromycin/lincomycin (1 μg $ml^{-1}$and 25 μg ml^{-1}, respectively).

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

Dr. Michiko M. Nakano generated NsrR fused to a Strep-tag at the C-terminus, by using plasmid pMMN771. 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*

pMMN810 (generated by Dr. Michiko M. Nakano), overproducing the C-terminal His_6-tagged NsrR in *B. subtilis*, was constructed using pHT01 plasmid (MoBiTec). The *nsrR* gene with the His_6-coding region was amplified from pMMN740 using oligonucleotides oMN06-311 (5´-GGAGGGATCCATGAAGTTAACCAATTATAC-3´) and oT7term (5´-GCTAGTTATTGCTCAGCG-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

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, pMMN810 was constructed using pHT01 plasmid (MoBiTec) (generated by Dr. Michiko M. Nakano). The *nsrR* gene with the His_6-coding region was amplified from pMMN740 using oligonucleotides oMN06-311 (5´- GGAGGGATCCATGAAGTTAACCAATTATAC-3´) and oT7term (5´-GCTAGTTATTGCTCAGCG-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 obtained by selection for chloramphenicol resistance.

Alanine substitutions of cysteine residues in NsrR

In order to substitute each cysteine in NsrR (amino acid residues 92, 100, and 106) and ectopically express the mutant *nsrR* genes in strain ORB6559 (*nsrR*:*spc*), the following plasmids were constructed using a *thrC* integration vector pDG795 (Guerout-Fleury *et al.* 1996). Plasmids pHG64 and pHG66 (constructed by Dr. Hao Geng), pUC18 derivatives carrying *nsrR* (C92A) and *nsrR* (C106A) respectively, were digested with EcoRI and BamHI and inserted into pDG795 digested with the same enzymes to generate pMMN666 and pMMN667, respectively. pDG795 carrying *nsrR* (C100A) was generated by two-step PCR as follows. Two PCR products amplified from pHG56 using oligonucleotide pairs – oHG89 (5'-AAGAATCTCGCTGTTATTTCCCCGGTT-3')/oHG94 (5'-GA*GGATCC*GCTTTTGACCTT -3': BamHI site is italicized) and oHG90 (5'-AACCGGGGAAATAACAGCGAGATTCTT-3')/oHG93 (5'-CG*GAATTC*GCACTTGCTTTC-3': EcoRI site is italicized). The PCR products were annealed and used as template in the second PCR with oHG93 and oHG94 as primers. The resultant PCR product was digested with EcoRI and BamHI and inserted into EcoRI/BamHI-cleaved pDG795, resulting in pMMN669. A pDG795 derivative carrying *nsrR* (wt), pMMN668, was constructed by subcloning the *nsrR* gene from pHG56 into pDG795 in a similar way described above. *nsrR* in each plasmid was verified by sequencing analysis. These four pDG795 derivatives were used to transform ORB6559 (*nsrR*::*spc*) with selection for erythromycin- and spectinomycin-resistance and Thr^- colonies generated by a double-crossover recombination were chosen as ORB6629 [*nsrR*::*spc thrC*::*nsrR* (C92A)], ORB6630 [*nsrR*::*spc thrC*::*nsrR* (C106A)], ORB6631 [*nsrR*::*spc thrC*::*nsrR* (wt)], and ORB6632 [*nsrR*::*spc thrC*::*nsrR* (C100A)]. Strains ORB6629 to 6632 were transduced by SPβ phage carrying *nasD-lacZ* (Nakano *et al.*

1998) to generate ORB6640 to 6643, respectively. pMMN666, pMMN667, pMMN668 and pMMN669 were constructed by Dr. Michiko M. Nakano.

β-Galactosidase activity measurement

Two independent isolates of each *B. subtilis* strain were cultured at 37°C under anaerobic conditions in 2x yeast extract-tryptone (YT) media (Nakano *et al.* 1988) with 0.5% glucose and 0.5% pyruvate supplemented with antibiotics, if necessary. Cells were harvested at 1-h intervals and β-galactosidase activity was measured as described (Miller 1972). The activity at T1 (1 h after the end of the exponential growth) was shown.

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).

Western blot analysis

Cells were cultured at 37°C in 2xYT supplemented with 0.5% glucose, 0.5% pyruvate, and appropriate antibiotics. When the optical density at 600 nm (OD_{600}) of the cultures reached approximately 0.4, cells were harvested and cell lysate was prepared by passing through a French press. Twenty μg of total protein was applied to sodium dodecylsulfate-polyacrylamide (15%) (SDS-PAGE) gel and western blot analysis was carried out using anti-NsrR antibody as described (Nakano *et al.* 2006).

Determination of cellular concentration of NsrR by quantitative western blot analysis

The intracellular concentration of NsrR was determined in JH642 cells grown anaerobically in 2xYT supplemented with 0.5% glucose and 0.5% pyruvate. Cells were harvested at OD_{600}=0.4 to 0.5, and cell lysate was prepared from the 45-ml cultures using the protoplast lysis method as previously described (Baruah *et al.* 2004). Around 15 μg of total protein in 15 μl was applied to a 15% SDS-PAGE. NsrR-His_6 was purified as described below and 0.0625 to 2 pmoles protein in 15 μl was used as standard. Detection of NsrR was carried out by western blot using anti-His_6-NsrR antibody as described (Nakano *et al.* 2006). Colony-forming units were determined from the cultures by serial dilution and plating on LB agar.

Anaerobic and aerobic purification of NsrR-His_6

The C-terminal His_6-NsrR protein was overproduced in *E. coli* BL21(DE3)/pLysS carrying pMMN740 as previously described (Yukl *et al.* 2008). For anaerobic protein purification, cell pellet was transferred to an anaerobic chamber (Coy Laboratory Products) containing 5% H_2 and 95% N_2, where the centrifuge tube was kept with its lid open until chamber oxygen level was below 5 ppm. Cell suspension was transferred into a French press cell and all French cell components were assembled in the anaerobic chamber. Cell lysate obtained by passage through the French press was collected into a centrifuge tube while being purged with N_2. Aerobic purification was performed outside the anaerobic chamber in the same way as anaerobic purification.

The NsrR(C100A) mutant protein was purified from BL21(DE3)/pLysS carrying pMMN805 under anaerobic conditions following the same procedure as for wild-type NsrR. To construct pMMN805, *nsrR* (C100A) was amplified by PCR using oMN0-296 (5'-GGCGCGGG*CATATG*AAGTTAACCAATTATAC-3': NdeI site is italicized) and oMN06-304 (5'-CGCT*CTCGAG*TTCCTTCATTTTTAAAAGC-3': XhoI site is italicized) with pMMN732 as template. The PCR product digested with NdeI and XhoI was inserted into pET23a(+) digested with the same enzymes to generate pMMN805. The *nsrR* coding sequence was confirmed by sequencing. pMMN805 was constructed by Dr. Michiko M. Nakano. Purification of NsrR under anaerobic conditions was performed with Jake Delepine's assistance.

Anaerobic and aerobic 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. For anaerobic preparations, 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. For aerobic preparations, cell lysis and protein purification steps were conducted under ambient conditions.

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/ml 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.

Purification of ResD, ResE, RNAP, and σ^A

ResD and ResE were overproduced in *E. coli* ER2566 (New England Biolabs) and purified as previously described (Geng *et al.* 2004) except that ResD protein was dialyzed against 25 mM Tris-HCl buffer (pH 8.0) containing 50 mM NaCl, 5 mM $MgCl_2$, 5% glycerol, and 5 mM β-mercaptoethanol before storage at -80°C. RNAP and σ^A were purified as previously described (Nakano *et al.* 2006).

Measurement of protein concentration and iron content

Protein concentrations were determined by Bio-Rad protein assay using bovine serum albumin as a standard. As seen with N-terminal His_6-NsrR (Yukl *et al.* 2008),

total amino acid analysis of the C-terminal His_6-NsrR (AAA Service Laboratory Inc., Damascus, Oregon) showed that the Bio-Rad protein assay overestimates NsrR concentrations by ~26%. [4Fe-4S]-NsrR concentrations were calculated accordingly. The iron content of NsrR at two different protein concentrations (1 and 4 μM for anaerobically purified protein and 10 and 20 μM for C100A mutant and aerobically purified protein) was determined with a ferene assay as described (Yukl *et al.* 2008) and QuantiChrom™ iron assay kit (BioAssay Systems). The minimum amount detected was around 0.45 μM. Both methods gave similar results.

Spectroscopic characterization of NsrR

The spectroscopic characterization of NsrR was performed by Dr. Erik Yukl and Dr. Takahiro Hayashi. The 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}.

Electrophoretic mobility shift assay (EMSA) of NsrR, ResD~P and RNAP

EMSA was carried out under anaerobic and aerobic conditions as previously

described except that the reaction buffer was slightly modified [50 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.5 mM DTT, 5 mM $MgCl_2$, 5 μg ml^{-1} BSA, 10 μg ml^{-1} poly(dI-dC), 10% glycerol] and native gels were run in TGE buffer (50 mM Tris, 0.38 M glycine, 2 mM EDTA; pH not adjusted). For anaerobic experiments, EMSA was performed in the anaerobic chamber as carried out with other Fe-S proteins (Reents *et al.* 2006; Edwards *et al.* 2010). The solutions and electrophoresis buffer were degassed by purging with N_2 and were transferred to the anaerobic chamber one day before the experiment. Polyacrylamide gels assembled with the electrophoresis apparatus were placed in the anaerobic chamber and allowed to stand for at least 2 to 3 h before the gel was pre-run 1 at 150 V for 1h. For EMSA using only NsrR, a shorter *nasD* probe (-45 to -20) was generated by annealing complementary 30-mer oligonucleotides oMN09-489 (5'-GCATAACATGTATCTTAAATATTCCTTTCG-3'; template strand) and oMN09-491 (5'-CGAAAGGAATATTTAAGATACATGTTATGC-3'; nontemplate strand). The annealed oligonucleotides cover the putative NsrR-binding site of *nasD* (Nakano *et al.* 2006). oMN09-489 (2 μM) was radiolabeled using 50 μCi [γ-^{32}P]-ATP (800 Ci/mmol) in the presence of T4 polynucleotide kinase at 37°C for 30 min. The reaction was stopped by heating at 65°C for 15min. The labeled oligonucleotide was purified using nucleotide removal kit (Qiagen) and was mixed with 2 μM of unlabeled oMN09-491 in New England Biolabs restriction enzyme buffer 2. To anneal the oligonucleotides, the mixture was heated at 90°C for 5 min in a heat block filled with water and the heat block was removed from the apparatus to slowly cool to the room temperature. Four probes (-114 to -85, -104 to -75, -93 to -63, and -71 to -40) used in the experiment of Figure 2.12 were generated similarly by annealing a labeled oligonucleotide shown in Figure 2.3 and the

complementary unlabeled nontemplate strand. Approximately 0.1 nM of the probe was used in 20 μl of the reaction buffer without or with NsrR.

For EMSA using ResD~P, RNAP, and NsrR, a longer *nasD* probe (-114 to -4) containing ResD-binding sites (Nakano *et al.* 2000; Geng *et al.* 2004) and the NsrR-binding site was generated as follows. Oligonucleotide oSK42 (5´-CCGTCCGAATCATACCTATT-3´) was radiolabeled using T4 polynucleotide kinase and [γ-^{32}P]-ATP. The probe DNA was generated by PCR from pMMN406 (Nakano *et al.* 2000) using labeled oSK42 and unlabeled oSK43 (5´-AGCGTAGCACAGCAAAAAGG-3´). The amplified PCR product was purified by PCR purification kit (Qiagen). A *nasD* probe (-114 to -40) that lacks the primary NsrR-binding site was similarly generated using oligonucleotides oSK42 and oSK79 (5´-GTTATAAAATGTAACAAAATATACG-3´). To determine the effect of NsrR on a preformed *nasD*-RNAP complex, the *nasD* probe (around 0.2 nM) and RNAP (15 nM) were mixed in 20 μl of the EMSA reaction buffer. To determine the effect on the *nasD*-ResD~P, or *nasD*-RNAP- ResD~P complex, ResD (0.5 μM) was phosphorylated with ResE (0.5 μM) in 20 μl of the EMSA reaction buffer, to which *nasD* probe and RNAP (for the ternary complex formation) were added. The reaction mixture containing the preformed binary or ternary complex was incubated for 10 min at room temperature. Increasing concentrations of NsrR were added to the reaction, which was further incubated for 15 min. To examine the effect of NO, Spermine NONOate (SperNO) (dissolved in 10 mM NaOH) was added to the reaction 10 min after the addition of NsrR, and incubated at room temperature for additional 20 min. As a control 10 mM NaOH at the same volume as the SperNO solution was included in the reaction. After complexes

were resolved by polyacrylamide gel electrophoresis, the gel was dried and radioactive bands were analyzed with a Typhoon Trio^{+} variable imager (GE Healthcare). The intensity of bands was quantified using ImageJ (NIH).

In vitro *transcription assay*

The effect of NsrR on ResD-activated *nasD* transcription was determined using *in vitro* transcription assays as previously described (Nakano *et al.* 2006) with minor modifications. In short, a *nasD* template (-170 to +96 with respect to the transcription start site) was generated by PCR using oSK-34 (5'-TTTAATCGGGGAAGCCTTAGA-3') and oHG-1 (5'-TATCTCTTCAATGGCCCTTA-3') and purified by low-melting agarose gel and PCR purification kit (Qiagen). As a control, an *rpsD* promoter template was generated by PCR using oSN03-86 and oSN03-87 (Nakano *et al.* 2006). All reactions were performed in the Coy anaerobic chamber except otherwise stated. ResD (1 μM) and ResE (1 μM) were mixed in the transcription buffer (Nakano *et al.* 2006) containing RNasin (Promega) and incubated at room temperature for 10 min. Then 5 nM of the *nasD* template, NsrR at different concentrations, RNAP (15 nM), and σ^{A} (7.5 nM) were added and incubated at room temperature for 10 min, followed by the addition of SperNO, where indicated. Transcription was initiated by adding [α-^{32}P]-UTP and NTPs. The reaction mixture was further incubated for 10 min. After the reaction was stopped, samples were taken out from the chamber, precipitated with ethanol, and separated on a pre-run 6% polyacrylamide-urea gel at 500 V for approximately 40 min. The gel was dried and analyzed with a Typhoon Trio^{+} variable imager and the intensity of bands was quantified using ImageJ (NIH).

2.3 RESULTS

2.3.1 Anaerobically purified NsrR either from aerobic or anaerobic *E. coli* cultures contains a [4Fe-4S] cluster

In order to eliminate the possibility that the [4Fe-4S] cluster detected with N-terminal and C-terminal His_6-tagged BsNsrR (Yukl *et al.* 2008) is from the result of the erroneous assembly of Fe-S cluster formation, we constructed a C-terminal Strep-tag BsNsrR, overproduced the protein in *E. coli*, and purified the protein under anaerobic conditions. The Strep-tag BsNsrR, like the C-terminal His_6-tagged BsNsrR (Yukl *et al.* 2008), is functional when introduced into and produced in *B. subtilis* (Figure 2.1). In collaboration with Dr. Pierre Moënne-Loccoz laboratory, the [Fe-S] cluster of NsrR was characterized using EPR spectroscopy and RR spectroscopy. Absorbance spectra in the visible region for Strep-tag NsrR showed that anaerobically purified protein has a [4Fe-4S] cluster like the His_6-tagged proteins (Figure 2.2). The presence of a [4Fe-4S] cluster was further confirmed by resonance Raman (RR) spectroscopy (Figure 2.2) and electron paramagnetic resonance (EPR) spectroscopy (data not shown). We also noted that even when cells were grown and handled aerobically prior to lysis, the [4Fe-4S] form of NsrR was observed if purified anaerobically, suggesting that native BsNsrR harbors a [4Fe-4S] cluster. These data ruled out any concern of interference from the His_6-tag on the Fe-S cluster, and subsequent experiments were carried out with the C-terminal His_6-NsrR (NsrR-His_6) protein.

2.3.2 The [4Fe-4S] cluster is required for high-affinity DNA-binding of NsrR

I examined the role of [4Fe-4S] cluster essentiality in binding of NsrR to *nasD*

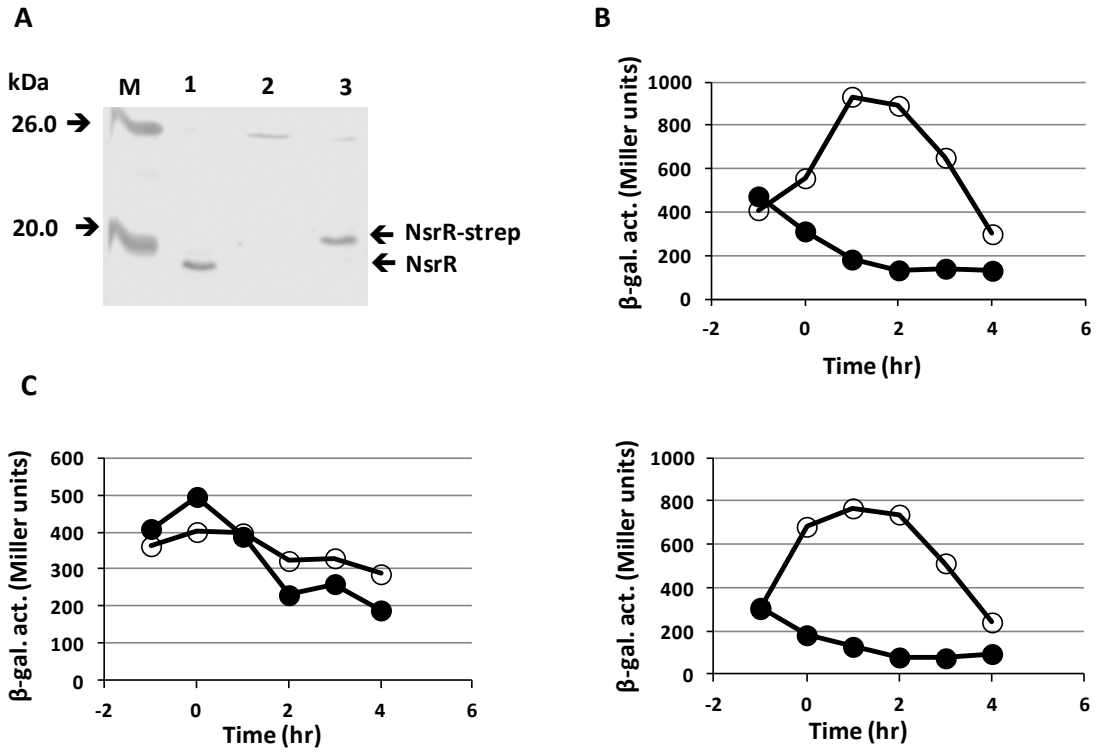

Fig. 2.1. Complementation analysis of Strep-tag NsrR in *B. subtilis*.
A. Western blot analysis of NsrR. Lane 1, LAB2854 (wild type); lane 2, ORB6188 (*nsrR*); lane 3, ORB7555 (*nsrR* carrying *nsrR*-strep). B to D. Expression of *nasD-lacZ* in LAB2854 (B), ORB6188 (C), and ORB7555 (D) cultured anaerobically 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.

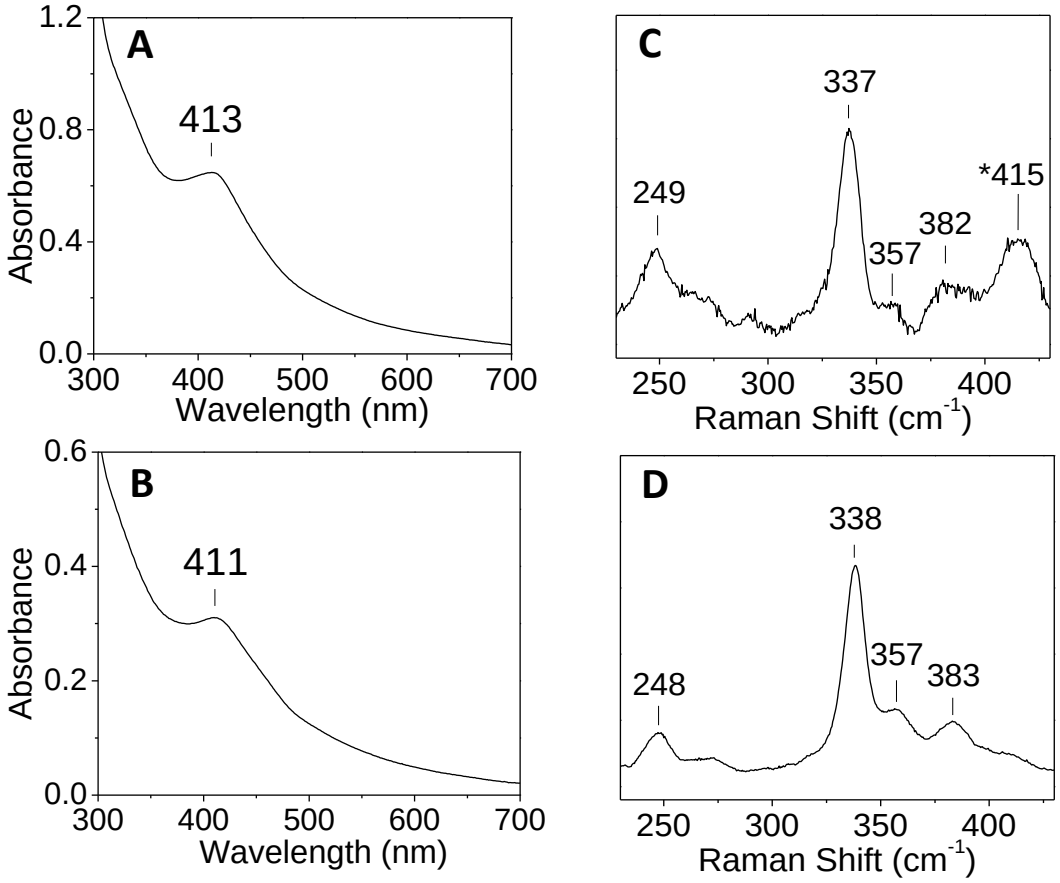

Fig. 2.2. Spectroscopic analysis of Strep-tag NsrR isolated from *E. coli* in comparison with His_6-tagged NsrR previously characterized (Yukl *et al.* 2008). UV-vis absorption spectrum of Strep-tag NsrR (A) and NsrR-His_6 (B) in the presence of 5 mM DTT. Room temperature resonance Raman spectrum of 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. Experiment performed and data analyzed by Dr. Erik Yukl and Dr. Takahiro Hayashi

promoter by electrophoretic mobility shift assays (EMSA). As a probe, we used a 30-base pair double-stranded *nasD* DNA carrying the putative NsrR-binding site previously identified (Nakano *et al.* 2006) (-44 to -19 in Figure 2.3). The double-stranded DNA was generated by annealing complementary oligonucleotides (Experimental procedures). Anaerobically purified [4Fe-4S]-NsrR bound to the double-stranded DNA (marked with *) and not to the single-stranded DNA (marked with **) (Figure 2.4A). [4Fe-4S]-NsrR at 0.5 nM (note that only around 28% of NsrR proteins contains the [4Fe-4S] cluster) began to bind *nasD* and the Kd that was observed to be between 2 to 8 nM depending on the extent of Fe-S cluster incorporation in each protein preparation. Two shifted bands with slightly different electrophoretic mobilities were observed and intensities of the faster migrating band increased as NsrR concentration increased. We assume that one of the shifted bands is likely the complex between target DNA and a heterodimer formed by [4Fe-4S]-NsrR and apo-NsrR for the following reasons. First, our previous result showed that NsrR forms a dimer regardless of the Fe-S cluster (Yukl *et al.* 2008). Second, both shifted bands are sensitive to sperNO (see Figure 2.6A), thus NsrR bound to the probe contains a Fe-S cluster, which is further supported by the finding that DNA-bound apo-NsrR migrates slower than these bands (data not shown). Excess amounts of cold *nasD* DNA competed for NsrR binding to the radioactive probe (Figure 2.4E).

The aerobically purified NsrR (apo-NsrR) contains essentially no Fe-S as judged by the measurement of Fe (less than 0.75% incorporation of the [4Fe-4S] cluster). The binding of apo-NsrR to *nasD* was much weaker compared to [4Fe-4S]-NsrR and the Kd was too high to be assigned at least within the range of concentrations used (Figure 2.4B). In contrast to the [4Fe-4S]-NsrR, only a single shifted complex was formed.

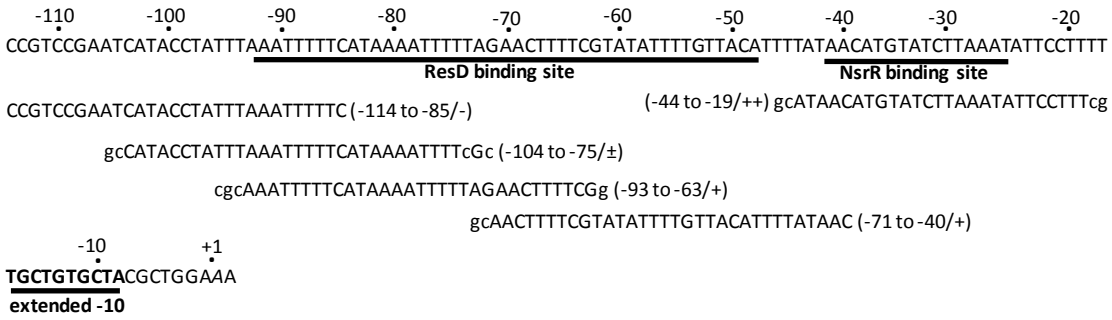

Fig. 2.3. Nucleotide sequence of the *nasD* regulatory region. The sequence of the coding strand from -114 to +1 (relative to the transcription start site) is shown. Underlined are the extended -10 element, the previously identified ResD-binding (Nakano *et al.* 2000; Geng *et al.* 2004) and NsrR-binding (Yukl *et al.* 2008) sites. The sequence of oligonucleotides used to generate EMSA probes are also listed. The numbers in the parentheses are relative to the transcription site. The oligonucleotide marked with ++ indicates the site where [4Fe-4S]-NsrR preferentially binds with high affinity. Apo-NsrR binds to the regions marked with +, weakly binds to the site marked with ±, does not bind to the site marked with -.

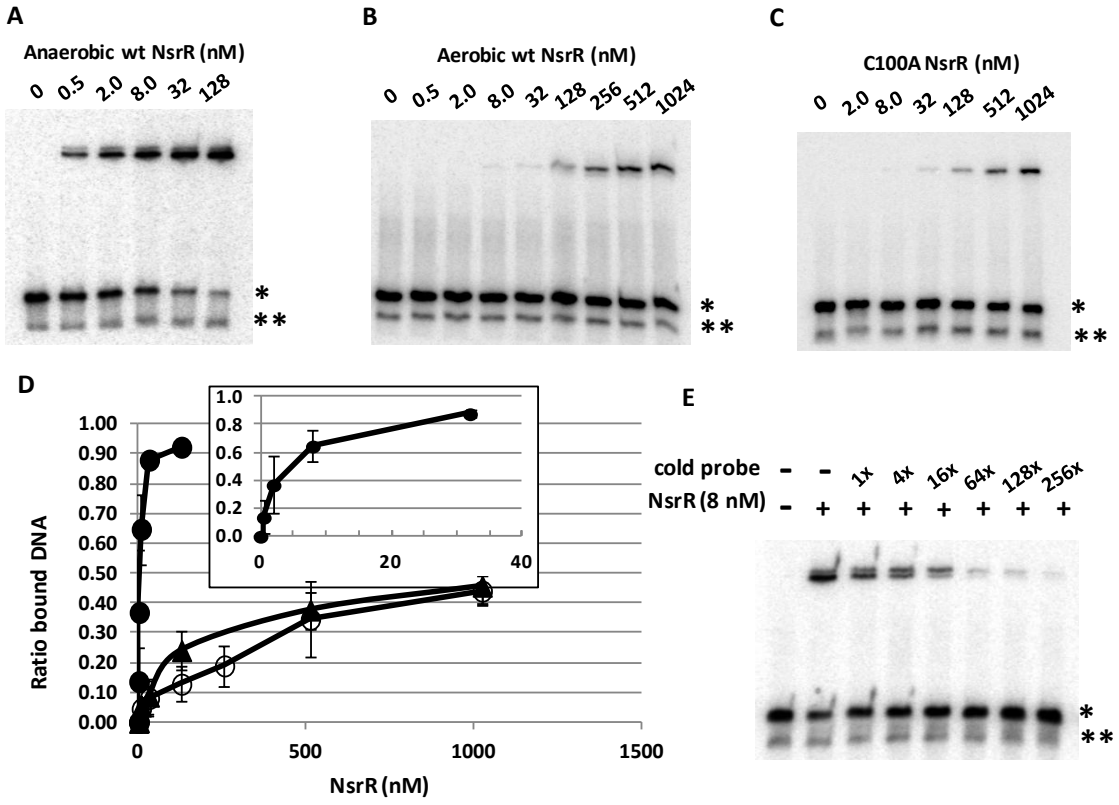

Fig. 2.4. Binding assay of NsrR to the *nasD* promoter. The sequence of the *nasD* probe (-44 to -19) that contains the proposed NsrR-binding site is shown in Figure 2.3. The radiolabeled probe (0.1 nM) was incubated with increasing concentrations of wild-type NsrR-His_6 purified under anaerobic conditions (A), aerobic conditions (B), or with the NsrR (C100A) mutant purified under anaerobic conditions (C). When anaerobically purified NsrR was used, the binding reaction as well as gel electrophoresis was carried out under anaerobic conditions as described in Experimental procedures. A single asterisk shows the double-stranded DNA and a double asterisk shows the single-stranded DNA, unannealed radiolabeled DNA oligonucleotide. D. ImageJ was used to quantify the ratio of shifted band to total double-stranded probe bands from multiple EMSA experiments (n=6 for A and n=3 for B and C) and the average values are shown with standard deviations. Symbols: closed circles, anaerobically purified NsrR; open circles, aerobically purified NsrR; closed triangles, anaerobically purified NsrR(C100A) mutant protein. The inset shows a binding curve with anaerobic NsrR at concentrations less than 32 nM. E. Competition assay. Anaerobically purified wild-type NsrR and radiolabeled *nasD* probe DNA were used with or without excess cold *nasD* DNA of the corresponding sequence.

These results demonstrated that the [4Fe-4S] cluster is essential for high-affinity binding of NsrR to the target promoter.

NsrR has three cysteines and it is very likely that these cysteines, together with a non-cysteinyl residue, serve to coordinate the [4Fe-4S] cluster. To assess the importance of cysteines for NsrR function, alanine codon substitutions at each cysteine codon position in *B. subtilis nsrR* were generated. In these constructs, the mutant alleles of *nsrR* transcribed from the native *nsrR* promoter were introduced into the *thrC* locus of an *nsrR* mutant. As shown in Figure 2.5A, the result of a western blot analysis showed that all mutant proteins are produced in *B. subtilis*. The effect of the cysteine substitutions on NsrR activity was determined by measuring β-galactosidase activity directed by a *nasD-lacZ* transcriptional fusion. When the wild-type *nsrR* was ectopically expressed, *nasD* expression was severely repressed in cells cultured anaerobically without nitrate (Figure 2.5B), indicating that NsrR repressed *nasD* transcription in the absence of NO. In contrast, *nasD* expression was derepressed in cells producing NsrR with the cysteine substitutions to a level similar to that in the *nsrR* null mutant, which demonstrated that the three cysteine residues are important for NsrR repressor activity. The effect of cysteine substitutions *in vivo* was determined by Dr. Michiko M. Nakano.

The C100A mutant protein was purified from *E. coli* to determine whether the loss of repressor activity was caused by a weaker binding affinity for the *nasD* promoter due to the lack of the [4Fe-4S] cluster. No measurable iron was detected in the mutant protein when purified anaerobically by the same protocol as used for wild-type NsrR purification, suggesting that the cysteine is important for ligation of the [4Fe-4S] cluster. NsrR(C100A) protein showed weak DNA-binding activity equivalent to the aerobically

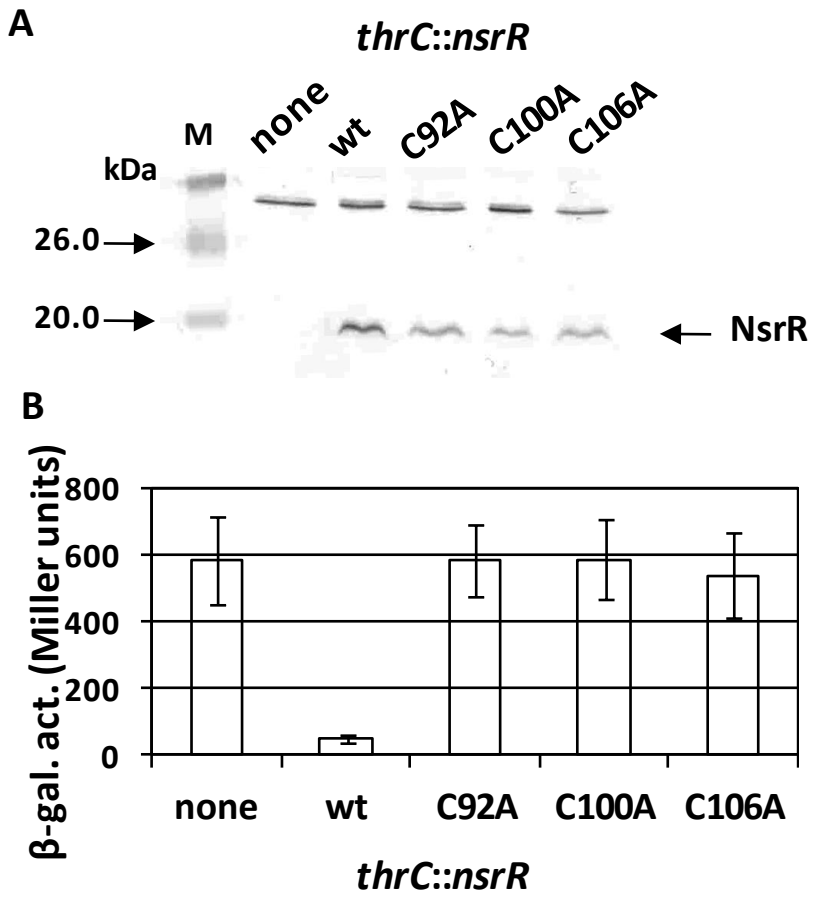

Fig. 2.5. Effect of cysteine mutations on *in vivo* NsrR activity.
A. Western blot analysis of the wild-type and mutant NsrR in *B. subtilis*. Cell lysate was prepared from a culture of an *nsrR* null mutant (none) as well as from the mutant carrying ectopically expressed wild-type *nsrR* (wt) or mutant alleles of *nsrR* (C92A, C100A, or C106A) at the *thrC* locus. The same amount of total protein from each lysate was resolved on an SDS-polyacrylamide gel and NsrR was detected by anti-NsrR antibody as described in Experimental procedures.
B. Strains carrying *nasD-lacZ* were grown in 2xYT supplemented with 0.5% glucose, 0.5% pyruvate, and appropriate antibiotics. Samples were collected at 1-h intervals to measure β-galactosidase activity and the activity at T1 (1 h after the end of exponential growth) is shown. The activities were measured at least three times in cultures of two independent clones and the average values of the data are presented along with standard deviations.

purified apo-NsrR (compare Figures 2.4B and 2.4C), confirming the requirement of the Fe-S cluster in efficient DNA binding to repress transcription.

2.3.3 Spermine NONOate relieves binding of NsrR to *nasD*

To address the effect of NO on binding of NsrR to *nasD,* I used a NO donor, SperNO. One mole of SperNO generates two moles of NO with a half-life of 39 min at 37°C and 230 min at 20-25°C (at pH 7.4, Cayman Chemical). Under our EMSA conditions, 1 μM or less NO is likely released from 10 μM SperNO solution. SperNO, at a concentration of 5 to 10 μM, inhibited the binding of [4Fe-4S]-NsrR (Figure 2.6A) as seen by an increase of the free probe. We noticed that a small amount of a *nasD*-NsrR complex with a slower electrophoretic mobility appeared at higher concentrations of SperNO (marked with an arrow in Figure 2.6A). This NO-induced complex migrated to the same position in a native gel as a complex formed with apo-NsrR (data not shown), suggesting that this complex was formed with *nasD* and apo-NsrR (Figure 2.10C). In contrast to [4Fe–4S]-NsrR, binding of apo-NsrR and NsrR(C100A) to *nasD* was not affected by SperNO (Figure 2.6B and 2.6C).Similarly, SperNO did not affect the binding of the NsrR(C100A) to *nasD* (Figure 2.5C). Taken together, these results clearly showed that the [4Fe-4S] cluster is essential for NO-responsive NsrR activity.

2.3.4 NsrR represses ResD-controlled *nasD* transcription *in vitro* and NO partially alleviates repression

To assess whether the EMSA results described above explain the negative effect of NsrR on *nasD* transcription as well as the antagonistic role of NO in NsrR-dependent

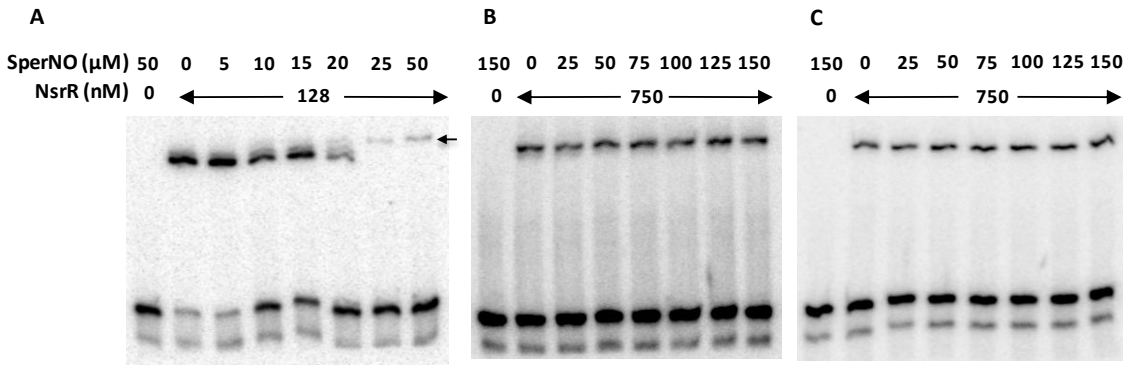

Fig. 2.6. Effect of spermine NONOate on DNA-binding activity of NsrR. The *nasD* DNA (-44 to -19) was incubated with indicated concentrations of either wild-type NsrR-His_6 purified under anaerobic conditions (A), aerobic conditions (B), or C100A mutant NsrR (C). Increasing concentrations of SperNO were added to the reaction as indicated. EMSA reactions and electrophoresis were carried out in an anaerobic chamber as described in Experimental procedures

repression, I performed *in vitro* transcription assays of *nasD*. We have previously shown that aerobically purified N-terminal His_6-tagged NsrR at 500 μM significantly represses *nasD* (and *hmp*) transcription *in vitro* (Nakano *et al.* 2006). A similar result was obtained with the C-terminal His_6-tagged apo-NsrR (Figure 2.7B) and NsrR(C100A) (Figure 2.7C), whereas [4Fe-4S]-NsrR was able to repress the transcription at much lower concentrations (Figure 2.7A). Quantitative analysis using ImageJ showed that minimal concentrations required for repression are slightly variable in the case of anaerobically purified NsrR, which is likely caused by variations in Fe-S cluster incorporation from prep to prep (Figure 2.7A). SperNO at 20 μM largely relieved NsrR-dependent *nasD* repression (Figure 2.7D), but had no significant effect on *nasD* transcription in the absence of NsrR, confirming that NO stimulates *nasD* transcription by alleviating NsrR-dependent repression. We have previously shown that transcription of *rpsD* encoding ribosomal protein S4 was not repressed by NsrR either *in vivo* or *in vitro* (Nakano *et al.* 2006). In the previous *in vitro* transcription study, we used aerobically purified NsrR. Therefore, I examined whether [4Fe-4S]-NsrR has any effect on *rpsD* transcription *in vitro*. As shown in Figure 2.7E, *rpsD* transcription was hardly affected by NsrR, indicating that NsrR-dependent repression is specific to *nasD*.

2.3.5 NsrR inhibits the interaction of RNA polymerase-ResD~P with the *nasD* promoter and NO relieves the inhibition

The EMSA results described above argue that the binding of NsrR to the -35 region plays a pivotal role in NsrR-dependent repression of *nasD*. We have previously shown that the ResD-ResE signal transduction proteins are essential for activation of

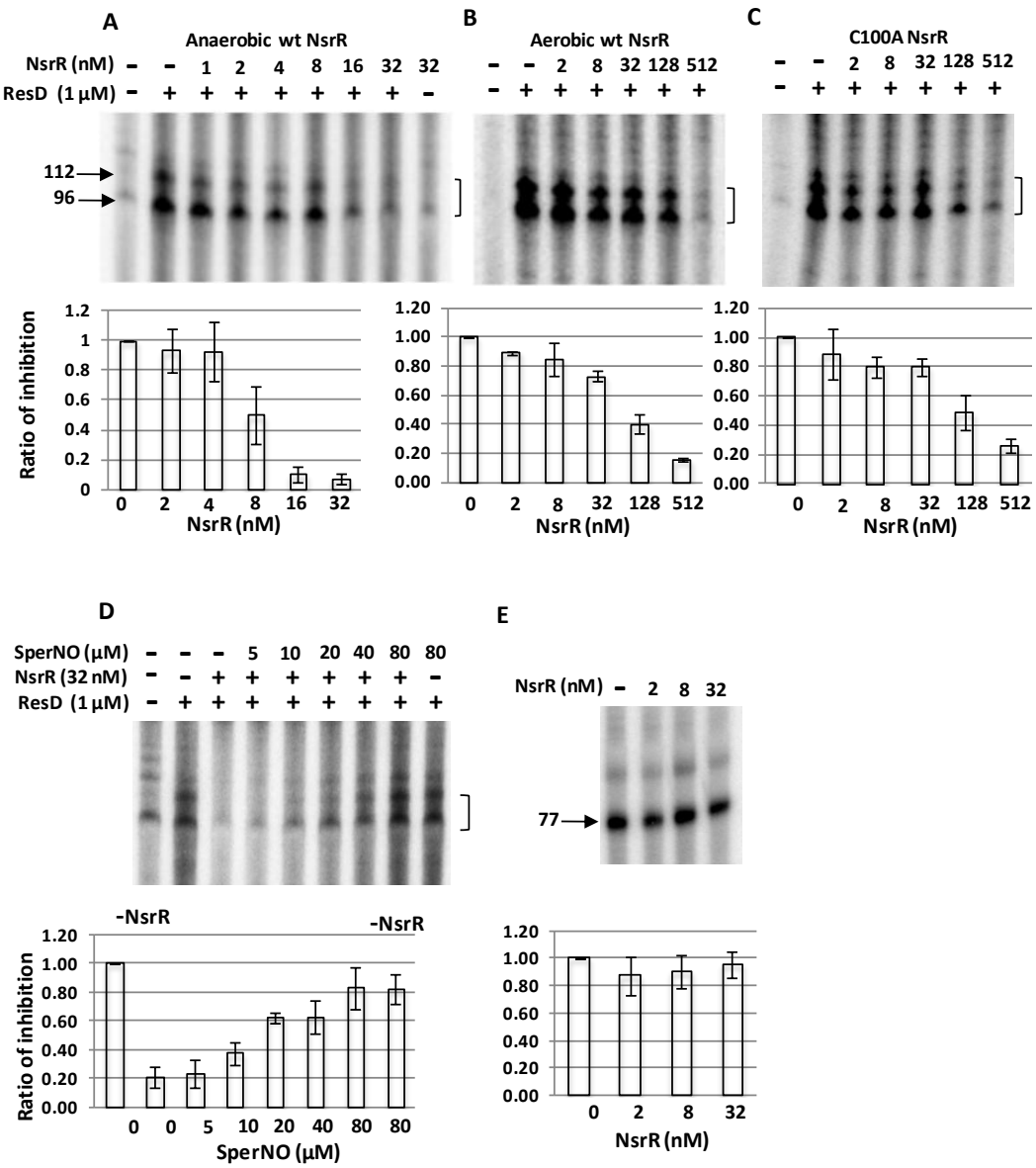

Fig. 2.7. *In vitro* transcription of *nasD*. The *nasD* template (-170 to +96) was incubated without or with σ^ARNAP, ResD~P (1 μM) and increasing concentrations of wild-type NsrR-His_6 purified under anaerobic conditions (A), aerobic conditions (B) or C100A mutant NsrR (C). An arrow with numbers shows the size of transcript in nucleotides. A bracket shows two *nasD* transcripts (96 and 112 base) that are generated by transcription *in vitro* as previously described (Geng *et al.* 2004) and the 96-base transcript corresponding to RNA transcribed *in vivo*. D. Effect of spermine NONOate on DNA binding activity of NsrR. SperNO at the indicated concentration was added in the reaction with anaerobically purified NsrR. E. *In vitro* transcription of *rpsD* using anaerobically purified NsrR. The 77-base transcript is marked. The intensity of the corresponding bands was quantified with ImageJ and is shown as the ratio of transcript detected in the absence of NsrR or SperNO. The average values (n=6 for A and n=3 for the rest) are shown with standard deviation.

nasD (Nakano *et al.* 1998). Therefore, in order to obtain a more complete picture of *nasD* transcriptional regulation, we need to elucidate the mechanism by which NsrR interferes with ResD-dependent transcription of *nasD*. To this end, anaerobic EMSA experiments were carried out with a longer *nasD* (-114 to -4) probe that includes the previously identified ResD-binding region (Nakano *et al.* 2000; Geng *et al.* 2004) and the NsrR-binding site (Figure 2.3). Figure 2.8A shows that ResD~P alone at 0.5 μM did not bind to the *nasD* promoter, whereas RNA polymerase (RNAP) as low as 15 nM interacted with the probe (shown by the arrow with #1 in Fig. 2.8). When RNAP was present, 0.5 μM ResD~P supershifted the *nasD*-RNAP complex (the arrow with #2). These data suggested that the role of ResD~P in *nasD* transcription is not simply to recruit RNAP to the promoter.

We next asked whether [4Fe-4S]-NsrR disrupts the preformed *nasD*-RNAP-ResD~P transcription initiation complex. As described in Experimental procedures, I allowed the *nasD*-RNAP-ResD~P ternary complex to form, followed by addition of NsrR. NsrR at 2 nM started to bind the probe and increasing concentrations of NsrR disrupted the preformed ternary complex, which was accompanied with formation of multiple *nasD*-NsrR complexes (the arrow with #3 in Figure 2.8B). These results indicated that [4Fe-4S]-NsrR efficiently competes with RNAP and ResD for binding to the *nasD* promoter and that the second and third NsrR-binding sites exist in the region between -114 and -3 (Figure 2.8B). The *nasD*-RNAP complex was also disrupted in the presence of 8 nM NsrR, and a small amount of a supershifted band appeared at higher concentrations of NsrR (the arrow with #2 in Figure 2.9A). This band could represent *nasD*-RNAP-NsrR complex resulted from binding of apo-NsrR to the upstream *nasD* as

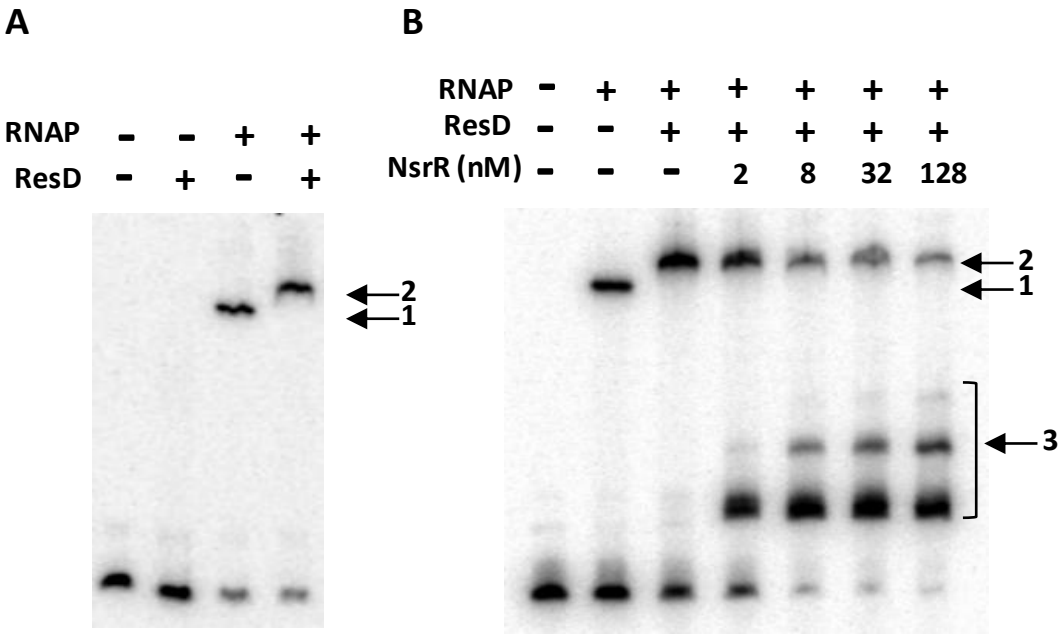

Fig. 2.8. Analysis of RNAP, ResD~P, and NsrR binding to the *nasD* promoter. The *nasD* probe encompassing -114 and -4 of the promoter region was used. All EMSA reactions and electrophoresis were carried out in an anaerobic chamber. A. The probe (0.2 nM) was incubated with 0.5 μM ResD phosphorylated with 0.5 μM ResE, 15 nM RNAP, or both. B. The probe was incubated with 15 nM RNAP in the absence or presence of 0.5 μM ResD~P and increasing concentrations of NsrR-His_6 purified under anaerobic conditions.

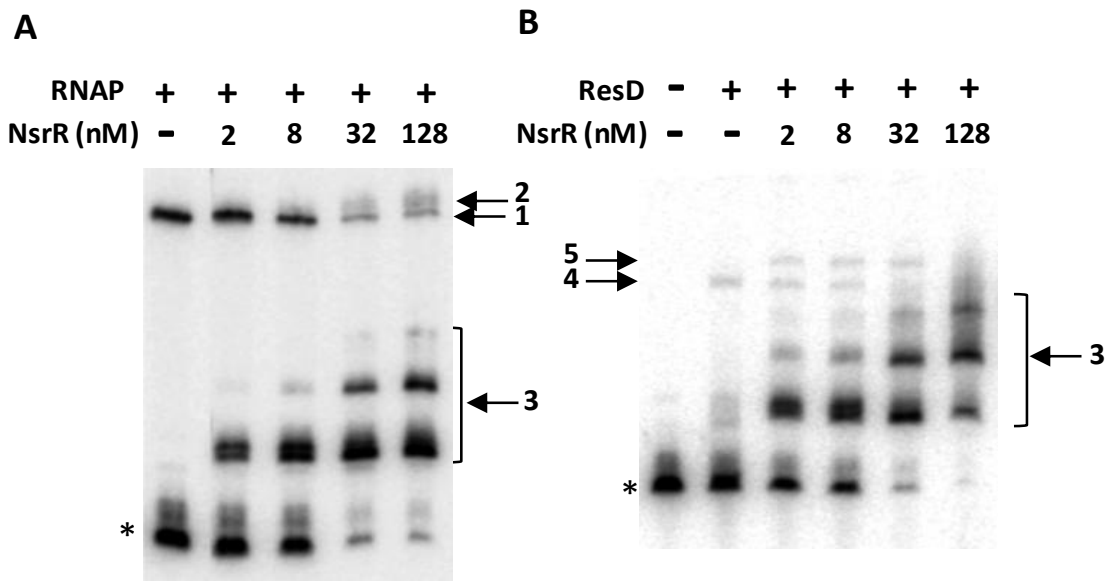

Fig. 2.9. Analysis of RNAP, ResD~P, and NsrR binding to the *nasD* promoter. The *nasD* (-114 to -4) was used as probe. All EMSA reactions and electrophoresis were carried out in an anaerobic chamber. A single asterisk shows the free probe. Arrows: 1, *nasD*-RNAP; 2, *nasD*-RNAP-NsrR; 3, *nasD*-NsrR; 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.

described later. Although ResD~P does not efficiently bind to *nasD* as shown in Figure 2.8A, it weakly binds at 1 μM (the arrow with #4 in Figure 2.9B). In the presence of 2 nM NsrR, a supershifted band appeared, which was likely formed by binding of ResD~P and [4Fe-4S]-NsrR to *nasD*.

We next examined the effect of SperNO on NsrR-dependent disruption of the *nasD*-RNAP-ResD~P ternary complex. As observed in EMSA using a shorter probe (Figure 2.6A), the presence of SperNO at concentrations of 20 to 40 μM and higher altered the electrophoretic mobility of the *nasD*-NsrR complexes (shown with solid arrows in Figure 2.10), generating new complexes (broken arrows) with slower mobilities. At these SperNO concentrations, we also observed a simultaneous increase in the amount of *nasD*-RNAP-ResD~P ternary complex and free probe (Figure 2.10A). Similarly, SperNO restored *nasD*-RNAP binary complex formation (Figure 2.10B). SperNO showed no significant effect on the binding of either RNAP alone or RNAP-ResD~P to *nasD* (data not shown), demonstrating that SperNO is directed only at [4Fe-4S]-NsrR.

We sought to uncover the nature of the *nasD*-NsrR complex generated by SperNO. We have previously shown that NsrR forms DNIC upon exposure to NO (Yukl *et al.* 2008). We compared how different *nasD*-NsrR complexes, which were formed with apo-NsrR, [4Fe-4S]-NsrR and SperNO-treated NsrR (DNIC-NsrR), migrate during electrophoresis through an SDS-polyacrylamide gel. Figure 2.10C shows that the complexes formed by anaerobically purified NsrR in the presence of SperNO migrated at the same position as those formed by aerobically purified apo-NsrR. In this experiment I used 128 nM NsrR, the concentration of which was shown to be sufficient for apo-NsrR

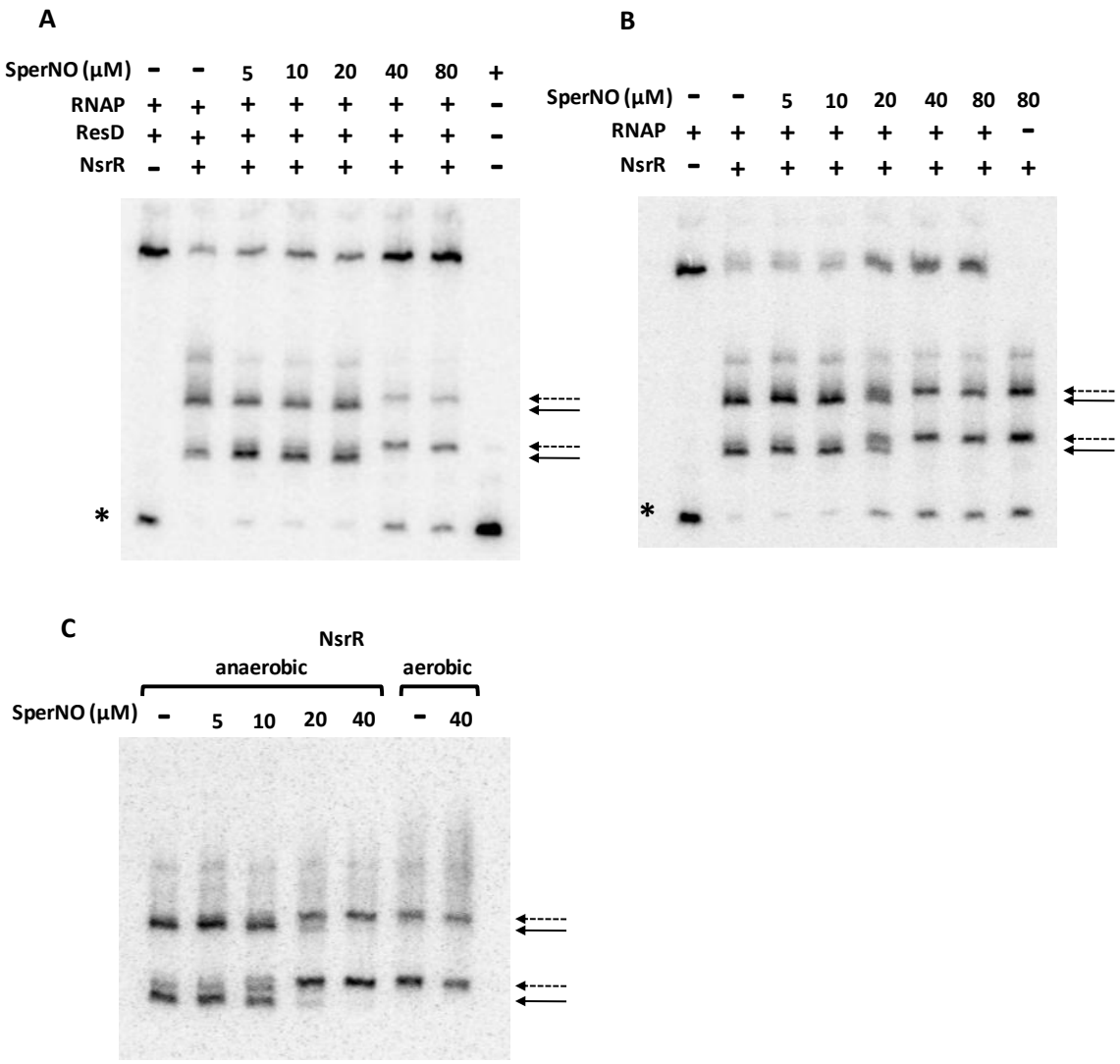

Fig. 2.10. Effect of spermine NONOate on DNA-binding activity of NsrR. The *nasD* (-114 to -4) probe was incubated with RNAP (15 nM), ResD~P (0.5 μM), and anaerobically purified NsrR-His_6 (128 nM) (A), or with RNAP and NsrR-His_6 (B) in the absence or presence of increasing concentrations of SperNO. Full arrows and broken arrows show the *nasD*-NsrR complex formed in the absence and presence of SperNO, respectively. A single asterisk shows the free probe.
C. The *nasD* probe was incubated with either anaerobically or aerobically purified NsrR-His_6 in the absence or presence of SperNO. The free probe ran off the gel. All EMSA reactions and electrophoresis were carried out in an anaerobic chamber.

to bind to *nasD* (Figure 2.4B). Then we asked whether a *nasD* complex formed with 8 nM NsrR could be supershifted by SperNO. As shown in Figure 2.4B, 8 nM apo-NsrR is unable to bind the *nasD* probe carrying only the primary NsrR-binding site. If the shifted band of the slowest mobility generated by SperNO is indeed the *nasD*-apo-NsrR complex, we expected that the band would not be generated when 8 nM NsrR was used. The result showed that it is indeed the case (data not shown). Therefore, we concluded the SperNO-induced *nasD*-NsrR complexes in Figures 2.6A and 2.10 are formed with apo-NsrR present in anaerobically purified NsrR protein or generated by SperNO, although we could not completely eliminate the possibility that the complex contains DNIC-NsrR.

2.3.6 Apo-NsrR binds to the ResD-binding region of *nasD*

The results described in Figures 2.8 and 2.10 suggested that NsrR interacts with *nasD* by binding to the second and third sites in addition to the primary site around the -35 region. To understand the role of the secondary binding sites in regulation of *nasD* transcription, we decided to determine the location of the secondary NsrR-binding sites and whether binding affinities of these *cis* elements are different between [4Fe-4S]-NsrR and apo-NsrR. To localize the secondary sites, I generated a probe (-114 to -40) that lacks the primary NsrR-binding site. Three important results were obtained by comparing EMSA results of NsrR between the full-length (-114 to -4) probe and the deleted (-114 to -40) probe. First, at higher concentrations of NsrR, three *nasD*-NsrR complexes were formed with the full-length probe, while only two complexes were

detected with the deleted probe (Figure 2.11). Based on this result, we concluded that the second and third NsrR-binding sites locate in the region between -114 and -40. Second, when the full-length probe was used, the *nasD*-NsrR complex with the fastest mobility (marked as #1 in Figure 2.11A) was formed with [4Fe-4S]-NsrR at much lower concentrations than with apo-NsrR (compare Figures 2.11A and 2.11B), whereas [4Fe-4S]-NsrR and apo-NsrR bound to the deleted probe with a similar affinity to form the first complex (marked #2/3, and compare Figures 2.11D and 2.11E). Furthermore, apo-NsrR binds similarly to these three sites (compare Figure 2.11B and 2.11E). This result argued that the NsrR-binding site around the -35 element is the only site that [4Fe-4S]-NsrR binds with a higher affinity than apo-NsrR does. Third, the *nasD* promoter lacking the primary NsrR-binding site does not generate NO-specific complexes with slow elecrophoretic moblities (compare Figures 2.11C and 2.11F), which is in good agreement with the interaction of apo-NsrR to the upstream binding sites.

To further define the location of the upstream NsrR-binding sites, I generated four overlapping short double-stranded oligonucleotides that cover the region between -114 to -40 of *nasD* (Figure 2.3). To determine with which probe apo-NsrR interacts, I performed EMSA experiment (Figure 2.12). The result clearly showed that the two binding sites reside in -93 to -63 and -71 to -40 of *nasD*. The probe from -104 to -75 showed a very weak activity, suggesting that the region between -93 and -75 serves as a core-binding site and DNA from -74 to -63 is required for stabilizing the *nasD*-NsrR complex. EMSA using [4Fe-4S]-NsrR showed an almost identical result (data not shown). Interestingly the region from -93 to -40 where apo-NsrR binds was identified as the ResD-binding site in our previous studies (Nakano *et al.* 2000; Geng *et al.* 2004).

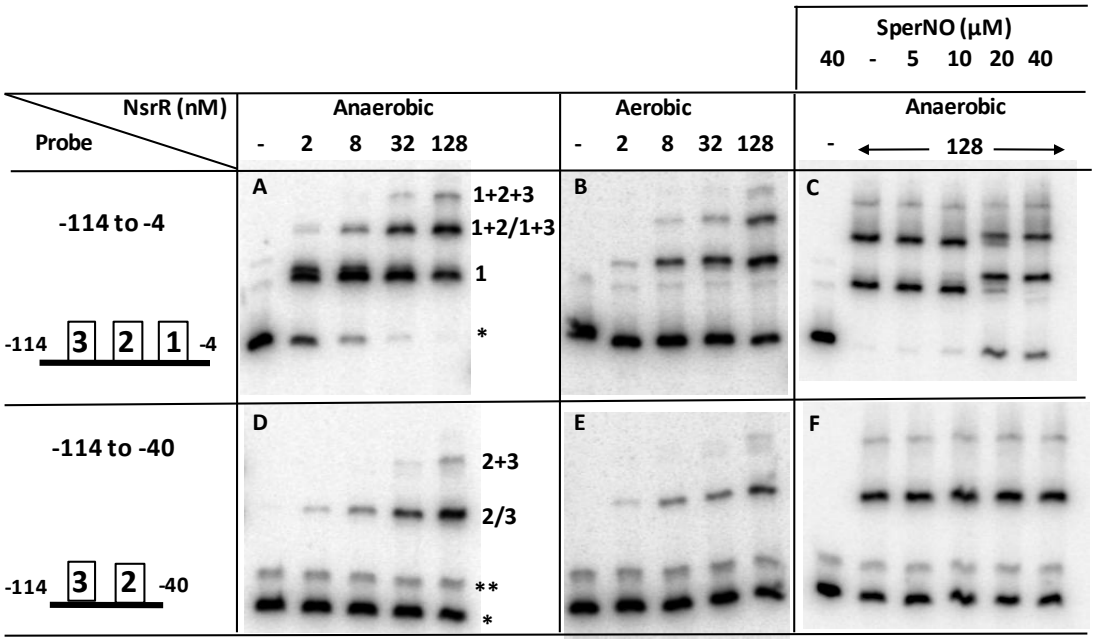

Fig. 2.11. Binding of NsrR to the *nasD* promoter with or without the primary NsrR-binding site. Schematic views of the probes are shown in the leftmost panels and include the primary NsrR binding site (marked with boxed 1) and secondary binding sites (marked with boxed 2 and 3).

A. The labeled *nasD* fragment (-114 to-4) containing the primary NsrR binding site was incubated with increasing concentrations of anaerobically purified NsrR-His_6. A single asterisk shows the free probe. The three shifted bands correspond to the NsrR complexes interacting with the binding site 1 (shown as 1), binding site 1 and 2 or 1 and 3 (1+2/1+3), and the binding site 1, 2, and 3 (1+2+3).

B. EMSA was carried out as described in A except aerobically purified NsrR-His_6 was used.

C. EMSA was carried out as described in A except the binding reaction was incubated in the absence or presence of increasing concentrations of SperNO.

D. The labeled *nasD* fragment (-114 to-40) lacking the primary NsrR binding site was incubated with increasing concentrations of anaerobically purified NsrR-His_6. A single asterisk shows the free probe and a double asterisk shows an unidentified DNA fragment generated in small amounts by PCR (note that this DNA does not bind to NsrR). The two shifted bands correspond to the NsrR complexes interacting with the binding site 2 or 3 (2/3), and the binding site 2 and 3 (2+3).

E. EMSA was carried out as described in D except aerobically purified NsrR-$His_6$was used.

F. EMSA was carried out as described in D except the binding reaction was incubated in the absence or presence of increasing concentrations of SperNO

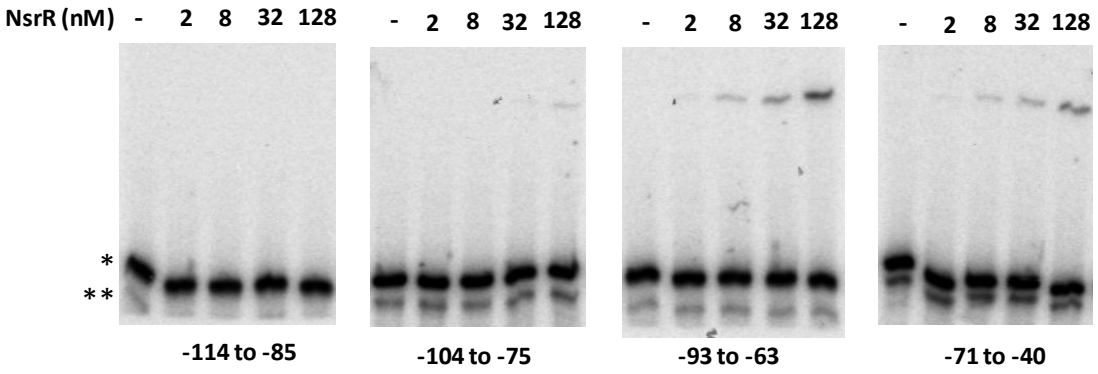

Fig. 2.12. Binding assay of NsrR to the *nasD* promoter. The sequence of the *nasD* fragments used as probes is shown in Figure 2.3. The radiolabeled probe (0.1 nM) was incubated with increasing concentrations of wild-type NsrR-His_6 purified under aerobic conditions. A single asterisk shows the double-stranded DNA and a double asterisk shows the single stranded DNA, unannealed radiolabeled DNA oligonucleotide.

2.3.7 A high concentration of NsrR is detected in *B. subtilis* cells

The results described above showed that apo-NsrR binds to the *nasD* regulatory region that overlaps with ResD-binding site. The question remains whether the interaction between apo-NsrR and *nasD* is physiologically relevant, particularly because the affinity of apo-NsrR to these sites is weaker than that of [4Fe-4S]-NsrR to the primary binding element. To evaluate whether there is good possibility that the apo-NsrR-DNA interaction occurs *in vivo*, Dr. Michiko M. Nakano measured NsrR concentration in *B. subtilis* cells using quantitative western blotting as previously described (Cai *et al.* 2002) with some modification (Experimental procedures) (Figure 2.13). As a standard, we used various concentrations of purified NsrR-His_6 protein. The cell lysate prepared from *B. subtilis* JH642 was resolved on an SDS-PAGE gel together with the standard NsrR protein. Polyclonal anti-His_6-NsrR antiserum (Nakano *et al.* 2006) was used to determine concentrations of NsrR in the western blot. We repeated the experiments using three independently prepared cell lysate and three different purification batches of NsrR proteins as standards. The aqueous volume of a *B. subtilis* cell was estimated as 1×10^{-14} liter by previous study (McCabe *et al.* 2004). By calculating with this value of cell volume, we found that *in vivo* NsrR concentration is 1.34 ± 0.21 μM (n=7), which corresponds to $8{,}066 \pm 1{,}264$ monomers in the cell. The result, together with the EMSA result in Figure 2.11, suggests that the cellular NsrR concentration is likely sufficient to allow NsrR to interact with low-affinity sites of *nasD*.

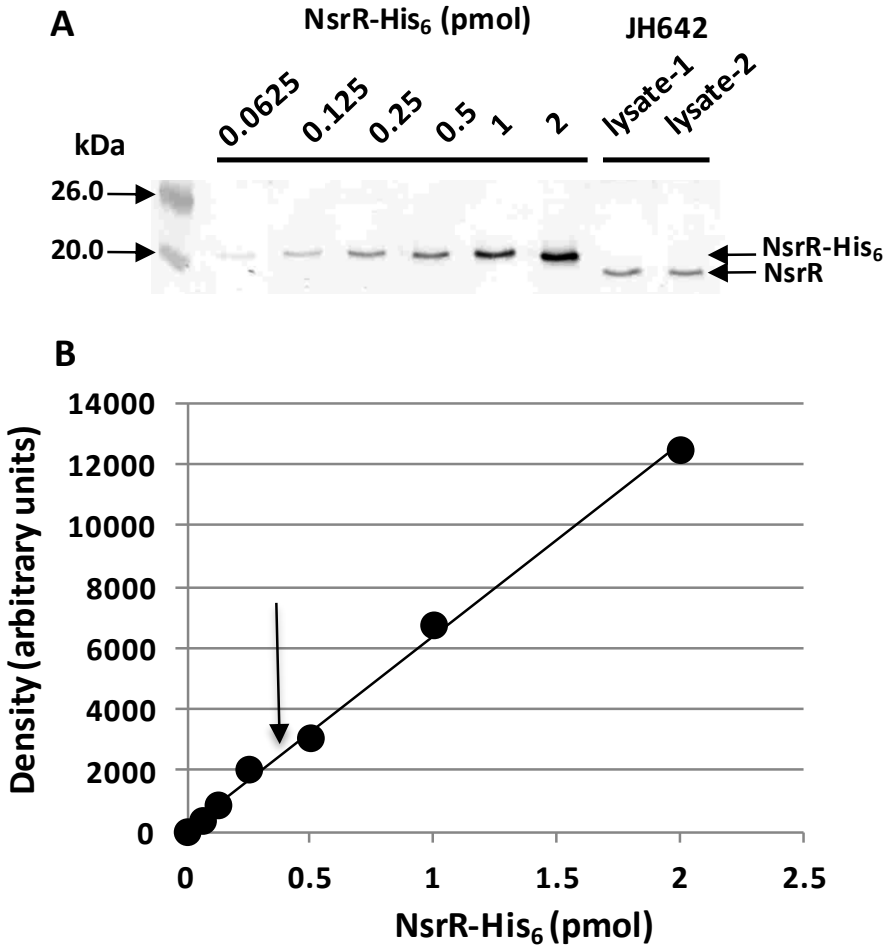

Fig. 2.13. Determination of *in vivo* NsrR concentration in *B. subtilis* cells using quantitative western blot analysis. A. Western blot of increasing amounts of purified NsrR-His_6 together with *B. subtilis* cell lysate pepared from independent cultures. Molecular weight markers are marked. B. The standard curve was plotted by quantifying band densitities of NsrR-His_6 shown in panel A. An arrow indicates the intensity of NsrR detected in the cell lysate shown in panel A.

2.4 DISCUSSION

The NO-sensitive transcription regulator NsrR plays an important role in the control of the NO stress response in a wide range of bacteria [reviewed in (Tucker *et al.* 2010)]. Although it is clear that NsrR is a Fe-S protein, studies to determine which form of Fe-S cluster it contains have been inconclusive. We have presented data here to reconfirm the previous study (Yukl *et al.* 2008) that NsrR proteins, when purified from either aerobic or anaerobic cultures of *E. coli*, carries a [4Fe-4S] cluster. In addition, we successfully overproduced the C-terminal His_6-NsrR in aerobically cultured *B. subtilis* and purified it anaerobically from the native host. The Fe-S cluster incorporation in NsrR purified from *B. subtilis* was higher (average 60%) than that of protein purified from *E. coli* (average 28%). UV-vis and resonance Raman spectra indicated that NsrR isolated from the native host also carries the [4Fe-4S] cluster (Figure 2.14). Preliminary results revealed that NsrR isolated from *B. subtilis* behaves similarly to the protein purified from *E. coli* with respect to DNA-binding activity and NO sensitivity (Figure 2.14). From these results we conclude that Bs-NsrR contains a [4Fe-4S] cluster in *B. subtilis*. The [4Fe-4S] cluster is maintained in aerobic cultures presumably because the concentration of intracellular oxygen remains sufficiently low. In addition, glutathione was postulated to be involved in stabilizing or reactivating oxidized Fe-S clusters (Ollinger *et al.* 2006) and a similar role might be played by low-molecular-weight thiols such as cysteine and bacillithiol (Newton *et al.* 2009) in *B. subtilis*, which lacks glutathione. In either case, the higher *in vivo* sensitivity of NsrR to NO than to oxygen is apparent from the previous *in vivo* result that

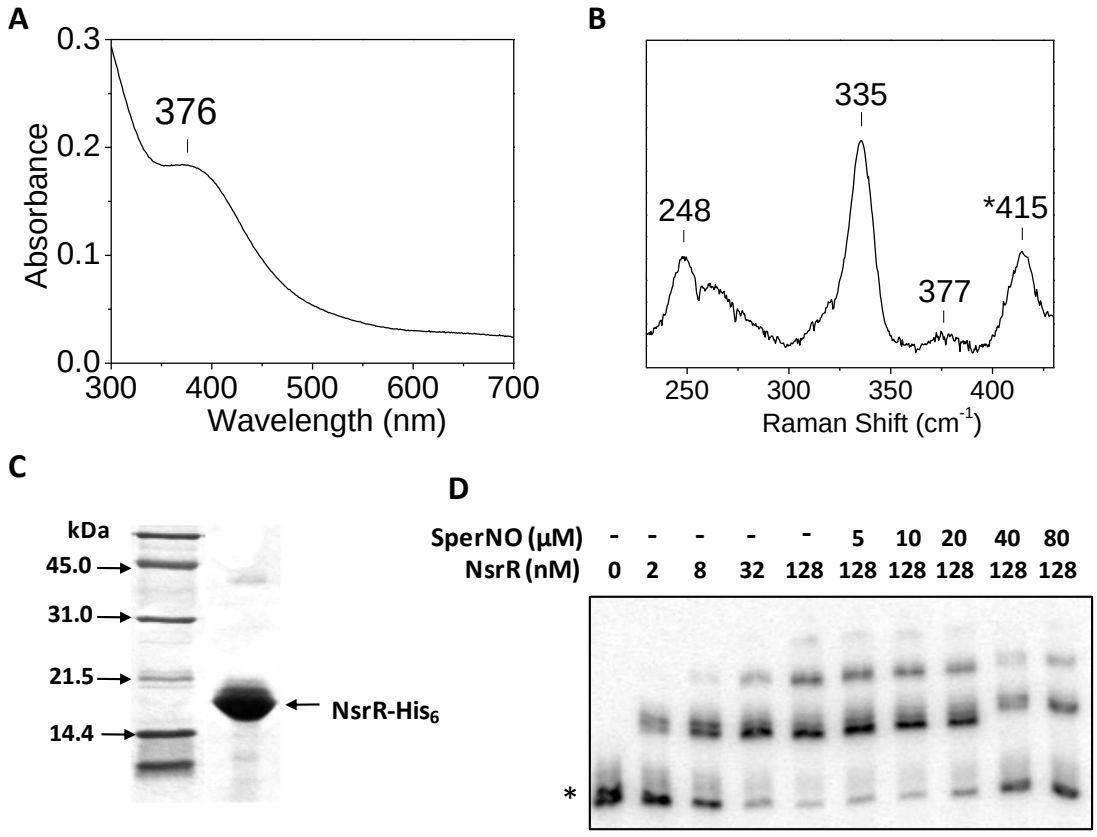

Fig. 2.14. Characterization of NsrR-His_6 isolated from *B. subtilis*.
A. UV-vis absorption spectrum in the presence of 5 mM DTT.
B. Resonance Raman spectrum. λ_{exc} = 488 nm, 70 mW. The peak arising from the presence of glycerol is marked with * .
C. Anaerobically purified NsrR-His_6 from aerobic *B. subtilis* cultures.
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.

ResD-independent *hmp* expression is repressed by NsrR in *B. subtilis* cells cultured aerobically and the repression is relieved by SperNO (Nakano *et al.* 2006).

Although NsrR-dependent repression of *nasD* is relieved by SperNO, NO-dependent upregulation of *nasD* transcription still requires ResD. We have previously shown that certain amino acid residues of the carboxy-terminal domain of the α subunit (αCTD) are required for ResD-dependent transcription of *fnr* (Geng *et al.* 2007) and *nasD* (H.G. and M.M.N. unpublished results). Transcriptional activators that require αCTD are known to bind upstream of the -35 element and recruit RNAP via interaction with αCTD (Busby *et al.* 1999). This recruitment model of gene activation could not explain how ResD~P plays a role in activation of *nasD*, as RNAP alone binds to *nasD* and stimulates binding of ResD~P to *nasD* (Figure 2.8A). We noticed that the *nasD* promoter contains 5′-TtTG-3′ sequence one base pair upstream of the -10 hexamer (5′-TGTGCT-3′). "Extended -10 promoters" carrying the 5′-TG-3′(and often 5′-TRTG-3′) motif have been found in *E. coli* (Mitchell *et al.* 2003) and are more common in *B. subtilis* (Helmann 1995; Voskuil *et al.* 1998). *E. coli* extended -10 promoters generally show weak matches to the consensus -35 element, whereas *B. subtilis* extended -10 promoters have highly conserved -35 sequences. Given that the *nasD* promoter contains a non-canonical -10 hexamer (TGTGCT) and lacks -35 hexamer, one could envisage that the interaction between ResD~P and αCTD assists in productive binding of RNAP to *nasD*.

We have shown here that the preformed *nasD*-RNAP-ResD~P complex is dissociated by NsrR (Figure 2.8B), partly, if not solely, by competing with RNAP for interaction with the high-affinity NsrR-binding site (-39 and -24) of *nasD* (Figure 2.9).

We observed that ternary complex formation is more strongly inhibited by the higher concentrations of NsrR that promotes secondary site binding (Figure 2.8B). The secondary sites were localized in two distinct regions, within -93 to -63 and -71 to -40, which overlap the previously identified ResD-binding region (around -91 to -46). Neither apo-NsrR nor [4Fe-4S]-NsrR bound the *nasD*(-114 to -85) fragment, indicating that apo-NsrR interacts with the secondary sites in a sequence-specific manner. Interestingly, anaerobically purified NsrR and aerobically purified NsrR bind with a similar affinity to these secondary sites, which is in sharp contrast to the primary NsrR-binding site where the [4Fe-4S] cluster is required for efficient binding.

There is a precedent for both holo- and apo-forms of Fe-S transcription regulator playing roles in gene regulation. *E. coli* IscR, a [2Fe-2S] cluster-carrying transcription regulator (Schwartz *et al.* 2001), and NsrR are similar in primary structure. IscR controls transcription of the *iscRSUA* and *sufABCDSE* operons that encode proteins that function in assembly of Fe-S clusters. Under oxidative stress and iron limiting conditions, apo-IscR activates *sufA* transcription (Yeo *et al.* 2006) and [2Fe-2S]-IscR-dependent repression of *iscR* is concomitantly relieved (Giel *et al.* 2006) in order to meet the cell's need for Fe-S reassembly. The two forms of IscR recognize sequences specific to promoters of each class (Giel *et al.* 2006; Nesbit *et al.* 2009). We have previously proposed a partial inverted repeat sequence ATRTATYTtAAATATAT as a putative *B. subtilis* NsrR-binding site (Nakano *et al.* 2006). Consistent with this notion, NsrR binds a 30-mer oligonucleotide that includes the putative binding site of 17 base pairs (Figure 2.3 and Figure 2.4). The EMSA results in Figure 2.12 (and see Figure 2.3 for sequence) indicated that apo-NsrR-binding elements within a 30 nucleotide region. We could not

find high sequence similarity between the sites recognized by apo-NsrR and the [4Fe-4S]-NsrR-binding element. The two regions recognized by apo-NsrR show some similarity (Figure 2.3), but further study is required to deduce the consensus binding sequence for apo-NsrR.

The observation that anaerobically and aerobically purified NsrR bound to the upstream sites with a similar affinity raises two alternative possibilities. One possibility is that apo- and [4Fe-4S]-NsrR indeed have a similar affinity to these sites. The other possibility is that apo-NsrR binds with a higher affinity than [4Fe-4S]-NsrR; however, a larger population of apo-NsrR present in anaerobically purified NsrR made it difficult to measure the binding affinity of [4Fe-4S]-NsrR in anaerobically purified protein sample. Although we could not eliminate the former possibility, we assume the latter possibility is more likely. The EMSA result with the probe (-114 to -40) lacking the high-affinity binding site indicated that the *nasD* complex formed by anaerobically purified NsrR did not promote the supershift usually observed in the presence SperNO (Figure 2.11F and compare with Figure 2.11C). The result suggests that NsrR molecules bound to the probe were almost exclusively apo-protein, in keeping with the preferential recognition of the two upstream binding sites by apo-NsrR. In the case of IscR, a recent study showed that, to the authors' surprise both apo- and [2Fe-2S]-IscR bind to *sufA*-class promoters in a sequence-specific manner (Nesbit *et al.* 2009).

To our knowledge, this is the first report suggesting that apo- and [Fe-S]-NsrR interact with two distinct classes of DNA targets that have different recognition sequences. Furthermore, instead of separate sets of genes, the two classes of NsrR-target sequences reside in the single *nasD* gene promoter region. It remains to be determined

what function these two NsrR-target sites perform in the regulation of *nasD* expression. Assuming that apo-NsrR concentration increases relative to [4Fe-4S]-NsrR under conditions that disrupt the Fe-S cluster formation, one could envisage apo-NsrR occupying the ResD-binding region. Simultaneously, RNAP might successfully compete with the -35 region to which apo-NsrR weakly binds. The outcome is that, instead of a *nasD*-RNAP-ResD complex, a *nasD*-RNAP-NsrR complex might be formed to initiate *nasD* transcription. This is a very speculative view but is worth examining in future. The results of this study also suggest that apo-NsrR might function independently of holo-NsrR in regulation of yet-unidentified genes.

CHAPTER 3

GLOBAL TRANSCRIPTIONAL CONTROL BY NSRR IN *BACILLUS SUBTILIS*[2]

3.1 INTRODUCTION

The NsrR transcriptional control plays a role in NO stress response as a repressor in gram-negative (Beaumont *et al.* 2004; Bang *et al.* 2006; Bodenmiller *et al.* 2006; Gilberthorpe *et al.* 2007; Rock *et al.* 2007; Wang *et al.* 2008; Isabella *et al.* 2009) and gram-positive (Nakano *et al.* 2006; Tucker *et al.* 2008) bacteria. Furthermore, recent studies showed that NsrR controls NO-induced expression of NO-resistant alternative oxidase (Aox) in *Vibrio fischeri*, thus supporting bacterial growth during NO stress (Dunn *et al.* 2010). The mechanism by which NsrR controls transcription of genes in response to NO has been investigated *in vitro*. NsrR from *N. gonorrhoeae* (Isabella *et al.* 2009) and *S. coelicolor* (Tucker *et al.* 2008) was shown to contain a [2Fe-2S] cluster when overexpressed in and purified aerobically from *E. coli*. The [2Fe-2S] cluster is necessary for NsrR to bind DNA and nitrosylation of Fe in the [2Fe-2S] cluster results in loss of DNA-binding activity. A similar result was observed in our previous study using *B. subtilis* NsrR purified anaerobically either from *E. coli* or *B. subtilis*, but unlike *N. gonorrhoeae* and *S. coelicolor* NsrR, *B. subtilis* NsrR contains a [4Fe-4S] cluster (Yukl *et al.* 2008).

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Kommineni S, Lama A, Popescu B, Nakano MM (2012) Global transcriptional control by NsrR in Bacillus subtilis. *J Bacteriol* 194**:** 1679-1688.

Detailed transcriptomic studies of the NsrR regulon were carried out in *E.coli* (Filenko *et al.* 2007), *N. gonorrhoeae* (Overton *et al.* 2006) and its close relatives *Neisseria meningitidis* (Heurlier *et al.* 2008). Flavohemoglobin Hmp is often specified by a gene controlled by NsrR among diverse bacteria (Rodionov *et al.* 2005). Hmp detoxifies NO by converting it to nitrate under aerobic conditions (Gardner *et al.* 1998; Hausladen *et al.* 1998). Other genes controlled by NsrR include those involved in NO metabolism and detoxification [summarized in (Tucker *et al.* 2010)] as predicted in a comparative genomics study (Rodionov *et al.* 2005). In *E. coli*, identification of significant overlap between NsrR regulon and the regulons of Fnr and nitrate-responsive regulator, NarL and NarP, revealed a possible complex control mechanism of gene expression in response to oxygen and various nitrogen compounds (Filenko *et al.* 2007). In Neisseria, apart from regulating NO damage repair system, NsrR is also involved in coordinating denitrification pathway to prevent toxic levels of NO in the cell (Rock *et al.* 2007; Heurlier *et al.* 2008). The AniA (membrane bound nitrite reductase) and the NorB (respiratory NO reductase) genes that are transcriptionally dependent on Fnr, are the two key components of denitrification pathway repressed by NsrR in *Neisseria* (Heurlier *et al.* 2008).

Until now, only *hmp* and the *nasDEF* operon encoding nitrite reductase were known as target genes of NsrR in *B. subtilis* (Nakano *et al.* 2006). Expression of both genes is induced upon oxygen limitation by the ResD-ResE two-component regulatory system (Nakano *et al.* 1995; LaCelle *et al.* 1996; Sun *et al.* 1996). In the previous study described in Chapter 2 (Kommineni *et al.* 2010), we found two different classes of NsrR-binding sites in the *nasD* promoter region. One site is located around the -35 region as

previously predicted (Nakano *et al.* 2006) and [4Fe-4S]-NsrR binds to this site with high affinity in an NO-sensitive manner [called class I site; (Kommineni *et al.* 2010)]. The other sites are located in two overlapping regions within -93 to -63 and -71 to -40 (with respect to the transcription start site), and binding of NsrR to these regions is weaker than that to the class I site and not enhanced by the [4Fe-4S] cluster coordination in NsrR, hence the binding is NO insensitive (class II sites). As the class I site in the *nasD* promoter has a major (if not the sole) role in NsrR regulation, we refer to *nasD* (and *hmp*) as members of the class I NsrR regulon.

In this study, we identify *cis*-acting sequences required for the two classes of NsrR binding. In addition, evidence is presented that genes carrying only class II sites (called the class II NsrR regulon) are abundant in *B. subtilis* and that expression of many genes under class II NsrR regulation is controlled by multiple transcription regulators including ResD.

3.2 MATERIALS AND METHODS

Bacterial strains and plasmids.

All *B. subtilis* strains used in this study are derivatives of *B. subtilis* JH642 and listed in Table 3.1. Plasmids and oligonucleotides are listed in Table 3.1 and Table 3.2, respectively. *E. coli* DH5α was used for transformation and plasmid propagation. *nsrR* mutant strains carrying various *lacZ* fusions were generated by transforming *lacZ*-bearing JH642 strains with chromosomal DNA prepared from ORB6179 (*nsrR*::*cat*) or *vice versa*. The *fur*::*kan* and *resD*::*spc* mutations were introduced into appropriate strains by transformation of chromosomal DNA prepared from HB2501 (Fuangthong *et al.* 2001)

Table 3.1. *B. subtilis* strains and plasmids

Strain list	Description	Reference or source
JH642	*trpC2 pheA1* (parental strain)	J. A. Hoch
HB2501	CU1065 *fur*::*kan*	(Fuangthong *et al.* 2003)
LAB2854	SPβc2del*2*::Tn*917*::pMMN392(*nasD-lacZ*)	(Nakano *et al.* 1998)
LAB2511	*resD*::*spc*	This study
MH5636	His_{10}-*rpoC*	(Qi *et al.* 1998)
ORB6179	*nsrR*::*cat*	(Nakano *et al.* 2006)
ORB6188	*nsrR*::*cat* SPβc2del*2*::Tn*917*::pMMN392 (*nasD-lacZ*)	(Nakano *et al.* 2006)
ORB6522	Spßc2del*2*::Tn*917*::pHG53	This study
ORB6544	*nsrR*::*cat* Spßc2del*2*::Tn*917*::pHG53	This study
ORB6864	*yvaW(sdpA)*::pMutin	This study
ORB6865	*nsrR*::*cat yvaW(sdpA)*::pMutin	This study
ORB 6910	SPßc2del*2*::*Tn917*::pMB4	This study
ORB 6911	*nsrR*::*cat* SPßc2del*2*::Tn*917*::pMB4	This study
ORB7838	pMMN810 (pHTo1-*nsrR*His_6)	This study
ORB7847	*thrC*::pMMN817 (*nsrR*)	This study
ORB7879	*thrC*::pALG22 (*tCys-nsrR*)	This study
ORB7886	*rok*::miniTn*10 yvaw*(*sdpA*)::pMutin	This study
ORB7887	*abrB*::*neo yvaw*(*sdpA*)::pMutin	This study
ORB7888	*rok*::miniTn*10 nsrR*::*cat yvaW*(*sdpA*)::pMutin	This study
ORB7889	*nsrR*::*cat abrb*::*neo yvaw*(*sdpA*)::pMutin	This study
ORB7898	*nsrR*::*cat yvaW*(*sdpA*)::pMutin *thrC*::pMMN817	This study
ORB7912	*yukE*::pMutin	This study
ORB7921	*nsrR*::*cat yukE*::pMutin	This study
ORB 7922	SPßC2del*2*::Tn*917*::*spoVG-lacZ*	This study
ORB7925	*nsrR*::*cat* SPßC2del*2*::Tn*917*::*spoVG-lacZ*	This study
ORB8008	*resD*::*spc yvaW(sdpA)*::*p*Mutin	This study
ORB8009	*nsrR*::*cat resD*::*spc yvaW(sdpA)*::pMutin	This study
ORB8012	SPßc2del*2*::Tn*917*::pSK17	This study
ORB8014	SPßc2del*2*::Tn*917*::pSK19	This study
ORB8015	*nsrR*::*cat* SPßc2del*2*::Tn*917*::pSK17	This study
ORB8017	*nsrR*::*cat* SPßc2del*2*::Tn*917*::pSK19	This study
ORB8018	SPßc2del*2*::Tn*917*::pSK13	This study

ORB8019	SPßc2del*2*::Tn*917*::pSK14	This study
ORB8021	*nsrR*::*cat* SPßc2del*2*::Tn*917*::pSK13	This study
ORB8022	*nsrR*::*cat* SPßc2del*2*::Tn*917*::pSK14	This study
ORB8026	*ykuN*::pMutin	This study
ORB8039	*nsrR*::*cat ykuN*::pMutin	This study
ORB8135	*nsrR*::*cat thrC*::pMMN817 *yukE*::pMutin	This study
ORB8152	*nsrR*::*cat resD*::*spc ykuN*::pMutin	This study
ORB8155	*resD*::*spc abrB*::*neo sdpA*::pMutin	This study
ORB8162	*nsrR*::*cat thrC*::pMMN817 *ykuN*::pMutin	This study
ORB8165	*resD*::*tet ykuN*::pMutin	This study
ORB8177	*nsrR*::*cat thrC*::pMMN817 SPßC2del*2*::Tn*917*::*spoVG-lacZ*	This study
ORB8187	*fur*::*kan ykuN*::pMutin	This study
ORB8189	*nsrR*::*cat fur*::*kan ykuN*::pMutin	This study
ORB8218	*fur*::*kan resD*::*tet ykuN*::pMutin	This study

Plasmid list

pDG1731	Vector for ectopic integration at the *thrC* locus in *B. subtilis*; Amp^R Spc^R	(Guerout-Fleury *et al.* 1996)
pHT01	Expression vector in *B. subtilis*	MoBiTec
pTK*lac*	ColE1 origin promoter-probe vector Amp^R Cm^R	(Tatti *et al.* 1991)
pMMN392	pTK*lac* with *nasD* promoter (-93 to +257)	(Nakano *et al.* 1998)
pMMN666	pDG795 with *nsrR* (C92A)	(Kommineni *et al.* 2010)
pALG22	pDG1731 with tCys-*nsrR* (C92A, C100A, C106A)	This study
pMMN817	pDG1731 with *nsrR*	This study
pMB4	pTK*lac* with *nasD*(T4 to A in class I)-*lacZ*	This study
pHG53	pTK*lac* with *nasD*(A5 T6 to CG in class I)-*lacZ*	This study
pSK13	pTK*lac* with *nasD*(T9 to Δ in class I)-*lacZ*	This study
pSK14	pTK*lac* with *nasD*(A12 T13 to CG in class I)-*lacZ*	This study
pSK17	pTK*lac* with *nasD*(A14 to T in class I)-*lacZ*	This study
pSK19	pTK*lac* with *nasD*(A5 to C in class I)-*lacZ*	This study

Table 3.2. Oligonucleotide primers used in this study

Oligonucleotide	Sequence (5' to 3')	Purpose
oMN97-15	CGGGATCCAATTGAATGTTGTT	pMMN392, (Nakano *et al.* 2000)
oMN09-489	GCATAACATGTATCTTAAATATTCCTTTCG	p*nasD* (class I -44 to -19), (Kommineni *et al.* 2010)
oMN09-491	CGAAAGGAATATTTAAGATACATGTTATGC	p*nasD* (class I -44 to -19) (Kommineni *et al.* 2010)
oMN09-493	GCATAACATGTATCTTAACGATTCCTTTCG	Class I A5 T6 to CG
oMN09-494	CGAAAGGAATATTTAAGCGACATGTTATGC	Class I A5 T6 to CG
oMN10-539	AAGAATCTCGCTGTTATTTCCCCGGTTGCCGGCTTAAA	pALG22
oMN10-540	TTTAAGCCGGCAACCGGGGAAATAACAGCGAGATTCTT	pALG22
oHG79	ATAACATGTCGCTTAAATA	pHG53
oHG80	TATTTAAGCGACATGTTAT	pHG53
oHG82	ATGCACCATTCCTTGCG	pMMN392
oHG93	CGGAATTCGCACTTGCTTTC	pALG22
oHG94	GAGGATCCGCTTTTGACCTT	pALG22
oMB07-3	CATTTTATAACATTTCTCTTAAATATTCC	pMB4
oMB07-4	GAATATTTAAGATTCATGTTATAAAATG	pMB5
oSK42	CCGTCCGAATCATACCTATT	p*nasD* (-114 to -4) (Kommineni *et al.* 2010)
oSK43	AGCGTAGCACAGCAAAAAGG	p*nasD* (-114 to -4) (Kommineni *et al.* 2010)
oSK44	GCATAACCTGTATCTTAAATATTCCTTTCG	Class I A1 to C
oSK45	CGAAAGGAATATTTAAGATACAGGTTATGC	Class I A1 to C
oSK46	GCATAACAGGTATCTTAAATATTCCTTTCG	Class I T2 to G
oSK47	CGAAAGGAATATTTAAGATACCTGTTATGC	Class I T2 to G
oSK48	GCATAACATTTATCTTAAATATTCCTTTCG	Class I G3 to T
oSK49	CGAAAGGAATATTTAAGATAAATGTTATGC	Class I G3 to T
oSK50	GCATAACATGAATCTTAAATATTCCTTTCG	Class I T4 to A
oSK51	CGAAAGGAATATTTAAGATTCATGTTATGC	Class I T4 to A
oSK52	GCATAACATGTCTCTTAAATATTCCTTTCG	Class I A5 to C
oSK53	CGAAAGGAATATTTAAGAGACATGTTATGC	Class I A5 to C
oSK54	GCATAACATGTAGCTTAAATATTCCTTTCG	Class I T6 to G
oSK55	CGAAAGGAATATTTAAGCTACATGTTATGC	Class I T6 to G

oSK56	GCATAACATGTATTTTAAATATTCCTTTCG	Class I C7 to T
oSK57	CGAAAGGAATATTTAAAATACATGTTATGC	Class I C7 to T
oSK58	GCATAACATGTATCGTAAATATTCCTTTCG	Class I T8 to G
oSK59	CGAAAGGAATATTTACGATACATGTTATGC	Class I T8 to G
oSK60	GCATAACATGTATCTGAAATATTCCTTTCG	Class I T9 to G
oSK61	CGAAAGGAATATTTCAGATACATGTTATGC	Class I T9 to G
oSK62	GCATAACATGTATCTTCAATATTCCTTTCG	Class I A10 to C
oSK63	CGAAAGGAATATTGAAGATACATGTTATGC	Class I A10 to C
oSK64	GCATAACATGTATCTTACATATTCCTTTCG	Class I A11 to C
oSK65	CGAAAGGAATATGTAAGATACATGTTATGC	Class I A11 to C
oSK66	GCATAACATGTATCTTAACTATTCCTTTCG	Class I A12 to C
oSK67	CGAAAGGAATAGTTAAGATACATGTTATGC	Class I A12 to C
oSK68	GCATAACATGTATCTTAAAGATTCCTTTCG	Class I T13 to G
oSK69	CGAAAGGAATCTTTAAGATACATGTTATGC	Class I T13 to G
oSK70	GCATAACATGTATCTTAAATTTTCCTTTCG	Class I A14 to T
oSK71	CGAAAGGAAAATTTAAGATACATGTTATGC	Class I A14 to T
oSK72	GCATAACATGTATCTTAAATAGTCCTTTCG	Class I T15 to G
oSK73	CGAAAGGACTATTTAAGATACATGTTATGC	Class I T15 to G
oSK74	GCATAACATGTATCTAAATATTCCTTTCG	Class I T to Δ
oSK75	CGAAAGGAATATTTAGATACATGTTATGC	Class I T to Δ
oSK76	GCATAACATGTATCTTAACGATTCCTTTCG	Class I A12 T13 to CG
oSK77	CGAAAGGAATCGTTAAGATACATGTTATGC	Class I A12 T13 to CG
oSK95	GCATAACATGACGCTTAAATATTCCTTTCG	Class I T4 A5 T6 to ACG
oSK96	CGAAAGGAATATTTAAGCGTCATGTTATGC	Class I T4 A5 T6 to ACG
oSK97	GCATAACATGTATCTTAACGTTTCCTTTCG	Class I A12 T13 A14 to CGT
oSK98	CGAAAGGAAACGTTAAGATACATGTTATGC	Class I A12 T13 A14 to CGT
oSK99	GCATAACATGTATCTTCCCTATTCCTTTCG	Class I A10 A11 A12 to CCC
oSK100	CGAAAGGAATAGGGAAGATACATGTTATGC	Class I A10 A11 A12 to CCC
oSK101	GCATAACATGTCGCTTAACGATTCCTTTCG	Class I A5 T6 to CG, A12 T13 to CG
oSK102	CGAAAGGAATCGTTAAGCGACATGTTATGC	Class I A5 T6 to CG, A12 T13 to CG
oSK103	GCATAACATGTATCGTCAATATTCCTTTCG	Class I T8 A10 to GC
oSK104	CGAAAGGAATATTGACGATACATGTTATGC	Class I T8 A10 to GC
oSK111	GCGTTTTTCATAAAATTTTTAGAACTTTTCG	Class II (-90 to -63)
oSK112	CGAAAAGTTCTAAAAATTTTATGAAAAACGC	Class II (-90 to -63)
oSK123	ATAACATGTATCTAAATATTCCTTTTTGC	pSK13
oSK124	GCAAAAAGGAATATTTAGATACATGTTAT	pSK13

oSK125	ACATGTATCTTAACGATTCCTTTTTGCTG	pSK14
oSK126	CAGCAAAAAGGAATCGTTAAGATACATGT	pSK14
oSK127	ACATGTATCTTAAATTTTCCTTTTTGCTG	pSK17
oSK128	CAGCAAAAAGGAAAATTTAAGATACATGT	pSK17
oSK129	CATTTTATAACATGTCTCTTAAATATTCC	pSK19
oSK130	GGAATATTTAAGAGACATGTTATAAAATG	pSK19
oSK135	AGTTCCCAAATTCAATTCTG	P*sdpA*(-1219 to +21)
oSK136	TAATAGGAAACATATAGTCATTACAG	P*sdpA*(-1219 to +21)
oSK137	GCGTTTTTAATAAAATTTTTAGAACTTTTCG	Class II C6 to A
oSK138	CGAAAAGTTCTAAAAATTTTATTAAAAACGC	Class II C6 to A
oSK139	GCGTTTTTCAGAAAATTTTTAGAACTTTTCG	Class II T8 to G
oSK140	CGAAAAGTTCTAAAAATTTTCTGAAAAACGC	Class II T8 to G
oSK141	GCGTTTTTCATAAAAGGTTTAGAACTTTTCG	Class II T13 T14 to GG
oSK142	CGAAAAGTTCTAAACCTTTTATGAAAAACGC	Class II T13 T14 to GG
oSK143	GCGTTTTTCATAAAATTTTTAAAACTTTTCG	Class II G19 to T
oSK144	CGAAAAGTTTTAAAAATTTTATGAAAAACGC	Class II G19 to T
oSK145	GCGTTTTTCATAAAATTTTTAGAAATTTTCG	Class II C22 to A
oSK146	CGAAAATTTCTAAAAATTTTATGAAAAACGC	Class II C22 to A
oSK147	GCGTTTTTCATAACATTTTTAGAACTTTTCG	Class II A11 to C
oSK148	CGAAAAGTTCTAAAAATGTTATGAAAAACGC	Class II A11 to C
oSK167	GCGTTTTTCATAAAATTTTAGAACTTTTCG	Class II T13 to Δ
oSK168	CGAAAAGTTCTAAAATTTTATGAAAAACGC	Class II T13 to Δ
oSK183	TGTACGGCGGCGTGACCAAC	C*nasD* (+1699 to +1877)
oSK184	CCGTACGCGTAGCCCGATGG	C*nasD* (+1699 to +1877)
oSK196	GTACAAGTGATAAAGGATATGCTGG	P*ykuN* (-156 to +30)
oSK197	GCTGGCATATGTAATCAAGGCTT	P*ykuN* (-156 to +30)

and LAB2511, respectively. LAB2511 was constructed from LAB2506 (Nakano *et al.* 1997) by replacing a chloramphenicol-resistance gene in *resD* with a spectinomycin-resistance gene using plasmid pJL62 (LeDeaux *et al.* 1995). Dr. Naotake Ogasawara and Dr. Kazuo Kobayashi at Nara Institute of Science and Technology (Japan), provided us with the pMutin libraries. Antibiotics were used at the following concentrations: Ampicillin, 50 μg/ml; chloramphenicol, 5 μg/ml; spectinomycin, 75 μg/ml; erythromycin/lincomycin, 1/25 μg/ml; neomycin, 5 μg/ml; kanamycin, 5 μg/ml.

Complementation analysis with the wild-type and a triple-Cys codon mutant allele of nsrR.

The mutant *nsrR* (tCys-*nsrR*) carrying alanine substitutions of three conserved cysteines was expressed from the native promoter at the *thrC* locus. For this purpose, pALG22 plasmid was constructed (by Dr. Amrita Lama) using an integration vector pDG1731 (Guerout-Fleury *et al.* 1996). To introduce C100A and C106A codon substitutions, mutagenic complementary oligonucleotides oMN10-539 and oMN10-540 were used in two-step PCR amplification using pMMN666 carrying the *nsrR* C92A mutation (Yukl *et al.* 2008; Kommineni *et al.* 2010) as a template. First, two PCR products were generated using oligonucleotide pair oHG93/oMN10-540 and oMN10-539/oHG94. The PCR products were annealed and used as a template in the second PCR with oHG93 and oHG94 (Kommineni *et al.* 2010). The resultant PCR product was digested with EcoRI/BamHI and cloned into pDG1731 digested with the same enzymes, resulting in pALG22. The *nsrR* gene in pALG22 was verified by sequencing. To introduce tCys-*nsrR* at the *thrC* locus, pALG22 was used to transform JH642 with

selection for spectinomycin resistance. Thr$^-$ colonies that were generated by a double-crossover recombination were chosen as ORB7879. To express the wild-type *nsrR* at the *thrC* locus, pMMN817 (by Dr. Michiko M. Nakano) was constructed by subcloning the *nsrR* fragment from pMMN668 (Kommineni *et al.* 2010) to pDG1731. pMMN817 was used to transform JH642 in a similar way as pALG22, resulting in ORB7847. Chromosomal DNA prepared from ORB7847 and ORB7879 was used to transform the *nsrR* mutant (*nsrR*::*cat*) carrying various *lacZ* fusions to introduce the wild-type and mutant *nsrR* at the *thrC* locus.

Construction of nasD promoter mutations.

All plasmids carrying mutant *nasD* promoters are derivatives of pMMN392 (Nakano *et al.* 1998; Nakano *et al.* 2000), which carries *lacZ* fused to the *nasD* promoter (-93 to +257). Base substitution and deletion mutations of the *nasD* promoter were constructed by two-step PCR using mutagenic complementary oligonucleotides. Each oligonucleotide pair was used for the first PCR together with either an upstream oligonucleotide oHG82 or a downstream oligonucleotide oMN97-15 (Nakano *et al.* 2000) and pMMN392 as a template. The annealed PCR products were used as a template for the second PCR with oHG82 and oMN97-15. The resultant PCR products were digested with EcoRI and BamHI and cloned into pTK*lac* (Kenney *et al.* 1991)digested with the same enzymes. Plasmids carrying the mutant *nasD* promoters (Table 3.1) were introduced into the SPβ prophage site of the wild-type and *nsrR* mutant strains as previously described (Nakano *et al.* 1998). Dr. Hao Geng and Mohamed Elbaz constructed plasmids pHG53 and pMB4, respectively.

Measurement of β-galactosidase activities.

B. subtilis cells were grown anaerobically in 2xYT supplemented with either 1% glucose and 0.2% nitrate, 0.5% glucose and 0.5% pyruvate, or 1% pyruvate, and harvested at 1 h intervals. For NO treatment, 100 mM stock solution of spermine NONOate (Cayman Chemical) (SperNO) was prepared by dissolving in 10 mM NaOH. One μl of SperNO was added to 2 ml cultures when optical density at 600 nm (OD_{600}) reached 0.3-0.4 and the same volume of 10mM NaOH was added for untreated cultures. Cells were harvested after 1 h and β-galactosidase activity was measured as described previously (Nakano *et al.* 1988).

Purification of proteins.

ResD and ResE were overexpressed and purified from *E. coli* ER2566 (New England Biolabs) as previously described (AEvarsson *et al.* 1994; Geng *et al.* 2007; Kommineni *et al.* 2010). RNA polymerase (RNAP) was purified from *B. subtilis* MH5636 (Qi *et al.* 1998) as described (Nakano *et al.* 2006). Purification of NsrR-His_6 from *B. subtilis* strain ORB7838 was performed aerobically (for apo-NsrR) or anaerobically (for [4Fe-4S]-NsrR or holo-NsrR) as described in a previous study (Kommineni *et al.* 2010). Iron content of each NsrR preparation was determined using ferene assay (Yukl *et al.* 2008; Kommineni *et al.* 2010). Protein concentrations were determined by the Bradford assay using BSA (Sigma-Aldrich) as a standard.

Electrophoretic mobility shift assay (EMSA) of NsrR.

EMSA reactions and gel electrophoresis were carried out under anaerobic or aerobic conditions in the reaction buffer [50 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.5 mM DTT, 5 mM $MgCl_2$, 5 μg/ml BSA, 10 μg/ml poly(dI-dC), 10% glycerol] and native gels were run in TGE buffer (50 mM Tris-HCl, 0.38 M glycine, 2 mM EDTA) (Kommineni *et al.* 2010). A short *nasD* probe carrying the class I NsrR-binding site (-44 to -19) was generated by annealing complementary 30-mer oligonucleotides oMN09-489 and oMN09-491 (for the wild-type promoter) and oligonucleotides carrying each mutation (for the mutant promoters) as previously described (Kommineni *et al.* 2010). A short *nasD* probe carrying one (-90 to -63) of the class II NsrR-binding sites was generated similarly by annealing oligonucleotides oSK111 and oSK112. Oligonucleotides corresponding to the coding strand were pre-labeled at their 5' ends with T4 polynucleotide kinase and [γ-^{32}P]-ATP before annealing with complementary oligonucleotides. To determine sites of NsrR interactions with a longer *nasD* promoter (-114 to -4) region, a *nasD* EMSA probe was generated by PCR with labeled oSK42 and unlabeled oSK43 oligonucleotides as previously described (Kommineni *et al.* 2010). A similar procedure was used to generate probes for *sdpA* (-219 to +21 relative to the translational start site) and *ykuN* (-156 to +30 relative to the translational start site) promoters and a segment of the *nasD* coding region (+1699 to +1877 relative to the translational start site) as a negative control.

To examine the effect of NO on NsrR binding to class I and class II sites, SperNO dissolved in 10 mM NaOH was added to the reaction 10 min after the addition of holo-NsrR in an anaerobic chamber, and incubated at room temperature for additional 20 min.

The same volume of 10 mM NaOH was added to the control reaction (Kommineni *et al.* 2010).

DNA microarray experiments.

The microarray experiments were carried out by Dr. Michiko M. Nakano in Dr. Alan Grossman's laboratory at MIT. RNA was prepared from JH642 and ORB6179 (*nsrR*::*cat*) cells cultured under the following three conditions. Cells were grown anaerobically in 2xYT supplemented with either 1% glucose and 0.2% nitrate (nitrate respiration) or 0.5% glucose and 0.5% pyruvate (fermentation conditions). Around early mid-exponential growth (OD_{600}=0.25), one set of the cultures with glucose and pyruvate were treated with 100 μM SperNO. After incubation for 1 h, cells were mixed with a half volume of cold methanol and harvested by centrifugation. RNA was isolated from three independent cultures and extracted using a Qiagen RNeasy kit according to the manufacturer's instructions.

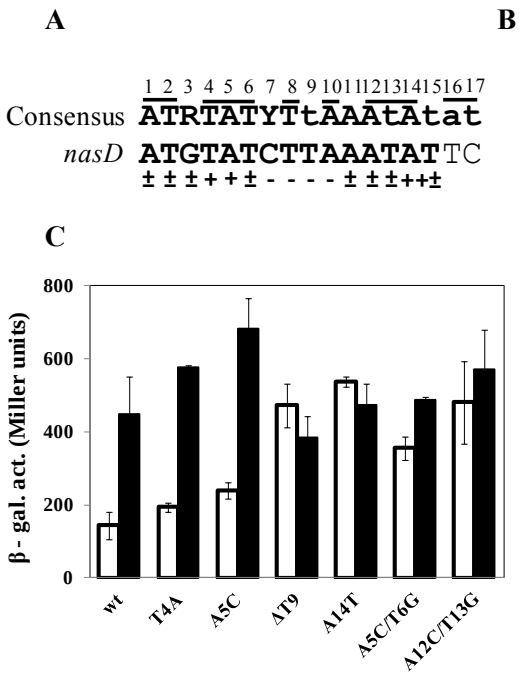
Labeling, hybridization, and data processing were carried out as previously described (Britton *et al.* 2002). Dr. Catherine Lee assisted in analyzing the transcriptome data. The expression ratio of the *nsrR* mutant to the wild-type strain from three independent experiments (i.e. independently cultured and prepared samples) were determined and averaged. The complete microarray data set is available at the NIH Gene Expression Omnibus (GEO) database under record number GSE33289.

3.3 RESULTS

3.3.1 NsrR recognizes different *cis*-acting sequences for class I and class II binding.

Our previous study suggested that NsrR binds two classes of DNA sites in the *nasD* promoter (Kommineni *et al.* 2010). EMSA showed that the class I site in *nasD* binds to [4Fe-4S]-NsrR with a higher affinity than the class II sites. Furthermore, binding of [4Fe-4S]-NsrR to the class I site is NO sensitive, whereas binding to the class II sites is NO insensitive (Kommineni *et al.* 2010). In order to determine whether the difference in binding characteristics is attributable to sequences of *cis*-acting sites targeted by NsrR, we carried out base substitution analysis of NsrR-binding sites in *nasD* and examined the effect of the mutations on NsrR binding by EMSA.

We previously proposed a 17-bp partial dyad symmetry ATRTATYTtAAAtAtat (underlined bases compose the symmetry) element as the consensus class I NsrR-binding sequence by an alignment of the *nasD* and *hmp* promoters [Figure 3.1A and (Nakano *et al.* 2006)] and by EMSA (Kommineni *et al.* 2010). The wild-type and mutant probes containing the class I site were generated by double-stranded oligonucleotides (-44 to -19) with and without base substitutions as described in Materials and Methods. Each probe was incubated in an anaerobic chamber with different concentrations of holo-NsrR (the Fe-S-cluster incorporation was around 65%) purified anaerobically from *B. subtilis*. Figure 3.1B shows percentages of each probe bound to NsrR at given concentrations (some of the EMSA gels are listed in Figure 3.2). Most severely affected single base substitutions are T4, A5 (category 3), and A14 (category 4) that reside at symmetrical positions TAT-N5-ATA (at positions 4 to 14). T4 and A5 in the left-half site are equally important for binding, but only a substitution of A14 in the right-half site showed a severe effect on binding, with an even more drastic effect than the T4 or A5 substitution. Substitutions of T6, A12, and T13, the residues constituting the other symmetrical



B

Probe	Percentage of probe bound by NsrR 2nM	8nM	32nM	128nM
Category 1				
WT	48 ± 15	68 ± 7	81 ± 5	92 ± 3
C7T	33 ± 10	46 ± 4	79 ± 7	94 ± 1
T8G	33 ± 9	56 ± 7	74 ± 5	88 ± 6
T9G	37 ± 8	53 ± 7	77 ± 1	86 ± 3
A10C	47 ± 9	67 ± 7	82 ± 3	89 ± 8
T8G/A10C	28 ± 9	59 ± 6	80 ± 5	91 ± 4
Category 2				
A1C	22 ± 5	36 ± 7	54 ± 5	65 ± 3
T2G	12 ± 2	41 ± 6	66 ± 5	73 ± 2
G3T	24 ± 4	32 ± 9	49 ± 1	61 ± 5
T6G	20 ± 8	45 ± 6	58 ± 8	65 ± 9
A11C	25 ± 6	48 ± 8	67 ± 9	78 ± 6
A12C	12 ± 10	24 ± 3	53 ± 6	78 ± 2
T13G	18 ± 3	26 ± 5	51 ± 6	76 ± 2
T15G	21 ± 9	43 ± 3	55 ± 1	69 ± 7
Category 3				
T4A	3 ± 3	6 ± 2	25 ± 2	62 ± 8
A5C	3 ± 2	10 ± 3	27 ± 8	45 ± 9
A5C/T6G	1 ± 1	5 ± 2	20 ± 13	44 ± 4
A12C/T13G	2 ± 1	6 ± 4	26 ± 11	52 ± 7
A12C/T13G/A14T	5 ± 8	9 ± 5	20 ± 7	46 ± 18
A10C/A11C/A12C	5 ± 2	12 ± 1	28 ± 2	47 ± 8
Category 4				
A14T	0 ± 2	5 ± 3	15 ± 4	25 ± 2
ΔT9	0 ± 0	0 ± 0	0 ± 0	0 ± 0
T4A/A5C/T6G	0 ± 0	2 ± 2	9 ± 3	17 ± 2
A5C/T6G/A12C/T13G	0 ± 0	1 ± 2	12 ± 6	22 ± 5

Fig. 3.1. *In vitro* and *in vivo* effects of mutations in the class I NsrR-binding site in the *nasD* promoter. (A) Alignment of a proposed consensus NsrR-binding site (Nakano *et al.* 2006) and the class I site (-39 to -23 relative to the transcription start site) in the *nasD* promoter. Underlined nucleotides involve in the formation of a partial dyad symmetry sequence. Abbreviations: R, A or G; Y, C or T. (B) Effect of the mutations on NsrR binding was determined by EMSA in the presence of holo-NsrR and ImageJ software was used to quantify the percentage of distinct shifted band to total (the shifted and the probe) band and the average value from three independent experiments is shown with standard deviation. Nucleotide numbers correspond to those marked in Figure 3.1A. Mutations were classified into four categories due to the effect on binding. Nucleotides in category 1, 2, 3, and 4 are marked with -, ±, +, and ++, respectively in Figure 3.1A. (C) Effect of selected *cis*-acting mutations on *nasD-lacZ* expression. The wild-type (open bars) and *nsrR* (closed bars) strains carrying *lacZ* fused to the wild-type and mutant *nasD* promoters were grown anaerobically in 2×YT with 0.5% glucose and 0.5% pyruvate. Samples were collected at 1 h intervals to measure β-galactosidase activity and the average activity at T1 (1 h after the end of exponential growth) from three independent experiments is shown with standard deviation.

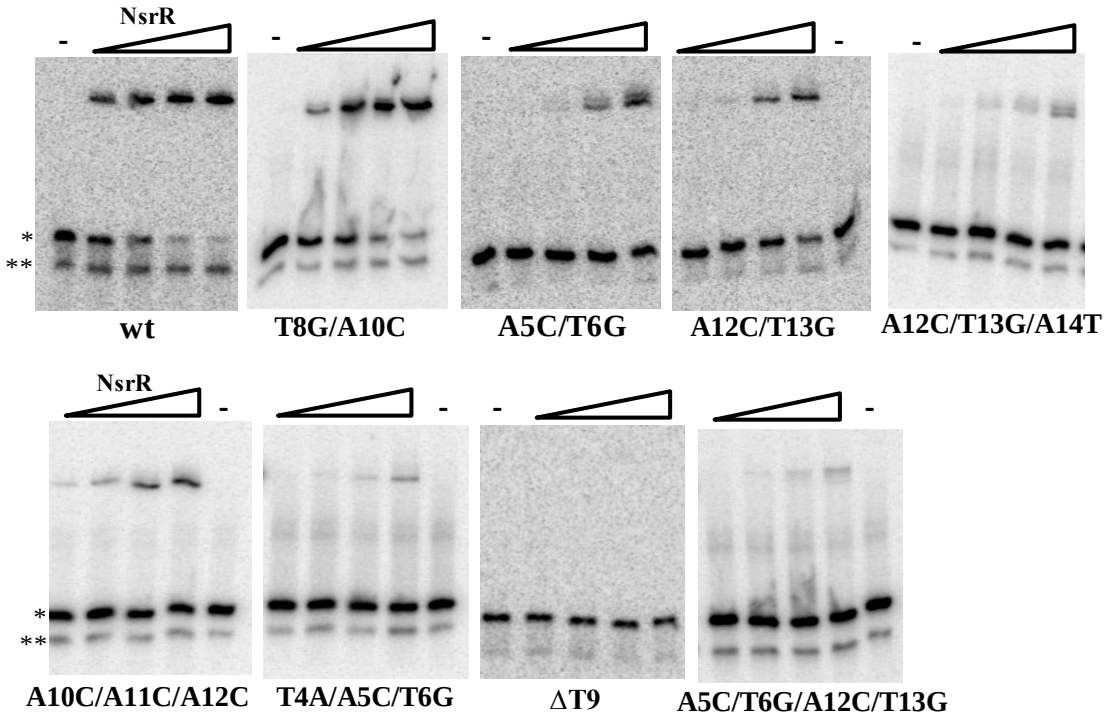


Fig. 3.2. Effect of nucleotide substitutions in the class I site on NsrR binding. EMSA gels of *nasD* carrying selected class I site mutations shown in Figure 3.1. The nucleotide positions are marked in Figure 3.1A. The radiolabeled wild-type and mutant *nasD* probes were incubated with increasing concentrations (0, 2, 8, 32, and 128 nM) of holo-NsrR. Single asterisks show double-stranded DNA and double asterisks show single-stranded unannealed oligonucleotides.

positions of TAT-N5-ATA, showed only moderate effects (category 2). Mutations in bases at positions 7 to 10 did not show any significant effect on binding (category 1). Although this result indicated that sequence specificity in a central part of the NsrR-binding site does not play a critical role, a deletion of T9 at the center of the dyad symmetry almost completely eliminated NsrR-binding activity (category 4). Furthermore, multiple substitutions showed that the T8G/A10C double mutation has no significant effect and that triple substitutions of TAT at positions 3-5 have more drastic effects than substitutions of ATA at positions 12-14, which is consistent with the results of single substitutions.

We next determined whether certain mutations that affect NsrR-binding *in vitro* also affect *in vivo* NsrR-dependent transcriptional regulation (Figure 3.1C). NsrR represses transcription of *lacZ* driven by the wild-type *nasD* promoter during anaerobic growth with glucose-pyruvate (Nakano *et al.* 2006; Kommineni *et al.* 2010). The transcription of *lacZ* from the *nasD* promoters carrying the T4A and A5C mutations in category 3 was similar to that from the wild-type promoter (Figure 3.1C). In contrast, NsrR repression was largely relieved by the A5C/T6G and A12C/T13G double mutations in category 3 and by the A14T and ΔT9 mutations in category 4. To assess why the A5C and A5C/T6G mutations that resulted in a similar reduction in NsrR binding displayed a different effect on NsrR-mediated repression *in vivo*, we examined whether NsrR inhibits RNAP-ResD complex formation with the mutant *nasD* promoters (Figure 3.3). Our previous study demonstrated that NsrR represses *nasD* transcription by displacing the transcription initiation complex (Kommineni *et al.* 2010). Binding of RNAP and ResD to

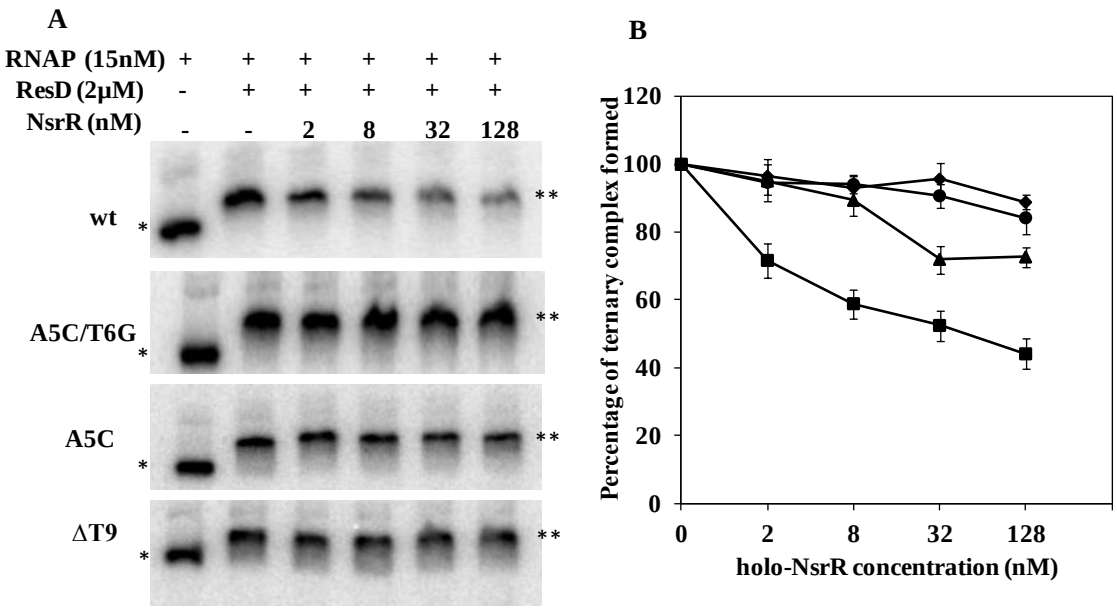


Fig. 3.3. Inhibition of the *nasD*-RNAP-ResD ternary complex formation by holo-NsrR. (A) The wild-type and mutant promoters of *nasD* (-93 to +4) were used as probes for EMSA with RNAP and ResD phosphorylated by ResE in the absence and presence of NsrR. The figures are shown only in regions where *nasD*-RNAP complex (shown by a single asterisk) and *nasD*-ResD-RNAP complex ran. (B) ImageJ software was used to quantify the percentage of the *nasD*-ResD-RNAP complex in the presence of holo-NsrR relative to the complex in the absence of NsrR. The average value from three independent experiments is shown with standard deviation. The wild-type promoter (square), A5C (triangle), A5C/T6G (circle) and ΔT9 (diamond).

the *nasD* promoter was not significantly affected by either the A5C, A5C/T6G, or ΔT9 mutation. Holo-NsrR displaced the wild-type *nasD*-RNAP-ResD complex but was not able to displace the ternary complex with the *nasD* promoter carrying the ΔT9 or A5C/T6G mutation. NsrR exhibited the effect on the ternary complex with *nasD* (A5C) albeit to a lesser extent compared to the wild-type *nasD* (Figure 3.3), which partly explains the effect of the mutations on *nasD* expression (Figure 3.1C).

The *in vitro* and *in vivo* results of mutational analysis indicated that the partial dyad symmetry is required for efficient [4Fe-4S]-NsrR binding to the class I site as previously predicted (Rodionov *et al.* 2005; Nakano *et al.* 2006). A sequence similar to the partial dyad symmetry is not detected in two class II NsrR-binding sites in the *nasD* promoter. Instead, we found that nucleotide sequences of the binding sites are A+T-rich and contain thrice-repeated T homopolymers [Figure 3.4 and (Kommineni *et al.* 2010)] and that holo-NsrR and apo-NsrR bind to the class II sites with the same affinity (Kommineni *et al.* 2010). Mutational analyses of a *nasD* fragment (-90 to -63) carrying one of the class II binding sites revealed that among single nucleotide substitutions tested so far, only C22A (marked in Figure 3.4) exhibits a slight but significant decrease in apo-NsrR binding. Taken together, these results demonstrated that class II binding of NsrR involves relaxed DNA sequence specificity.

3.3.2 Transcriptome analysis unveiled a global NsrR class II regulation.

It is difficult to characterize how the class II sites play roles in NsrR control of *nasD* transcription because the *nasD* promoter also contains the high-affinity class I

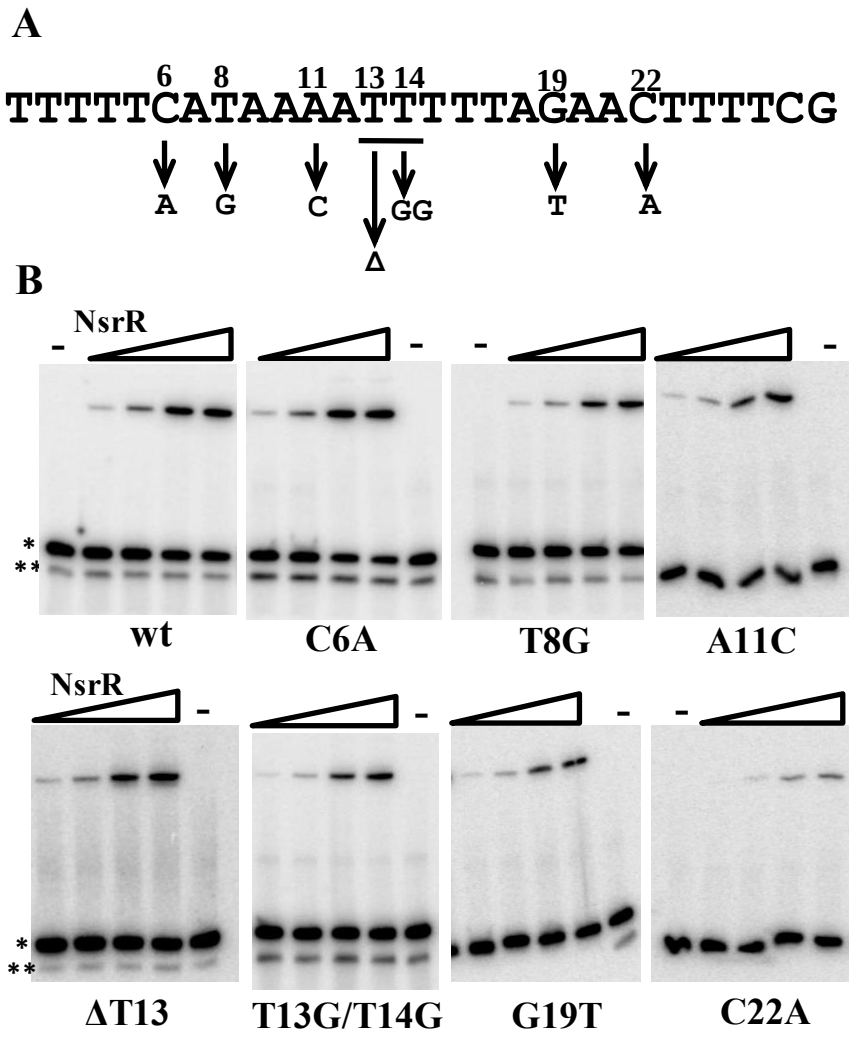


Fig. 3.4. Effect of mutations in a class II NsrR-binding site in the *nasD* promoter. (A) Single nucleotide substitutions and a deletion were made in one (-90 to -63 relative to the transcription start site) of the class II binding sites. (B) Effect of the mutations on NsrR binding was examined by EMSA in the presence of increasing concentrations (32, 128, 512, and 1024 nM) of apo-NsrR. Single asterisks show double-stranded oligonucleotide probes and double asterisks show single-stranded unannealed oligonucleotides.

NsrR-binding site. As A+T-rich sequences, a characteristic of class II sites, are abundant in the *B. subtilis* genome, we assumed that significant numbers of genes could be under the control of class II NsrR regulation. As NsrR binds to the class II *cis*-acting sites in *nasD* with a low affinity in an NO-insensitive manner (Kommineni *et al.* 2010), we thought that class II genes, which contain only class II NsrR-binding site(s), could be found among those moderately repressed by NsrR and/or those repressed even when cells encounter NO stress. A transcriptome experiment was conducted in which RNA was isolated from the wild-type and *nsrR* mutant strains cultured under anaerobic conditions in 2xYT medium supplemented with (1) glucose and nitrate (nitrate respiration), (2) glucose and pyruvate (fermentation), and (3) glucose and pyruvate followed by the treatment with 100 μM SperNO for 1 h. The first and third culture conditions provide endogenous and exogenous NO, respectively.

The results in Table 3.3 showed that transcription of known class I NsrR regulon genes, *nasDEF* and *hmp*, was highly repressed by NsrR (14-fold and 121-fold, respectively) in cells grown in glucose-pyruvate media, but the repression was not observed in the other two media in which NO is generated, confirming NO-sensitive repression. The *ykjA* gene downstream of *hmp* was also regulated in a similar way but to a lesser extent (13-fold repression) than *hmp*, which is consistent with a previous finding that *ykjA* (unknown function) is transcribed from the *hmp* promoter despite the presence of a possible factor-independent transcription terminator between *hmp* and *ykjA* (LaCelle *et al.* 1996). The data also revealed that Fur (ferric uptake repressor) regulon genes show a pattern similar to NO-sensitive NsrR repression, but less pronounced. Fur is a repressor for genes involved in siderophore biosynthesis and uptake under iron-replete conditions,

Table 3.3. Genes regulated by NsrR

Gene[d]	Gene function	Ratio of Gene expression (*nsrR*/wt)[c]		
		Nitrate	Pyruvate	NO
Class I				
hmp(*ykjA*)	Flavohemoglobin	0.8	121.0	1.0
nasD(*EF*)	nitrite reductase	1.0	14.0	0.9
Class II				
Cell surface and Extracellular function				
sdpA(*BC*)[a,b]	sporulation delaying protein	1.4	2.6	1.5
yukE(***D***/*C/B/A*)***yueB***(***C***/*D*)	SPP1 phage receptor, ESAT-6	4.4	1.2	1.6
ylqB[b]	extracellular protein	1.2	1.8	1.4
yqxI(*J*)[b]	extracellular protein, skin element	1.7	2.1	1.7
yybM(*L/K,J*)[a]	similar to ABC transporter	1.2	1.4	1.2
yydF(*G/**H**/I/J*)[a,b]	antimicrobial peptide production and processing	1.2	1.8	1.7
sboA[a,b]	subtilosin A precursor	1.4	1.8	1.9
yvcA(*B*)[b]	membrane protein	1.0	1.8	0.8
yoqM[b]	SPβ prophage	2.0	1.5	1.7
sunT(*bdbA*/*yolJ*/*bdbB*)[a,b]	sublancin 168 production and processing	1.3	1.7	1.4
Fur regulon				
dhbA(*CEBF*)[b]	Biosynthesis of siderophore bacillibactin	0.8	4.3	0.9
besA[b]	trilactone hydrolase	0.9	4.0	0.9
ykuP(***ON***)	Flavodoxin	0.7	3.2	0.8
yclN(*OPQ*)	petrobactin ABC transporter	0.9	2.0	0.9
feuA(*BC*)	ABC transporter for siderophores	0.6	2.5	0.6
fhuD(*G*)	hydroxamate siderophore ABC transporter	1.0	3.8	0.9
fbpB	RNA chaperone	0.9	3.5	1.2
yusV	ABC transporter for siderophores	1.0	2.8	0.9
yxeB	hydroxamate siderophore ABC transporter	1.0	2.9	1.0
yfmC	iron/citrate ABC transporter	1.0	2.6	0.9
yfhC	Putative NAD(P)H nitroreductase	1.0	2.3	0.9
yfiY(*Z*)	ABC transporter for siderophores	0.9	2.2	0.9
hmoA	heme monooxygenase	1.5	4.1	1.2
Others				
sspB[b]	small acid-soluble spore protein	2.9	3.0	3.6
spoVG[b]	spore cortex synthesis	1.7	1.6	1.8
ymfJ[b]	Unknown	1.1	1.7	1.7

[a] Indicates genes repressed by Rok

[b] Indicates genes repressed by AbrB

[c] Shown are genes repressed by NsrR in at least one of the culture conditions tested. Fold changes ≥1.4 or <1.4 are shown.

[d] Genes in parenthesis are those that appear to be regulated in a similar way as the first gene of the operon. NsrR repression of genes in bold were confirmed by assays using transcriptional *lacZ* fusions,

thus controlling iron homeostasis (Baichoo *et al.* 2002). Thirteen Fur-controlled genes (and operons) were repressed more than 2-fold by NsrR only during fermentative growth (except *hmoA* that was repressed under all conditions), meeting the criterion for the class I regulon in terms of NO sensitivity; however, promoter regions of Fur regulon genes do not show any similarity to the consensus class I NsrR-binding sequence, and hence these genes likely belong to the class II NsrR regulon.

In order to identify genes under class II NsrR control, we searched for genes repressed by NsrR regardless of the medium conditions. We found that over 150 genes belong to this category using 1.4-fold change as a cut-off. The cut-off number of 1.4-fold was provisionally chosen because NsrR control was later confirmed in genes that show similar levels of change in the transcriptome experiment. Genes relevant to this study are listed in Table 3.3. Among these genes, we found 24 late sporulation genes and operons including those coding for small acid soluble proteins and spore coat proteins. The *sspB* gene is only listed in Table 3.3 as an example. Expression of these sporulation genes is under a complex control mediated by multiple alternative sigma factors and transcriptional regulators (Errington 2003). Although late sporulation genes were upregulated in the absence of NsrR, we have not observed sporulation of the *nsrR* mutant under the same conditions; hence, the physiological significance (if any) of the upregulation of late sporulation genes remains mysterious. We also found that genes repressed by AbrB (23 genes) or by both AbrB and Rok (14 genes) are among possible class II NsrR-controlled genes.

Using transcriptional *lacZ* fusions, we validated the microarray data (validated genes are marked in Table 3.3) and Figure 3.5 shows the effect of *nsrR* on transcription

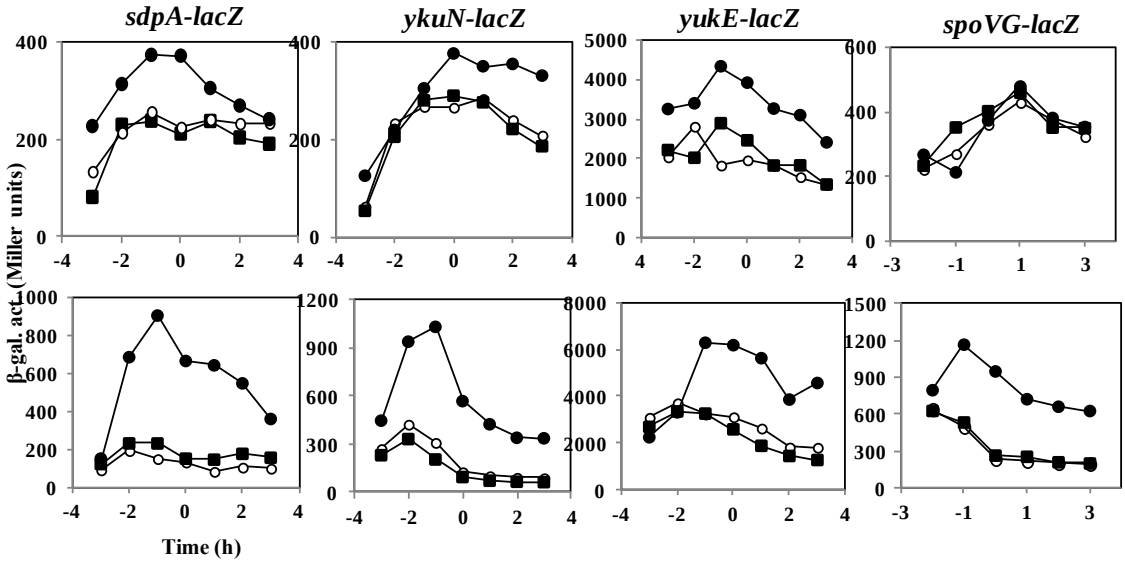


Fig. 3.5. Effect of the *nsrR* mutation on anaerobic expression of *sdpA*, *ykuN*, *yukE*, and *spoVG*. *B. subtilis* strains were grown anaerobically in 2×YT supplemented with 1% glucose and 0.2% nitrate (top row) or with 0.5% glucose and 0.5% pyruvate (bottom row). Open circle, wild type; closed circle, the *nsrR* mutant; closed square, the *nsrR* mutant carrying *nsrR* at *thrC*. Time zero is the end of the exponential growth. The figures are representatives of at least three independent experiments using different isolates of the indicated strains.

of *sdpA* (responsible for delaying sporulation) (Ellermeier *et al.* 2006), *ykuN* (flavodoxin gene) (Baichoo *et al.* 2002; Wang *et al.* 2007), *yukE* (encoding an ESAT-6-family protein) (Burts *et al.* 2008), and *spoVG* (σ^H-dependent gene). The *nsrR* knockout mutation had no effect on *spoVG* and a minor effect (around 50% to two-fold) on transcription of other genes in glucose-nitrate medium. In contrast, derepression of these genes, particularly *sdpA* and *ykuN*, was more substantial in glucose-pyruvate medium (Figure 3.5). If NsrR is the sole regulator of class II gene transcription and class II NsrR repression is as sensitive to NO as class I repression, we would expect that NO generated by nitrate respiration inactivates NsrR repressor activity, leading to complete derepression in the wild-type strain. As expression of these genes in the wild-type strain during nitrate respiration was lower than those in the *nsrR* mutant during fermentative growth, the NsrR repressor activity for class II genes might be less sensitive to NO (at least NO levels produced internally during nitrate respiration) than class I genes. Alternatively, a regulator(s) other than NsrR might also repress transcription during nitrate respiration, which is consistent with the notion that the increase in transcription by the *nsrR* mutation is lower in the nitrate medium than the pyruvate medium. Another possibility is that complete derepression by the *nsrR* mutation might require another factor, which is active only during fermentation. These possibilities are not mutually exclusive. Transcription of these genes in the *nsrR* mutant was fully complemented by ectopic expression of *nsrR* from its own promoter at the *thrC* locus (Figure 3.5).

3.3.3 NsrR directly interacts with promoter regions of class II genes in an NO-insensitive manner.

The results described above indicated that NsrR directly or indirectly exerts transcriptional repression of class II genes. We performed EMSA to examine whether NsrR directly interacts with the promoter regions of *sdpA* and *ykuN* (Figure 3.6A). We used [4Fe-4S]-NsrR (65% Fe-S cluster incorporation) and apo-NsrR purified from *B. subtilis*. Holo-NsrR bound to a longer *nasD* promoter DNA carrying the class I and two class II sites with much higher affinity (K_d between 0.25 to 0.5 nM, data not shown) than apo-NsrR (K_d around 32 nM, Figure 3.6A). We also observed that *nasD*, when it formed complexes with holo-NsrR, showed mobilities faster than those with apo-NsrR as previously detected (Kommineni *et al.* 2010). Binding to the *sdpA* and *ykuN* promoters was observed with an approximate K_d between 32nM to 128 nM and binding was only weakly enhanced by the presence of the [4Fe-4S] cluster in NsrR. We consider that NsrR interaction with the class II genes is likely specific, as a less A+T-rich DNA fragment from the *nasD* coding region displayed extremely weak binding (Figure 3.6A). Second, the excess amounts of cold *sdpA* promoter DNA competed with the radioactive *nasD* promoter probe for NsrR binding, although the competition was not as efficient as the cold *nasD* promoter (Figure 3.6B). In contrast, 256-fold excess concentration of the cold *nasD*-coding DNA was not able to compete with the radioactive probe. Lastly, AbrB and Rok, *in vivo* binding of which to *sdpA* was confirmed (Smits *et al.* 2010; Chumsakul *et al.* 2011), bound to the *sdpA* promoter *in vitro* with K_d similar to that of NsrR (data not shown). NO affected complex formation between holo-NsrR and *nasD* as previously shown [Figure 3.7 and (Kommineni *et al.* 2010)]. Upon exposure to 40 µM NO, holo-NsrR-*nasD* complexes converted either to a free probe or new complexes with mobilities slower than those with holo-NsrR. As discussed before (Kommineni *et al.* 2010), the

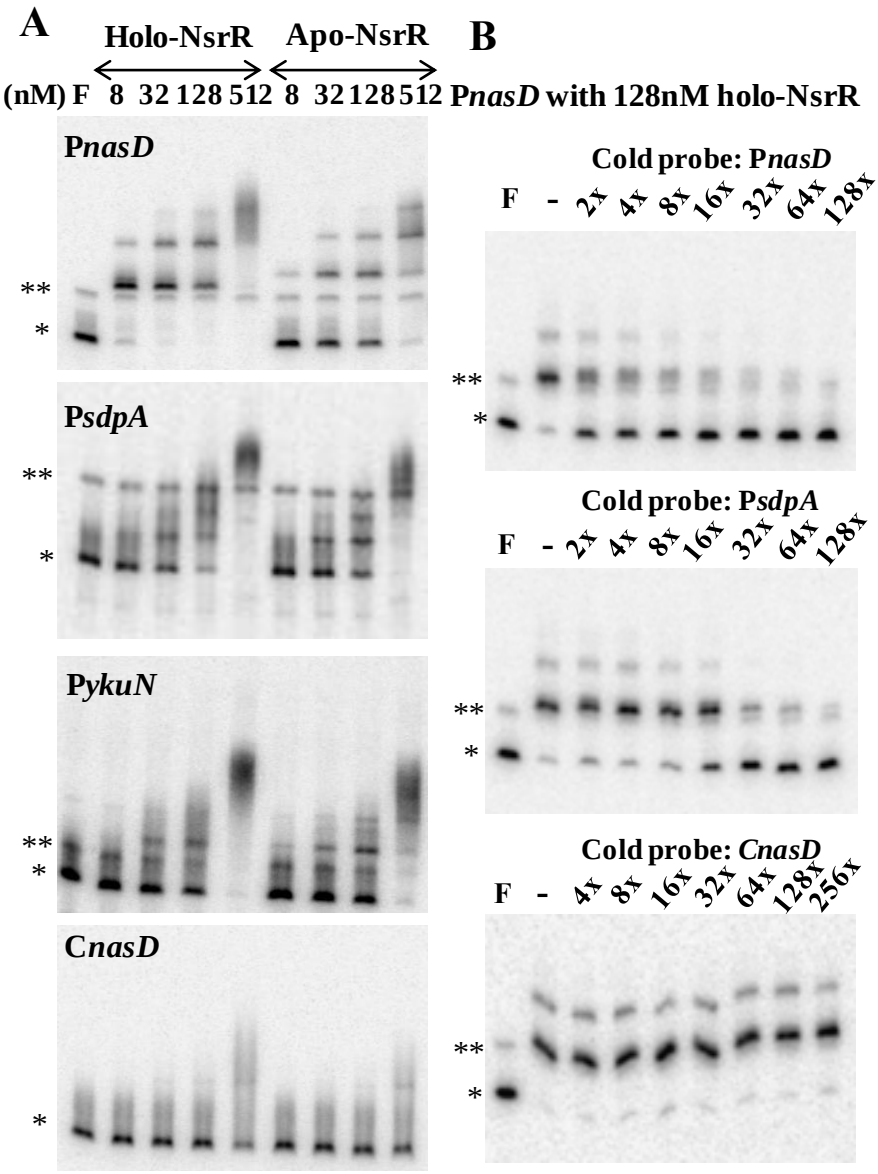


Fig. 3.6. Binding assay of NsrR to the *nasD* promoter and class II promoters. (A) Radiolabeled probes were incubated with increasing concentrations of NsrR purified under anaerobic (holo-NsrR) and aerobic (apo-NsrR) conditions. P*nasD*, the *nasD* promoter that contains the class I and class II binding sites; P*sdpA*, the *sdpA* promoter; P*ykuN*, the *ykuN* promoter; C*nasD*, a fragment in the *nasD*-coding region. (B) Competition assay of cold probes with radiolabeled P*nasD*. 128 nM of holo-NsrR and the radiolabeled P*nasD* probe were used with or without excess cold P*nasD*, P*sdpA* and C*nasD* DNA. Single asterisks represent free probes and double asterisks represent unidentified DNA fragments generated during PCR.

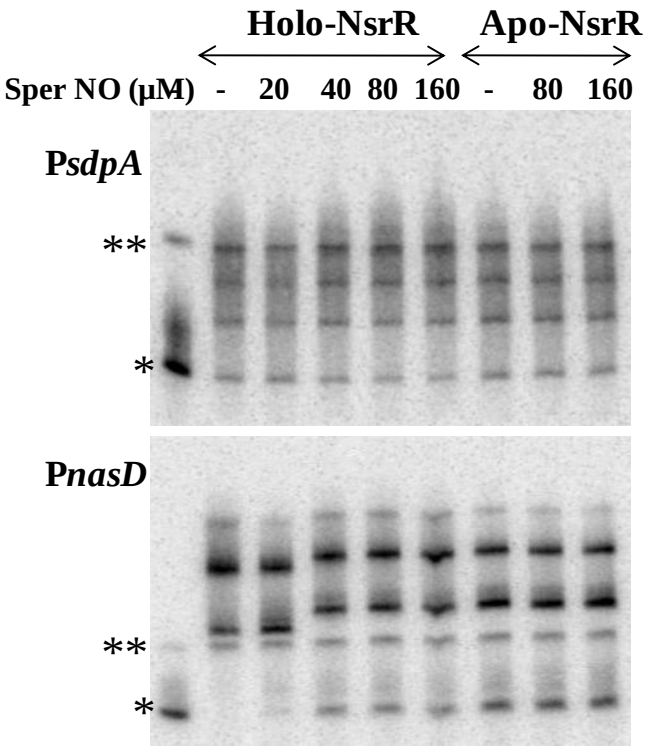


Fig. 3.7. Effect of spermine NONOate on DNA-binding activity of NsrR to class I and class II promoters. The radiolabeled *nasD* promoter (-114 to +4 relative to the transcription start site) or *sdpA* promoter (-219 to +21 relative to the start codon) DNA was incubated with 128nM of holo-NsrR or apo-NsrR. Increasing concentrations of SperNO were added to the reactions as described in Materials and Methods. Single asterisks represent free probes and double asterisks represent unidentified DNA fragments generated during PCR.

slow-mobility complexes are likely formed with apo-NsrR. In contrast, the NsrR complex with *sdpA* or *ykuN* was insensitive to NO concentrations at least up to 160 μM (Figure 3.7 and data not shown).

3.3.4 NsrR participates in transcriptional control of genes repressed by both AbrB and Rok.

Previous studies showed that genes involved in cell surface and extracellular functions such as *sdpA*, *sboA* (for subtilosin A) (Zheng *et al.* 2000), and *yydH* (for an ABC transporter) are repressed by both AbrB and Rok (Table 3.3) (Albano *et al.* 2005; Strauch *et al.* 2007). AbrB is a transition-state regulator involved in controlling postexponential processes in *B. subtilis* (Trowsdale *et al.* 1979; Strauch *et al.* 1989) and Rok was originally identified as a negative regulator of *comK* that encodes a transcription factor required for competence development (Albano *et al.* 2005). Eight genes (or operons) were shown to interact with both AbrB (Chumsakul *et al.* 2011) and Rok (Smits *et al.* 2010) *in vivo*. Based on the microarray result, among the eight genes (or operons) only *rok* and *comK* were not repressed by NsrR. *comK* is not expressed in cells grown in 2xYT rich medium from which RNA was isolated for the transcriptome analysis. Although it was shown that AbrB represses *sboA* transcription both under aerobic and anaerobic conditions (Nakano *et al.* 2000; Zheng *et al.* 2000), the repression of *sdpA* and *yydH* transcription by AbrB and Rok has been reported only under aerobic conditions. Therefore, we examined whether *abrB* and *rok* null mutations affect anaerobic expression of *sdpA* (Figure 3.8). In glucose-nitrate medium, the *nsrR* mutation resulted in only ~2-fold increase in *sdpA* transcription, whereas the *abrB* mutation caused a dramatic increase

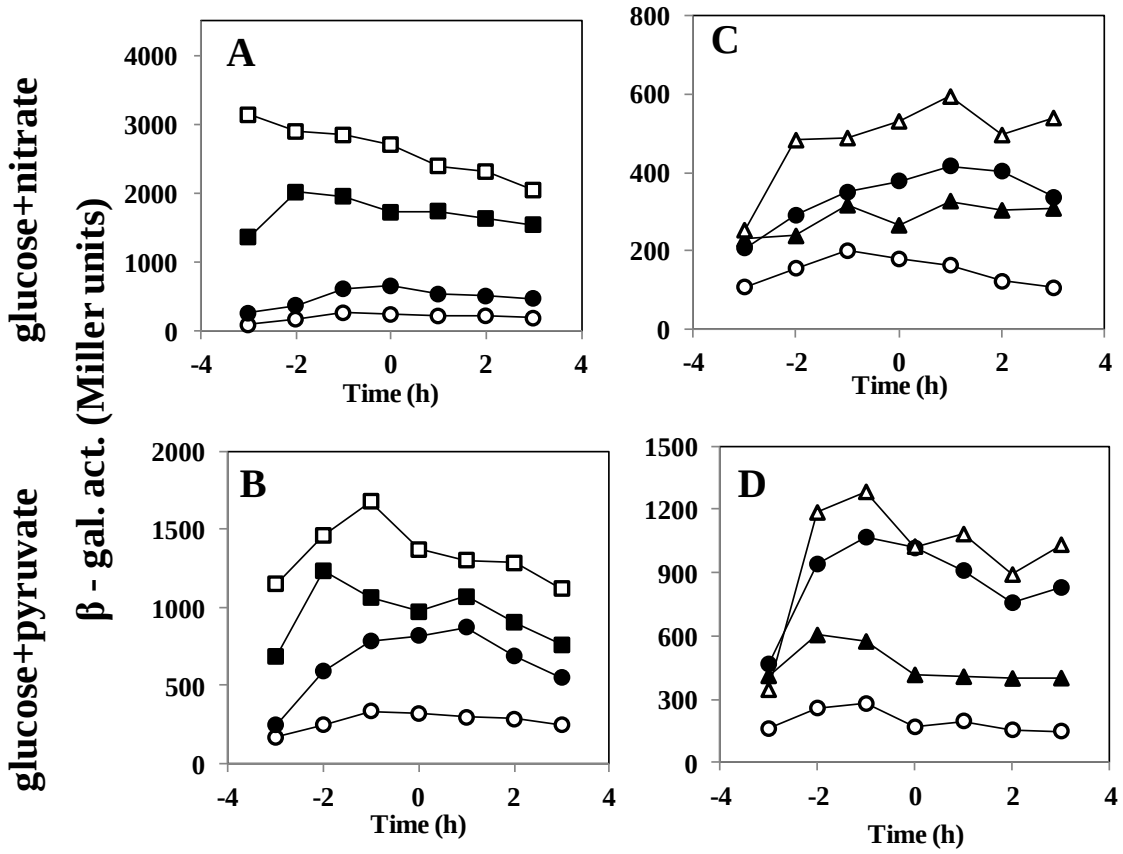


Fig. 3.8. Effect of the *nsrR*, *abrB* and *rok* mutation on anaerobic expression of *sdpA-lacZ*. *B. subtilis* strains were grown anaerobically in 2xYT supplemented with 1% glucose and 0.2% nitrate (top row) or with 0.5% glucose and 0.5% pyruvate (bottom row). (A and B) open circle, wild type; closed circle, *nsrR*; closed square, *abrB;* open square, *nsrR abrB*. (C and D) Open circle, wild type; closed circle, *nsrR*; closed triangle, *rok*; open triangle, *nsrR rok*. Time zero represents the end of exponential growth phase. The figures are representatives of at least three independent experiments using different isolates of the indicated strains.

in *sdpA* transcription. In glucose-pyruvate medium, derepression by *abrB* and *nsrR* was comparable, indicating that AbrB is a major repressor for *sdpA* transcription during nitrate respiration, whereas both AbrB and NsrR are equally involved in the repression during fermentative growth. The effect of *abrB* and *nsrR* mutations was additive under either growth condition. Repression of *sdpA* by Rok under anaerobic conditions was relatively low and *sdpA* expression was derepressed only ~2-fold in the *rok* mutant compared to the wild-type cells. The effect of AbrB and NsrR on *yydH* transcription was similar to that on *sdpA* but the *abrB* mutation had a more drastic effect than the *nsrR* mutation even in glucose-pyruvate medium (data not shown). These results indicate that NsrR, AbrB and, to a lesser extent, Rok repress transcription of these genes and that NsrR repression has a stronger effect on anaerobic regulation when *B. subtilis* is grown under fermentative conditions than during nitrate respiration.

3.3.5 *In vivo* effect of NO on transcription of Fur-controlled genes is mediated by NsrR and Fur under anaerobic conditions.

This study indicated that anaerobic expression of Fur-controlled genes is repressed by NsrR during fermentative growth. As shown in Figure 3.5, transcription of *ykuN* and other Fur regulon genes (data not shown) was high in exponential growth in 2xYT medium supplemented with 0.5% glucose and 0.5% pyruvate, and the level of transcription sharply decreased thereafter. *ykuN-lacZ* in the medium supplemented with 1% pyruvate remained high even in the stationary-phase growth (Figure 3.9A). In contrast, addition of 1% glucose to 2xYT did not increase a basal level of *ykuN* expression observed in unsupplemented 2xYT (data not shown). Therefore, we used

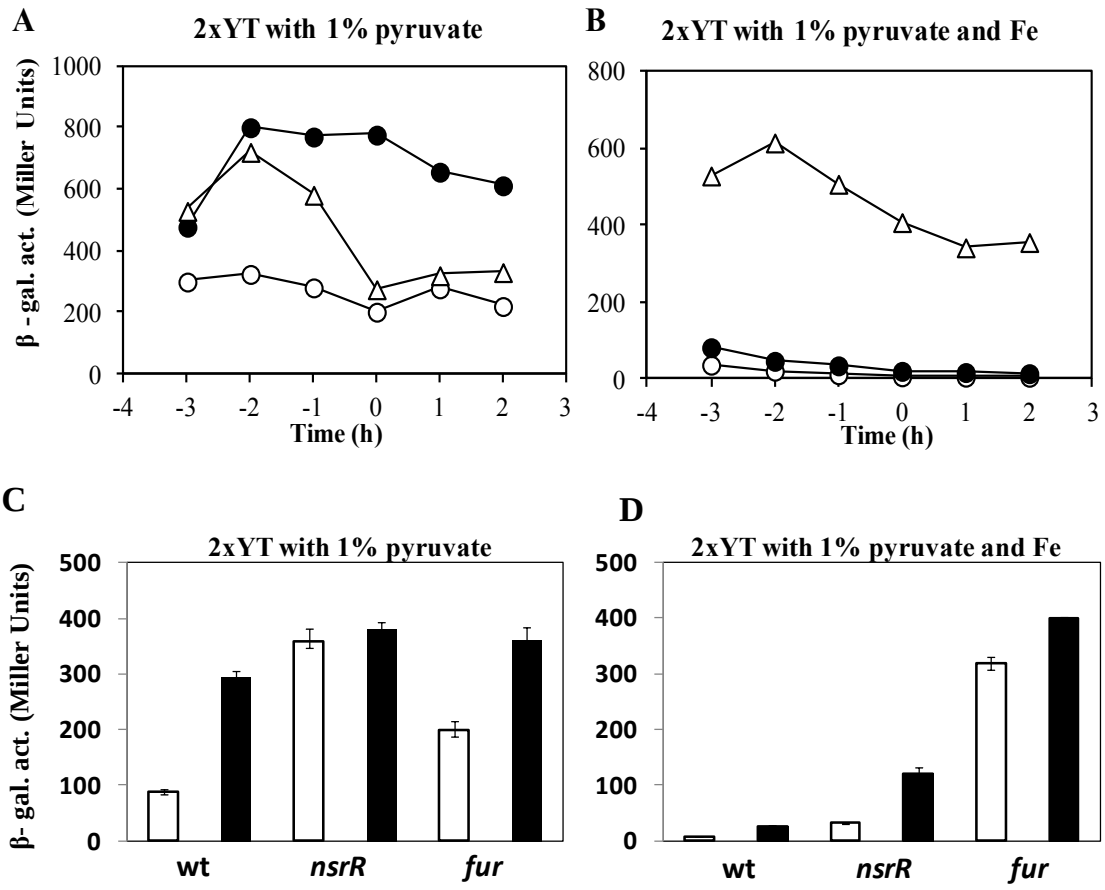


Fig. 3.9. Effect of the *nsrR* and *fur* mutations on *ykuN* expression. Wild type (open circle), the *nsrR* mutant (closed circle), and the *fur* mutant (open triangle) carrying *ykuN-lacZ* were grown anaerobically in 2xYT supplemented with 1% pyruvate (A) or 2xYT supplemented with 1% pyruvate and 0.004% $FeCl_3$/citrate (B). Time zero is the end of exponential growth. The figures are representatives of at least three independent experiments using different isolates of the indicated strains. The wild- type and mutant strains carrying *ykuN-lacZ* were grown in 2xYT supplemented with 1% pyruvate (C) or supplemented with 1% pyruvate and 0.004% $FeCl_3$/citrate (D). When OD_{600} of cultures reached around 0.3-0.4, spermine NONOate dissolved in 10 mM NaOH was added at the final concentration of 50 μM (filled column). The same volume of 10 mM NaOH was added to control cultures (open column). After incubation of 1 h, samples were harvested for measurement of β-galactosidase activities. Values were shown as the average of three samples with standard deviation.

2xYT supplemented with 1% pyruvate to examine the effect of the *nsrR* and *fur* mutations on expression of *ykuN-lacZ*. The *nsrR* mutation led to 4-fold increase in *ykuN* expression and the activity remained high in stationary phase (Figure 3.9A). The *fur* mutant also showed a similar level of derepression in exponential growth phase but the expression dropped to the wild-type level after T_0 (the end of the exponential growth). In the same medium supplemented with 0.004% $FeCl_3$/citrate, *ykuN* expression was strongly repressed in the presence of excess iron and the *nsrR* and *fur* mutation led to 3-fold and around 50-fold derepression, respectively (Figure 3.9B). These results showed that both NsrR and Fur function in repression of *ykuN* transcription under iron-limited conditions, whereas Fur plays a major role in repression in the presence of excess iron.

Previous study showed that Fur-repressed genes are induced by NO, suggesting that direct nitrosylation of the Fe(II) center in Fur inhibits its DNA-binding activity (Moore *et al.* 2004), the mechanism of which was uncovered in a study of *E. coli* Fur repressor (D'Autreaux *et al.* 2002). Therefore, we examined whether NO induces transcription of the Fur regulon and which repressor, NsrR or Fur, responds to NO stress. Cells at OD_{600}=0.3-0.4 were treated with 50 μM SperNO for 1 h as described in Materials and Methods. Expression of *ykuN* was derepressed 2.4-fold by the *nsrR* mutation and 1.3-fold by the *fur* mutation in the iron-depleted medium (Figure 3.9C). *ykuN-lacZ* was induced 2-fold by NO in the wild-type strain and around 1.8-fold in the *fur* mutant. On the contrary, further induction of *ykuN* transcription by NO was not detected in the *nsrR* mutant. In the iron-replete medium, transcription was derepressed 4-fold by the *nsrR* mutation and 43-fold by the *fur* mutation (Figure 3.9D). Exposure to NO increased the

expression 3.5-fold in the wild type, 3.7-fold in the *nsrR* mutant, and 1.2-fold in the *fur* mutant. These results demonstrated that anaerobic upregulation of *ykuN* and likely other Fur-repressed genes in response to NO is caused by inactivation of the NsrR or Fur repressor depending on iron concentrations. Expression of other class II genes such as *sdpA* and *spoVG* was moderately induced by NO in an NsrR-dependent manner (data not shown). These results indicated that NsrR represses transcription of these genes in an NO-sensitive manner, although NsrR binding to class II genes *in vitro* is NO insensitive.

3.3.6 ResD is required for transcriptional derepression in the *nsrR* mutant during fermentation.

As described above, the NsrR class II-binding sites in *nasD* overlap with the ResD-binding region (Kommineni *et al.* 2010). If NsrR directly interacts with class II genes to regulate transcription, *cis*-acting sites for NsrR in other class II genes may also be targets of ResD control. Therefore, we examined whether ResD controls transcription of class II genes. The *resD* mutation completely abolished the derepression of *sdpA* observed when NsrR is absent (Figure 3.10A). As AbrB represses transcription in the *nsrR* mutant (Figure 3.8B), the reduced expression in the *nsrR resD* mutant was likely mediated by AbrB (Figure 3.10A). Taken together, this result indicated that ResD antagonizes AbrB repression in the absence of NsrR. In contrast, the elevated transcription in the *abrB* mutant was not dependent on ResD (Figure 3.10B), suggesting that ResD was unable to antagonize NsrR repression. A similar antirepression of ResD against Fur was detected, as elevated expression of *ykuN-lacZ* resulting from the *nsrR* mutation was eliminated by the *resD* mutation (Figure 3.10C). The effect of *resD* is

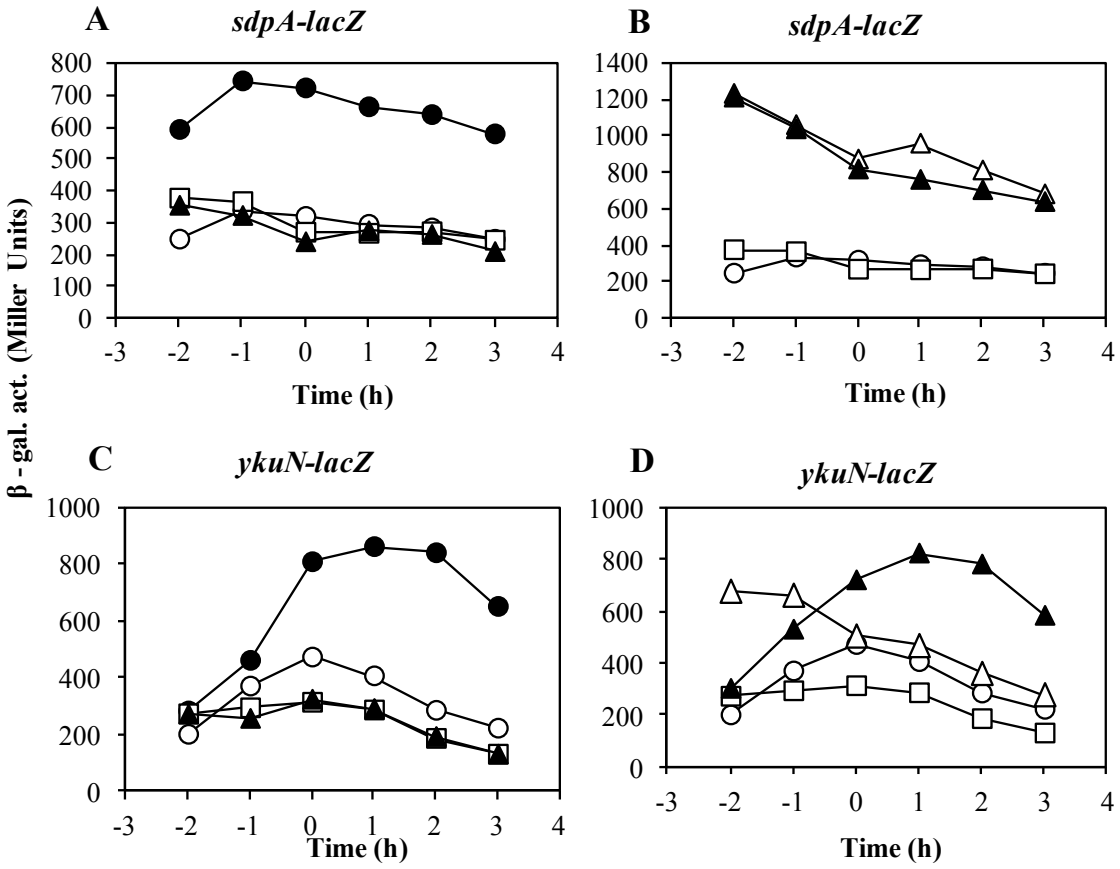


Fig. 3.10. ResD is required for upregulation of *sdpA* and *ykuN* caused by the *nsrR* mutation. *B. subtilis* strains carrying *sdpA-lacZ* were grown anaerobically in 2×YT supplemented with 0.5% glucose and 0.5% pyruvate (A and B). Symbols: (A) Wild type (open circle), the *nsrR* mutant (closed circle), the *resD* mutant (open square), and the *nsrR resD* mutant (closed triangle); (B) Wild type (open circle), the *abrB* mutant (open triangle), the *resD* mutant (open square), and the *abrB resD* mutant (closed triangle). *B. subtilis* strains carrying *ykuN-lacZ* were grown anaerobically in 2xYT supplemented with 1% pyruvate (C and D). Symbols: (C) Wild type (open circle), the *nsrR* mutant (closed circle), the *resD* mutant (open square), and the *nsrR resD* mutant (closed triangle); (D) Wild type (open circle), the *fur* mutant (open triangle), the *resD* mutant (open square), and the *fur resD* mutant (closed triangle). The figures are representatives of at least three independent experiments using different isolates of the indicated strains.

more complex in the case of *ykuN* expression in the *fur* mutant. Although ResD has a positive role in transcription throughout growth in the *fur*$^+$ background, ResD controls transcription positively in the exponential phase of growth and negatively in stationary phase in the *fur* background (Figure 3.10D). The temporal pattern and level of expression was similar between the *nsrR* mutant (Figure 3.10C) and the *fur resD* mutant (Figure 3.10D) mutant. That NsrR was unable to repress the transcription in the latter mutant and yet NsrR represses transcription in the *fur* mutant during stationary phase (Figure 3.10D) suggests that ResD is required for repression by NsrR at the *ykuN* promoter.

We next examined whether the stimulatory effect of the *nsrR* mutation on transcription of other genes is also dependent on ResD. All class II NsrR-controlled genes tested, namely, *sdpA*, *yydH*, *yukE*, *ylqB*, *ykuN*, *yetG*, and *spoVG*, showed that the elevated transcription caused by the *nsrR* mutation was abolished in the *nsrR resD* mutant (data not shown). Based on these results, we propose that ResD controls most (and probably all) of NsrR-repressed genes. There are two types of ResD-controlled genes in the NsrR regulon; one is dependent on ResD regardless of NsrR (like *nasD*) and the other is conditionally dependent (like *sdpA*) in that the effect of the *resD* mutation is only observed when NsrR repression is relieved. These findings have uncovered a second class of "hidden" genes within the ResD regulon.

3.4 DISCUSSION

Our current study presented evidence that NsrR exerts more global transcriptional control in *B. subtilis* than originally thought (Rodionov *et al.* 2005; Nakano *et al.* 2006).

Transcriptome analyses in diverse bacteria have indicated that the regulon of NsrR is relatively small (less than 10 genes) in the case of *N. meningitidis* (Heurlier *et al.* 2008) and *M. catarrhalis* (Wang *et al.* 2008), moderate (20 genes downregulated by NsrR) in *E. coli* (Filenko *et al.* 2007), and large in *Streptomyces* [over 300 predicted target genes, unpublished but cited in (Tucker *et al.* 2010)]. Previous studies have presented evidence that NsrR transcriptional regulation is modulated at the posttranslational level, namely, modification of Fe-S clusters in NsrR by NO (Isabella *et al.* 2009; Kommineni *et al.* 2010). Although *B. subtilis* expresses an NO synthase (Adak *et al.* 2002), generation of NO by NO synthase requires oxygen. Hence NO synthase does not play a role in NsrR control of either class I or class II genes under anaerobic conditions (Kommineni and Nakano, unpublished results).

This study showed that *B. subtilis* NsrR represses transcription of genes in two different classes, which is somewhat reminiscent of the dual transcriptional control by *E. coli* IscR. IscR, like NsrR, belongs to the Rrf2 transcriptional family and contains three conserved cysteines in its C-terminal region. [2Fe-2S]-IscR and apo-IscR function as a transcriptional repressor and activator, respectively (Schwartz *et al.* 2001; Yeo *et al.* 2006). The involvement of apo-IscR in transcription is unambiguously shown both *in vitro* and *in vivo* using cysteine substitution mutants (Yeo *et al.* 2006; Nesbit *et al.* 2009). Complementation experiments with ectopically expressed tCys-NsrR indicated complicated and yet unexplainable results such as a partial complementation (for *ykuN* and *yukE*), no complementation (*spoVG*), and a higher expression than the *nsrR* mutant (*sdpA*) (data not shown). In contrast, the wild-type NsrR in the same context was able to fully restore the repression of these genes (Figure 3.5 and data not shown). Although

tCys-NsrR binds to class II genes, it shows a different binding pattern compared to the wild-type protein at higher concentration. The mutant protein exhibited a multiple ladder-like binding profile with *sdpA* and other class II genes even at high concentrations of 512 and 1024 nM (Figure 3.11), whereas wild-type holo- and apo-NsrR generated shifted smeary band with slower migration at 512 nM (Figure 3.6A). It is unknown if the binding pattern unique to tCys-NsrR has any relevance to unexplained *in vivo* results. Although these results did not provide any conclusive answer whether apo-NsrR exerts class II gene regulation, another *in vivo* study showed that NO moderately upregulates class II gene expression and the expression is constitutively active in the *nsrR* mutant (Figure 3.9C and data not shown). This result suggested that NsrR binding to the class II genes could be NO sensitive in the context of *in vivo* DNA structure, thus implicating the participation of [4Fe-4S]-NsrR in the control of class II genes. As the upregulation of these genes by NO requires ResD (data not shown), the possibility that ResD activity is regulated through NO could not be ruled out, however. IscR binds both classes of IscR-regulated promoters in a sequence specific manner (Lee *et al.* 2004; Giel *et al.* 2006; Nesbit *et al.* 2009). Although NsrR recognizes a partial dyad symmetry sequence in the class I *cis*-acting site with critical residues therein, a similar sequence is not found in promoter regions of class II genes. A MEME motif search (Bailey *et al.* 2009) was performed to identify an overrepresented motif among class II DNA regions of *nasD* *sdpA*, *ykuN*, *sboA*, *yukE,* and *yydH*, which were shown to interact with NsrR by EMSA (Fig. 3.6 and data not shown). As a negative control, we used the *nasD* coding region that does not interact with NsrR (Figure 3.6). Figure 3.12 shows the motif enriched in all genes except *ykuN*. It remains to be elucidated whether NsrR specifically binds to this

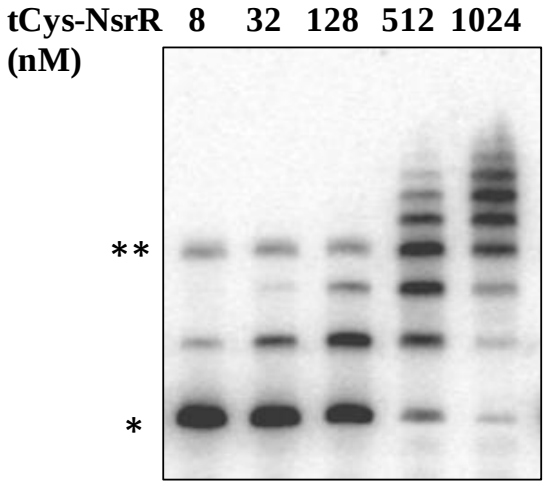


Fig. 3.11. Binding assay of tCys-NsrR to the sdpA promoter. The radiolabeled *sdpA* probe used in Fig. 3.6 was incubated with increasing concentrations of tCys-NsrR under aerobic conditions. Note the difference in the binding pattern between tCys-NsrR at 512 and 1024 nM and the wild-type holo- and apo-NsrR at 512 nM shown in Fig. 3.6. A single asterisk represents the free probe and a double asterisk represents an unidentified DNA fragment generated by PCR.

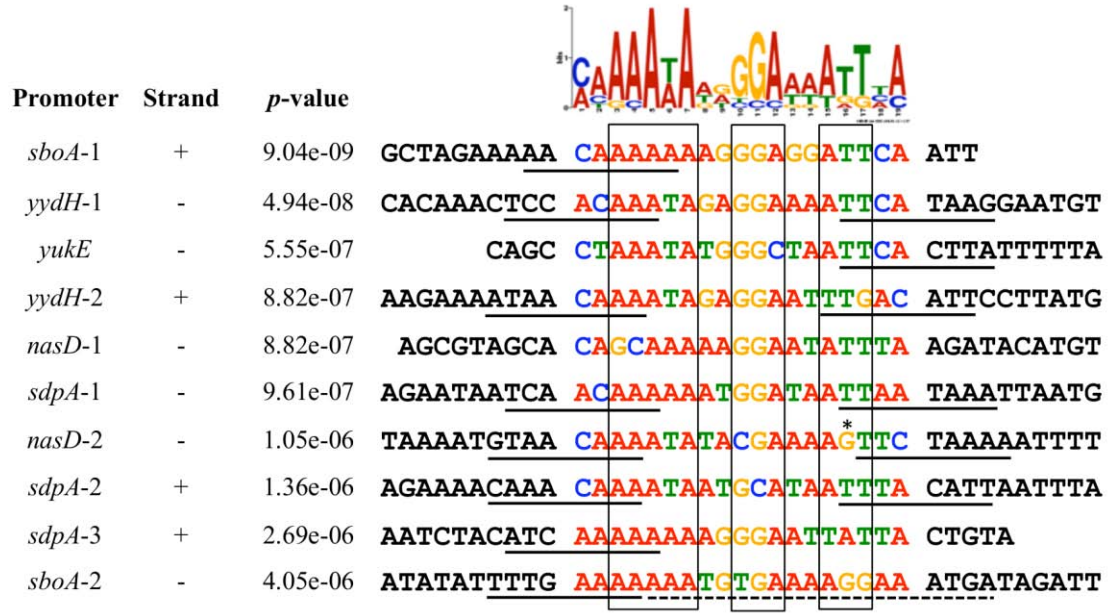


Fig. 3.12. Sequence LOGO of the motif enriched in the sequences that bind NsrR in vitro. Sequence LOGOs are derived from a MEME search (http://meme.nbcr.net) using the NsrR binding probes used in EMSA analysis (Fig. 3.6 and data not shown). The *nasD*-coding region showed in Figure 3.6 was used as a negative control. Note that the *nasD* sequences are complementary to those shown in Figures 1 and 2. P-values are calculated by MEME. G shown by an asterisk in *nasD* promoter corresponds to the C nucleotide, the substitution of which caused a significant decrease in apo-NsrR binding (Fig. 3.4). Solid underlined sequences show at least five of eight matches to the complementary sequence (except *sboA* in the last row that shows a direct match) to a half-site (TTTGTGAA) of a proposed ResD-binding site (Geng *et al.* 2007). A dashed underline sequence was reported previously as an overrepresented motif in some Rok-binding regions (Smits *et al.* 2010).

region, as class II regulation is under complex transcriptional control by multiple transcription factors including AbrB, Rok, Fur, and ResD.

AbrB and Rok bind to A+T-rich sequences both *in vitro* (Strauch 1995) and *in vivo* (Smits *et al.* 2010; Chumsakul *et al.* 2011), suggesting that class II NsrR-binding sites might overlap with AbrB and/or Rok-binding sites. In fact, a previous study showed that a MEME search using a few Rok-binding regions with relatively higher G+C content than average binding regions identified AAATGT (in $AAATGTN_7AAATGT$) as an overrepresented motif, one of which was identified in *sboA-2* (Smits *et al.* 2010) shown in Figure 3.12. In addition, *sdpA-1*, *sdpA-2*, and *sboA-2* sequences in Figure 3.12 reside within regions where AbrB was shown to bind by DNase I footprinting (Strauch *et al.* 2007). Genome-wide binding profiles of AbrB revealed that three out of four binding patterns contain TGGNA motifs connected by A+T-rich sequence (Chumsakul *et al.* 2011). Similar NGGAA motifs are present in the center of the A+T-rich sequence in the sequence logo in Figure 3.12. Although the MEME search did not identify the overrepresented motif in *ykuN*, it is worth noting that the GGAAAA sequence in the motif identified in the NsrR-binding regions is somewhat similar to the half site (TGATAA) of the Fur-binding dyad-symmetry sequence (Fuangthong *et al.* 2003). Base substitution analysis demonstrated that the GA sequence in the half site is the key for recognition by the Fur repressor (Fuangthong *et al.* 2003), which is also well conserved in the overrepresented motif of the NsrR-binding regions.

Lastly, we found that ResD is indispensable for derepression of class II genes caused by the *nsrR* mutation (Figure 3.10). That ResD exerts control over such a diverse collection of genes might be related to the observation based on proteomic study of *B.*

subtilis RNA polymerase holoenzyme, that ResD is a binding partner of RNA polymerase, an attribute not detected for other response regulator proteins (Delumeau *et al.* 2011). Sequences similar to a previously proposed half ResD-binding site (Geng *et al.* 2007) appear to partially overlap with the identified motif in the NsrR-binding regions (Figure 3.12). In summary, the current study presented evidence that transcriptional control can be governed by multiple transcription factors that bind to A+T-rich regions.

We have previously shown that ResD is an activator of *nasD* and NsrR displaces the *nasD*-RNAP-ResD transcription initiation complex (Kommineni *et al.* 2010). The current study uncovered other roles of ResD associated with NsrR regulation. ResD, in the absence of NsrR, likely acts as an antirepressor of AbrB and Fur at the *sdpA* and *ykuN* promoter, respectively. In addition, ResD reinforces NsrR-dependent repression in stationary phase in the absence of Fur at the *ykuN* promoter. It was shown that ComK functions as a transcriptional activator for late competence genes but acts as an antirepressor of Rok and CodY repressors at the *comK* promoter (Smits *et al.* 2007). Interestingly, ComK antirepression occurs without preventing binding of the repressors and the authors postulated that antirepression is likely achieved by modulating DNA topology. ResD might antagonize AbrB and Fur through a similar mechanism postulated for ComK. In addition, ResD is required for NsrR repression of the Fur regulon (Figure 3.10D), which might explain why repression by NsrR is stronger in stationary phase (Figure 3.10C). Increased levels of phosphorylated ResD in later growth stage could enhance NsrR repression. The interplay of these transcription factors and the mechanism for their recognition remain to be elucidated.

Recent chromatin immunoprecipitation and microarray analysis (ChIP-chip) revealed genome-wide identification of direct targets of NsrR in *E. coli* (Partridge *et al.* 2009). A similar approach both for ResD and NsrR will be useful to assess these possibilities to obtain a better picture of ResD/NsrR regulation in *B. subtilis*.

CHAPTER 4

IN VIVO BINDING OF RESD AND NSRR UNDER ANAEROBIC CONDITIONS

4.1 INTRODUCTION

The ResDE two-component signal transduction system plays a vital role in upregulating genes required for anaerobic respiration in *B. subtilis* (Nakano *et al.* 1997). However, this system is also indispensible for aerobic respiration (Sun *et al.* 1996). Therefore, an additional regulatory mechanism was postulated to exist that specifically activates genes involved in nitrate respiration (Nakano *et al.* 2006). Our previous work identified that upregulation of ResD-dependent transcription of *hmp* and the *nasDEF* operon during nitrate respiration is due to the inactivation of transcription repressor NsrR by endogenously generated NO (Chapter 2).

Mutational studies on the *nasD* regulatory region identified a partial dyad symmetry sequence that is required for [4Fe-4S]-NsrR interaction with the promoter (class I NsrR-binding site) (Chapter 3). A genome-wide transcriptome analysis identified many genes repressed by NsrR and the transcription of these genes was more elevated in the *nsrR* mutant during anaerobic fermentative growth with pyruvate (Kommineni *et al.* 2012). Holo- and apo-NsrR bind to the promoter regions of these genes similarly (class II NsrR-binding site) and the binding is NO insensitive *in vitro*. Mutational analysis showed that interaction of NsrR with A+T-rich class II sites involves more relaxed sequence specificity than class I sites. The class II NsrR regulon includes genes controlled by both AbrB and Rok repressors that also recognize A+T-rich sequences and genes repressed by the Fur repressor. Interestingly, in all NsrR-repressed genes tested,

the loss of NsrR repressor activity was not sufficient to induce transcription of these genes, as induction required ResD. Therefore the previous study discussed in Chapter 3 showcased multiple roles of ResD, depending on the promoters. Complex interwoven regulation governed by multiple transcription regulators at a single promoter has been reported in *E. coli* and *Salmonella* under anaerobic conditions (Grainger *et al.* 2008; Squire *et al.* 2009; Xu *et al.* 2009; Browning *et al.* 2010; Walthers *et al.* 2011). For example, the *E. coli nrf* promoter was shown to exhibit high complexity, by possessing binding sites for at least six transcription factors: Fnr, NarL, NarP, IHF, Fis, and NsrR (Browning *et al.* 2010). It was speculated that some (Fis, IHF, and NsrR) of these regulators are involved in changing the topology of the surrounding DNA, thereby influencing the binding of other regulators and ultimately affecting the transcription (Browning *et al.* 2010). A novel regulatory mechanism was hypothesized that explained how NsrR regulates the *nrf* promoter activity in *E. coli*. In this scenario, NsrR might function in a fine-tuning mechanism rather than simply switching the regulation on to off (Browning *et al.* 2010). The NsrR-binding site overlaps with the IHF binding site and both regulators were shown to repress the transcription of *nrf* (Browning *et al.* 2010). In this chapter, I will briefly introduce some of the transcription regulators that are designated as the nucleoid associated proteins in bacteria as they are known to contribute to both nucleoid structure and gene regulation simultaneously.

I will also discuss the study that is currently ongoing in our laboratory to identify the diverse roles of ResD in transcriptional regulation of class I and class II genes using ChAP-qPCR analysis. The study will likely unravel the complex regulation governed by and influenced between the transcriptional regulators, ResD and NsrR in *B. subtilis*.

4.2 NUCLEOID ASSOCIATED PROTEINS (NAPs)

Transcription in bacteria is a well-orchestrated event that involves complex network of regulators for controlling the promoter activity in response to different living conditions. Transcription is often regulated by two or more factors and is affected by their interaction with promoters (Lloyd *et al.* 2001). Recent studies have shed light on certain regulators that influence transcription by stabilizing DNA structures, a task similar to that performed by histones of eukaryotes. Some proteins that were originally identified as gene-specific transcriptional regulators are now being acknowledged as global regulators that have a significant role in organization of genomic DNA within the nucleoid (Azam *et al.* 1999; Lloyd *et al.* 2001). Bacterial NAPs are DNA-binding proteins that affect the shape of the DNA upon their interaction, thus influencing the transcription of neighboring genes (Dillon *et al.* 2010). NAPs were identified and well studied in *E. coli* and its closest relatives among γ proteobacteria (Ohniwa *et al.* 2006). These proteins include FIS (Factor for inversion stimulation), H-NS (Histone like nucleoid structuring protein), HU (Histone like protein), StpA (suppressor of *td* mutant phenotype A), Dps (DNA-binding proteins from starved cells), IHF (integration host factor), CbpA (curved DNA-binding protein), and Lrp (leucine-responsive regulatory *protein*) in gram-negative bacteria (Ali Azam *et al.* 1999). Almost all NAPs identified so far lack a clear consensus DNA sequence with an exception of IHF (Azam *et al.* 1999). Most *cis*-regulatory sites targeted by NAPs are A+T-rich (Dorman *et al.* 2009). NAPs are capable of regulating gene expression by altering the curvature of the DNA molecule by bending, wrapping or bridging activities (Zimmerman 2006; Dorman *et al.* 2009; Dillon *et al.* 2010; Graham *et al.* 2010). Many NAPs were also shown to influence the

binding of transcriptional factors to a promoter region either in a positive or a negative manner (Dillon *et al.* 2010; Kahramanoglou *et al.* 2011). They are mostly categorized based on their abundance inside the bacterial cell at given growth conditions rather than their function (Ali Azam *et al.* 1999; Dillon *et al.* 2010). For example H-NS is present at a constant H-NS:DNA ratio and, its function is modulated by the presence of its paralogue, StpA (Deighan *et al.* 2003). StpA and H-NS interact with each other to form heteromers, and the changes in the heteromer amounts exert different effects on transcription (Dorman 2009). Fis concentrations are high during the exponential phase of the cell growth curve, while Dps concentrations are reciprocal to that of Fis in *E. coli* (Grainger *et al.* 2008). IHF also shows growth-phase-dependent availability, whereas HU function is regulated by its subunit composition (Dillon *et al.* 2010). The requirement of NAPs for transcriptional regulation was mostly attributed to their role in silencing of horizontally acquired genes that became integrated into the preexisting regulatory networks (Jain *et al.* 2002). Comparative genomic analysis revealed that *Salmonella* (Porwollik *et al.* 2003) and *E. coli* (Lawrence *et al.* 1998; Jain *et al.* 2002) have acquired a considerable amount of new genomic DNA through horizontal gene transfer during the course of evolution. Though the new extracellular DNA might bring a survival advantage to bacteria by providing virulence factors and for extracellular functions during certain harsh environmental conditions, they are more likely to decrease the fitness of the bacteria if their transcription is not controlled (Jain *et al.* 2002; Navarre *et al.* 2007). For example integration of phage DNA, transposon insertions, and conjugative plasmids can compromise the surviving capability when they become integrated into essential regions of the genome (Buckling *et al.* 2002). One of the best

studied examples is H-NS in protecting the cell from the detrimental effects of low-GC horizontally acquired DNA regions. H-NS represses or silences the transcription of newly acquired DNA under unfavorable conditions (Navarre *et al.* 2006).

A search for homologs of the above mentioned NAPs in *B. subtilis* did not yield any candidate with the exception of HU (Kawamura *et al.* 1995). The HU protein comprises two subunits, HUα and Huβ, which are capable of forming homodimers or heterodimers based on their relative abundance in the cell. HU protein influences DNA superhelicity and flexibility. Studies on the transcription regulators, AbrB and Rok made investigators to speculate their role as NAPs in *B. subtilis* (O'Reilly *et al.* 1997; Smits *et al.* 2010; Chumsakul *et al.* 2011).

4.2.1 AbrB

AbrB belongs to a unique class of transcription factors, the transition-state regulators (TSRs). AbrB is a 10.4 kDa protein that is comprised of an N-terminal DNA-binding domain and a C-terminal multimerization domain that contributes to its homo-tetramer configuration (Yao *et al.* 2005). The *abrB* gene (antibiotic resistant protein B) was identified as a pseudorevertant obtained in the *spo0A* mutant background (Trowsdale *et al.* 1979). The AbrB regulon includes genes that function in biofilm formation, antibiotic production, motility, competence development, sporulation, carbon utilization, and extracellular enzyme synthesis (Hamon *et al.* 2004; Chumsakul *et al.* 2011). AbrB is responsible for restructuring the gene expression of nearly 100 genes during post-exponential cell growth (Chumsakul *et al.* 2010). Orthologs of AbrB are present in all

Bacillus, *Clostridium* and *Listeria* species. In *B. cereus* and *B. anthracis* AbrB also regulates the induction of virulence factors (Saile *et al.* 2002; Lucking *et al.* 2009).

In *B. subtilis*, AbrB protein levels are high during exponential phase, which contributes to the regulation observed during the exponential phase of the cell growth curve (Banse *et al.* 2008). AbrB binds to its own promoter and autoregulates transcription (Strauch *et al.* 1989). The decrease in the AbrB levels after the entry into stationary phase is regulated by Spo0A, the master regulator for sporulation initiation (Fujita *et al.* 1998; Banse *et al.* 2008). AbrB is also regulated via Spo0A-regulated AbrB-specific inhibitor, AbbA (Strauch *et al.* 1990; Greene *et al.* 1996; Banse *et al.* 2008). Interaction of AbbA with AbrB prevents AbrB from binding its DNA target (Banse *et al.* 2008). To date, over 60 genes have been identified as being regulated by AbrB. Despite many studies on AbrB as a TSR and several biochemical studies on its structure (Klein *et al.* 2002), it is still unclear how AbrB recognizes the nucleotide sequences of its target DNA. DNase I footprinting experiments revealed that this small protein of 10.4 kDa binds to DNA ranging from 30 to 120 base pairs (Strauch 1995). It has been hypothesized that AbrB binding requires a specific three-dimensional conformation of the DNA helix rather than nucleotide sequence itself (Bobay *et al.* 2004).

Structural and NMR spectroscopy studies conducted on the N-terminal 53 residues of AbrB (AbrBN) revealed that arginine residues at 8, 15, 23 and 24 are responsible to its interaction with DNA and are highly conserved among the orthologs and paralogs of AbrB (Bobay *et al.* 2006). The conserved arginines are responsible for docking the protein to the negatively charged phosphate backbone and for hydrogen bond

formation with specific base pairs in the DNA (Bobay *et al.* 2006; Sullivan *et al.* 2008). NMR studies also revealed that AbrB binds to the major and minor groove of the single faced B-form DNA (Bobay *et al.* 2004; Bobay *et al.* 2006). The AbrB paralogs in *B. subtilis*, Abh and SpoVT (Yao *et al.* 2005; Sullivan *et al.* 2008), are remarkably similar in structure to AbrB, but their C-terminal multimerization domains are only functionally similar (Yao *et al.* 2005; Sullivan *et al.* 2008). Studies with the fusion proteins between AbrB and the paralogs have shown that the C-terminal domain is essentially interchangeable between the three, with no change in DNA binding specificity (Yao *et al.* 2005). Abh regulates some of the AbrB regulon *in vivo* and binds to those promoters *in vitro* (Strauch *et al.* 2007; Chumsakul *et al.* 2011). Genome-wide binding and transcriptome profile of AbrB and Abh *in vivo* during exponential growth phase extended the roles of AbrB and Abh as both transcriptional regulators and possibly nucleoid structuring proteins (Chumsakul *et al.* 2011). AbrB and Abh form functional heteromers *in vivo* (Chumsakul *et al.* 2011). Quantitative comparisons of AbrB/Abh-binding sites *in vivo* did not reveal any strict consensus sequence, but rather suggested certain motif requirements for homomers and heteromers to bind DNA with different specificities and affinities (Chumsakul *et al.* 2011). *In vitro* and *in vivo* studies suggested that AbrB or AbrB/Abh binds to the TGGNA motif mostly with relaxed specificity (Xu *et al.* 1996; Banse *et al.* 2008).

sdpABC, which we have studied as an example of class II NsrR regulon genes, is also regulated by AbrB. Transcriptional regulation of the *sdpABC* operon occurs indirectly by phosphorylated Spo0A via repression of AbrB (Fujita *et al.* 2005). The *sdpABC* operon is responsible for the synthesis and the export of an extracellular factor,

SdpC, that results in a delay in the sporulation process (Gonzalez-Pastor *et al.* 2003). Repression of the *sdpABC* transcription by AbrB was verified using a *lacZ* transcriptional fusion to the *sdp* promoter and EMSA showed direct binding of AbrB to the promoter region (Strauch *et al.* 2007). As discussed in the next section, the *sdp* operon also belongs to the Rok regulon, though the effect of *rok* mutation on the sporulation phenotype has not been determined (Albano *et al.* 2005).

4.2.2 Rok

The *rok* (repressor of *comK*) gene product of *B. subtilis* was identified as a negative regulator of competence development (Hoa *et al.* 2002). It represses the transcription of *comK* (Hoa *et al.* 2002). ComK is a transcriptional activator for genes that code for the competence machinery (Hamoen *et al.* 1998). Transcriptome analysis identified genes/operons such as those that encode extracellular function proteins to be regulated by Rok (Albano *et al.* 2005). Rok represses the endogenous excision of the ICE*Bs1* (integrative and conjugative element inserted into *trnS-leu2* (tRNA-Leu2) in the chromosome of *B. subtilis*) (Smits *et al.* 2010). Rok repressor is found in several closely related *Bacillus* species. Attempts to identify the consensus sequence required for Rok binding had not been successful (Albano *et al.* 2005). Recent *in vivo* binding study has found that Rok binds to extended regions that might have been acquired through horizontal gene transfer to the *B. subtilis* genome (Smits *et al.* 2010). Deletion analyses of Rok coupled with ChIP-qPCR identified the significance of the C-terminal region in DNA binding. The N-terminal region of Rok is suspected to be involved in dimerization and/or multimerization (Smits *et al.* 2010). When expressed in *E. coli*, Rok was

associated with A+T-rich DNA. Rok autorepresses its own transcription, the function of which was hypothesized as a necessity for the cell to allow adjustment of Rok concentrations in response to extracellular A+T-rich DNA acquisition (Smits *et al.* 2010). These results suggest that Rok is an NAP-like protein that functions in repressing the expression of A+T-rich genes (Smits *et al.* 2010).

From various comparative and genetic studies, AbrB appears to be a functional analog of Fis in *E. coli* (Klein *et al.* 2002) and Rok appears to be functionally analogous to H-NS in gram-negative bacteria (Smits *et al.* 2010). Our recent examination of the NsrR microarray data revealed some of the class II NsrR regulon genes are controlled by both AbrB and Rok repressors. Most of the regulators (AbrB, Rok, NsrR and ResD) described in this thesis follow a common theme of relaxed DNA specificity for interacting with the target DNA. Hence it is likely that these regulators affect transcription by altering DNA topology.

4.3 MATERIALS AND METHODS

Bacterial strains and growth conditions

B. subtilis strains used in this study are the derivatives of 168 and JH642 strains and are listed in the Table 4.1. Oligonucleotides are listed in Table 4.2. Two 168 derivatives were used for ChAP-qPCR experiments. The *B. subtilis* strain, OC0010 expressing NsrR C-terminally tagged with 12 histidine residues, was constructed by Dr. Onuma Chumsakul at NAIST, Japan as previously described (Chumsakul *et al.* 2011). *B. subtilis* strain ORB8238 expressing ResD C-terminally tagged with 12 histidines was constructed by Dr. Michiko M. Nakano using a marker-free mutant construction method

Table 4.1. *B. subtilis* strains and plasmids

Strains	Description	Reference or source
168	*trpC2*	Shu Ishikawa
JH642	*trpC2 pheA1*	J. A. Hoch
LAB2511	*trpC2 pheA1 resD*::*spec*	(Kommineni *et al*. 2012)
ORB6179	*trpC2 pheA1 nsrR*::*cat*	(Nakano *et al.* 2006)
ORB7963	*trpC2* SPß*c2del2*::Tn*917*::pMMN392(*nasD-lacZ*)	This study
ORB7964	*trpC2* SPß*c2del2*::Tn*917*::pMMN392(*nasD-lacZ*), *nsrR*::*nsrR-his*$_{12}$ (*tet*)	This study
ORB7967	*trpC2* SPß*c2del2*::Tn*917*::pMMN392(*nasD-lacZ*), *nsrR*::*cat*	This study
ORB7968	*trpC2 sdpA*::pMUTIN	This study
ORB7969	*trpC2 sdpA*::pMUTIN, *nsrR*::*nsrR-his*$_{12}$ (*tet*)	This study
ORB7972	*trpC2 sdpA*::pMUTIN, *nsrR*::*cat*	This study
OC0010	*trpC2 nsrR*::*nsrR-his*$_{12}$ *tet*	This study
ORB8238	*trpC2 resD*::*resD-his*$_{12}$	This study
ORB8264	*trpC2 resD*::*resD-his*$_{12}$ *nsrR*::*cat*	This study
ORB8265	*trpC2 resD*::*resD-his*$_{12}$ *abrB*::*neo*	This study
ORB8266	*trpC2 resD*::*resD-his*$_{12}$ *fur*::*neo*	This study

Table 4.2. Oligonucleotide primers used in this study

Oligonucleotide	Sequence (5' to 3')	Purpose
oSK-199	TGGTTCGAAAGTCTTGATTTAAAAG	*abrB* template
oSK-200	ATAACAAGGTTTCCAATAATCGTCA	*abrB* template
oSK-203	TAAGTCCAATCCAAATGGTTGAATA	*sdpA* template
oSK-204	TTTGATGTAGATTACCTCCTCTAAGC	*sdpA* template
oSK-211	TCAGGAAAATCCATTTTAAAGACAG	*ykuN* template
oSK-212	TAGCCATGTTATCACCCCATTAGTT	*ykuN* template
oSK-213	GTAAAATGCCCGGTTTTAAGGTATG	*nasD* template
oSK-214	GCACAGCAAAAAGGAATATTTAAGA	*nasD* template
oSN03-86	CATGTTTTTATCACCTAAAAGTTTACCAC	*rpsD* template
oSN03-87	CGATACACCTTATTGATAAGGAACAAAATG	*rpsD* template

as described previously (Morimoto *et al.* 2011). Antibiotics were used at the following concentrations: chloramphenicol, 5 μg/ml; spectinomycin, 75 μg/ml; tetracycline, 10 μg/ml; erythromycin/lincomycin, 1/25 μg/ml; neomycin, 5 μg/ml.

ChAP-qPCR analysis

The sample for ChAP-qPCR was processed as described before (Chumsakul *et al.* 2011) with slight modifications (Figure 4.1). An overnight aerobic culture of OC0010 or ORB8238 in 2×YT liquid medium at 37˚C was used as a preculture. The preculture was transferred (the starting OD_{600}= 0.06) to 250 ml glass bottles filled with 2xYT supplemented with either 1% glucose and 0.2% nitrate or 0.5% glucose and 0.5% pyruvate. Cells were harvested at T_1 (1 hour after the end of exponential growth) for crosslinking. For NO treatment, 100 mM stock solution of spermine NONOate (Cayman Chemical) (SperNO) was prepared by dissolving in 10 mM NaOH. SperNO was added at the final concentration of 50 μM to the cultures when OD_{600} reached 0.5, and the same volume of 10 mM NaOH was added for untreated cultures. After 1 hr, cells were harvested. Harvested cells were treated with formaldehyde (1% final concentration) for 30 min, washed with TBS buffer (50 mM Tris, 150 mM NaCl, pH 7.6), and stored at -80˚C until further use. The frozen pellet is treated with RNaseA at a final concentration of 200 μg/ml in 3 ml of buffer containing 0.1 M Tris-HCl (pH 7.5), 0.2 M NaCl, 1% (v/v) Triton X-100, 0.1% (w/v) Na-deoxycholate, 0.2% (w/v) Brij 58, and 20% (v/v) glycerol, for 30 minutes at 37˚C with shaking at 230 rpm, followed by the centrifugation at 5500 g.

The suspended cells were disrupted by sonication on ice in 3 ml of UT buffer

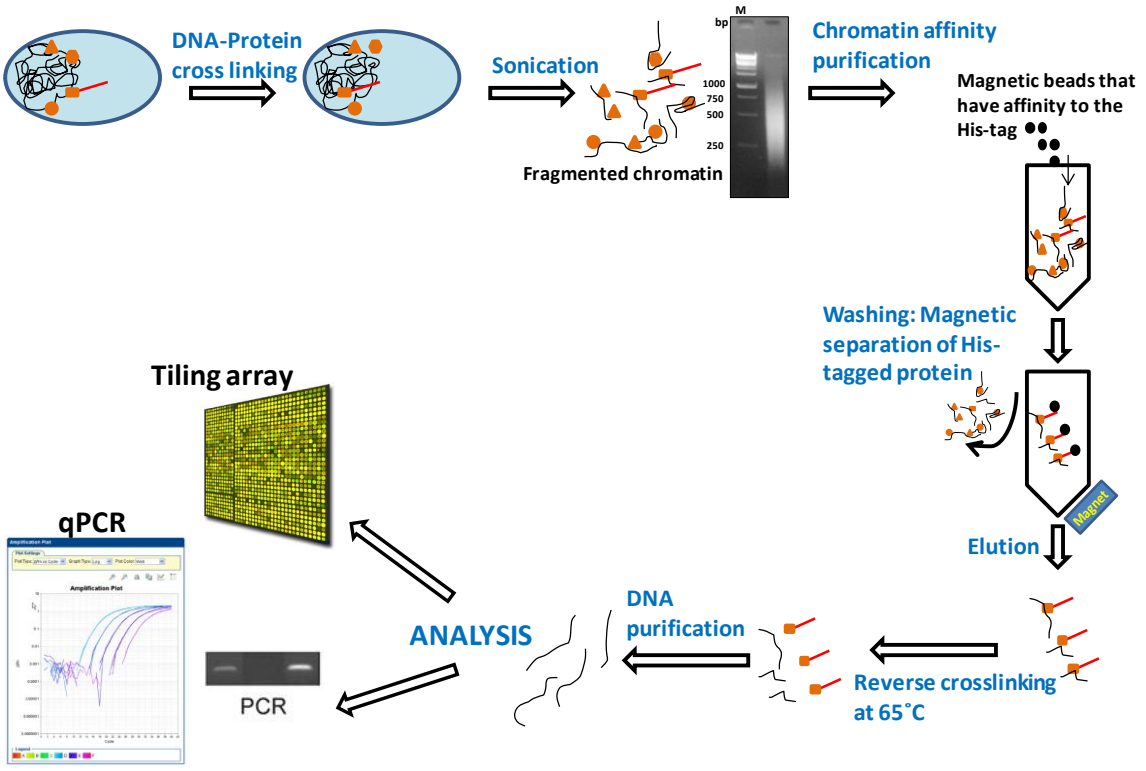


Fig 4.1 Outline of the ChAP-qPCR procedure

(100 mM HEPES, 10 mM imidazole, 8 M urea, 0.5 M NaCl, 1% Triton X-100, 1 mM PMSF, pH 7.4). Sonication was performed by Branson sonifier using the following settings: amplitude: 45% over 10 min (4 sec 'on' and 10 sec 'off'). After centrifugation at 6000 g for 20 minutes, 50 µl of Dynabead Talon (Invitrogen) magnetic beads were added to the cleared supernatant (Input), followed by an overnight incubation at room temperature with gentle shaking. The incubated magnetic beads in the cleared lysate were washed ten times with UT buffer, and bound proteins were eluted with 400 µl of elution buffer (100 mM Tris–HCl, pH 7.5, 0.5 M imidazole, 1% SDS, 10 mM DTT). The eluate (ChAP sample) was diluted by 1 ml of M-wash buffer (100 mM Tris–HCl, pH 7.5, 1% SDS, 10 mM DTT) and subjected to the reverse cross-linking by heating at 65˚C overnight. DNA was purified using phenol/chloroform/isoamyl alcohol DNA purification method and precipitated with ethanol in the presence of 0.1 µg/µl glycogen. Additionally, 10 µl of cleared cell lysate (Input) was also subjected to reverse crosslink as described above for the purpose of quantitating the enrichment in the ChAP sample. Precipitated DNA in the purified ChAP and input sample was used for ChAP-qPCR.

Quantitative PCR (qPCR) was performed on an Applied Biosystem step-one-plus RT-PCR machine. Two microliters of 10-fold diluted ChAP and 100-fold diluted input samples were analyzed in triplicate in a 20 µl reaction volume that contained fast Sybr Green master mix (Applied Biosystems) and promoter-specific primers. The lists of primers and the promoter regions used for detecting the enrichment are listed in Table 4.2. To detect *ykuN* (-90 to +46), *nasD* (-141 to -10), and *sdpA* (-189 to -21) enrichment, oSK-211/oSK-212, oSK213/oSK214, and oSK-203/oSK-204 oligonucleotide pairs were used respectively (Figure 4.2). Primers specific to the *rpsD* and *abrB* promoters were

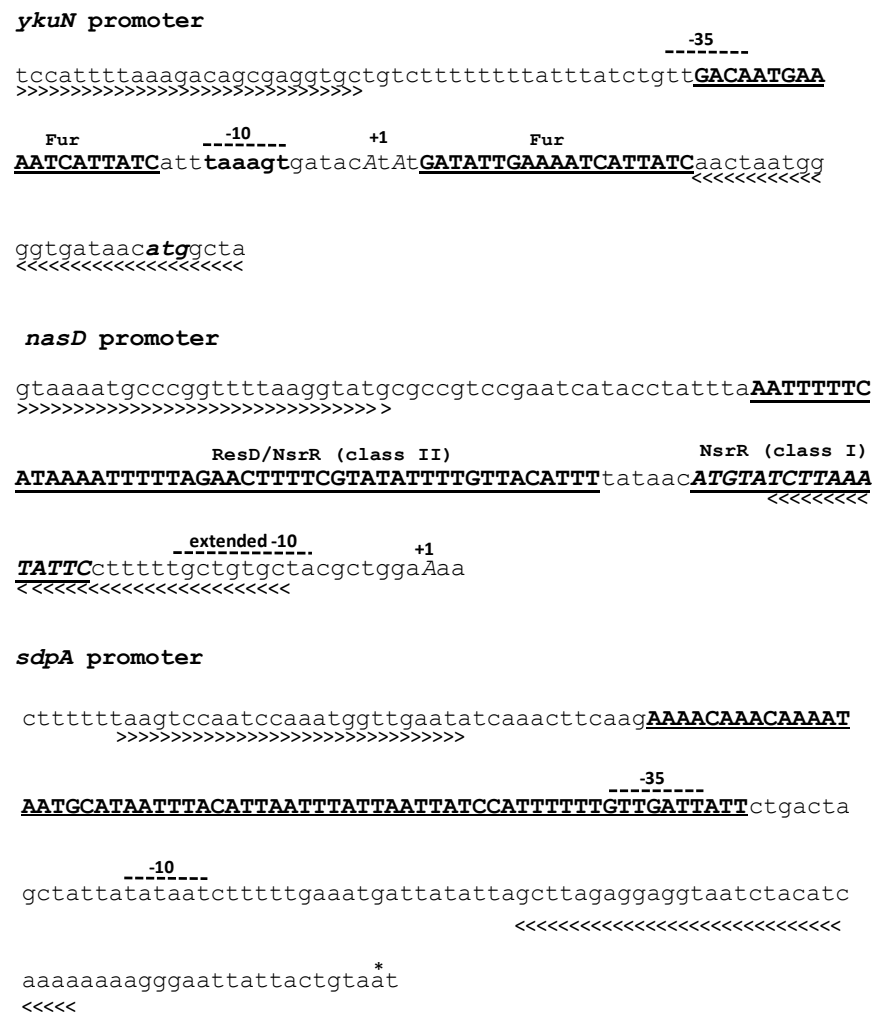


Fig. 4.2. qPCR analysis of the regulatory regions in the *ykuN*, *nasD*, and *sdpA* promoters. The oligonucleotides listed in the Table 4.2 are shown by the arrows underneath the sequence for the amplification of the *ykuN* (-90 to +46 with respect to the transcription start site), *nasD* (-141 to -10 with respect to the transcription start site), and *sdpA* (-189 to -21 relative to the translation start site) promoters. The nucleotide sequence in uppercase indicate the binding regions by each regulator. (A) The *ykuN* promoter region contains two Fur boxes (bold and underlined) as identified previously (Baichoo *et al.* 2002). -35 and -10 regions (dashed line) and two transcription start sites (uppercase and italicized) are indicated. (B) The *nasD* promoter region consists of an overlapping ResD and NsrR class II binding sites (bold and underlined (dash line). Also shown are NsrR class I binding site (bold and underlined) and -10 extended region, and the transcription start site (upper case and italicized). (C) The *sdpA* promoter region showing AbrB protected region (underlined and bold) previously shown by DNase I footprinting. -10 and -35 regions are indicated by dashed line. * denotes the translation start site (Strauch *et al.* 2007).

used as controls that do not show association with ResD and NsrR. The final fold-enrichment of qPCR was calculated using previously a described normalization method (Merrikh *et al.* 2011). In short, the quantity of amplification for each sample was determined using the standard curve obtained from the same primer set with chromosomal DNA as a template. Data obtained for ChAP and input samples of a tested target promoter were first normalized to the quantity of ChAP and input samples of the *rpsD* promoter, respectively. The final-fold enrichment was calculated as the ratio of normalized ChAP to the normalized input of the target promoter. Hence, the formula for determining the final fold enrichment is: (*x* ChAP/*rpsD* ChAP) / (*x* input/*rpsD* input), where *x* represents the promoter of interest. All the data presented are the average of at least three biological replicates ± standard error.

Western blot analysis and detection of ResD phosphorylation in vivo

B. subtilis cells were grown anaerobically in 2xYT supplemented with either 1% glucose and 0.2% nitrate or 0.5% glucose and 0.5% pyruvate. Cells were harvested at post exponential growth (T_1), washed with 50 mM Tris buffer (pH 7.5), and stored at -80˚C. The frozen pellet was suspended in 200 µl of 50 mM Tris buffer (pH 7.5) and was broken on a vortex mixer with 0.1 mm glass beads. The cell debris was removed by centrifugation at 20000 g to obtain clear lysate. Fifteen micrograms of the total protein was applied to 15% SDS-PAGE gel and Western blot analysis was carried out using anti-ResD antibody as described (Nakano *et al.* 2000).

ResD phosphorylation levels were measured using Phos-tag ™ - acrylamide gel electrophoresis (Wako Chemicals) and subsequent western-blot analysis. Phos-tag gels

were prepared according to the instructions described by the manufacturer with minor modifications as suggested (Kinoshita *et al.* 2006). 10% gels were prepared by copolymerizing 25 μM Phos-tag™ acrylamide with 50 μM $MnCl_2$. The cell lysates prepared above were run for analysis of ResD phosphorylation with a mixture of ResD and ResD~P as a marker prepared as follows. ResD protein purified in Chapter 2 was phosphorylated using 20 μM of lithium potassium acetyl phosphate (Sigma-Aldrich) in the reaction buffer [50 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.5 mM DTT, 5 mM $MgCl_2$, 5 μg ml^{-1} BSA, 10% glycerol]. After incubation at 37°C for 30 min, 4.5 μl of 5xSDS loading buffer (60 mM Tris-HCl [pH 6.8], 25% glycerol, 2% SDS, 0.3 M DTT, 0.1% bromophenol blue) was added to the reaction. Phos-tag™ acrylamide gels were run at 4 °C under constant voltage (150 V). Gels were fixed for 10 min in standard transfer buffer, 20% (v/v) methanol, 50 mM Tris, and 40 mM glycine, except supplemented with 1 mM EDTA to remove Mn^{2+} from the gel. Gels were incubated for an additional 20 min in the transfer buffer without EDTA to remove the chelated metal. Western blot analysis was performed as described above.

4.4 RESULTS

4.4.1 NsrR association with class I and class II promoters is NO sensitive *in vivo*

Our previous study showcased the NsrR regulon to be widespread and overlap with the AbrB, Rok and Fur regulons. It seems from the *in vivo* transcription assays that NsrR mediates changes in *B. subtilis* transcriptome in response to NO (Chapter 3, Figure 3.9). On the contrary, *in vitro* binding experiments suggested an NO-insensitive interaction of NsrR to regulatory regions in the class II regulon (Chapter 3, Figure 3.7). Based on these

results it is difficult to draw a solid conclusion about NO sensitivity of NsrR binding (if any) to class II promoters. In addition, it became of considerable interest to identify whether the transcriptional changes observed in class II genes are due to a direct or indirect effect of NsrR. As NsrR recognizes A+T-rich sequences with a relaxed sequence specificity, the recognition likely requires distinct DNA topology. Therefore, we chose chromatin affinity purification with quantitative PCR (ChAP-qPCR) to identify direct interactions of the class II promoters with NsrR under anaerobic conditions. We constructed a strain producing NsrR with a 12-histidine tag at its native locus. The tagged NsrR is functional *in vivo* as it represses *nasD-lacZ* and *sdpA-lacZ* and the repression is sensitive to NO, as observed in strains expressing the untagged NsrR (Figure 4.3 and data not shown). In this experiment, we chose two class II genes, *sdpA* as a member of the AbrB regulon and *ykuN* for the Fur regulon. As a positive control, the class I *nasD* gene was used. ChAP-qPCR was analyzed in cells cultured under fermentation conditions. The *rpsD* and *abrB* promoters were hardly enriched in the NsrR-His_{12}-ChAP samples, as only less than 5% increase was detected compared to the input. Therefore in the following experiments, *rpsD* was used as a negative control to eliminate the background noise and to obtain a comparable fold-change in the interaction of NsrR. Figure 4.4 confirmed that NsrR is associated with the *nasD* promoter under these conditions as described in Chapter 2. Furthermore, SperNO and endogenous NO generated from nitrate respiration caused 6-fold reduction in NsrR binding to the promoter. ChAP-qPCR analysis also showed that the *ykuN* promoter DNA was enriched by NsrR-His_{12} (Figure 4.4A). The observed enrichment was reduced around 3-fold in the presence of NO. There is no significant enrichment found for the *sdpA* promoter in either

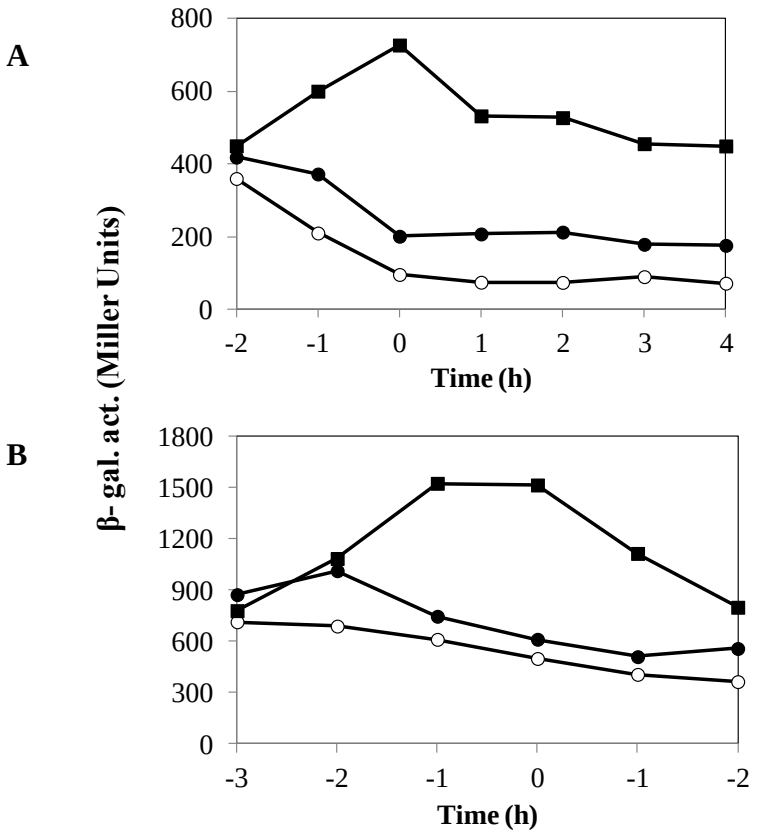


Fig 4.3 NsrR-His$_{12}$ is functional in *B. subtilis*.
(A) Expression of *nasD-lacZ* in the wild-type (ORB7963), *nsrR* mutant (ORB7967) and *nsrR-his$_{12}$* (ORB7964) strains cultured anaerobically in 2xYT supplemented with 0.5% glucose and 0.5% pyruvate. T_0 represents the end of exponential growth. (B) Expression of *sdpA-lacZ* in the wild-type (ORB7968), *nsrR* mutant (ORB7972), and *nsrR-his$_{12}$* (ORB7969) strains cultured anaerobically in 2xYT supplemented with 0.5% glucose and 0.5% pyruvate. Open circle, wild type; closed square, the *nsrR* mutant; closed circle, the strain carrying *nsrR-his$_{12}$* at the *nsrR* locus.

fermentation nor nitrate respiration conditions (Figure 4.4B).

This result confirmed NO-sensitive interaction of NsrR to the *nasD* promoter, which is consistent with *in vitro* binding results (Chapter 2, Figure 2.9). In addition, it also showed that NsrR was associated with the *ykuN* regulatory region in an NO-sensitive manner (Figure 4.4A) contrary to the binding results observed *in vitro* (Chapter 3, Figure 3.7). However, the *in vivo* interactions observed herein is in good agreement with the result that NO relieves repression of *ykuN-lacZ* around T_1 during fermentative growth (Chapter 3, Figure 3.9). In contrast, NsrR was not found associated with the *sdpA* promoter in the wild-type background under any of the conditions tested (Figure 4.4B).

4.4.2 ResD interaction with NsrR class I and class II regulon gene promoters

As ResD was shown to be required for the derepression of class II genes in the *nsrR* mutant (Chapter 3, Figure 3.10), we want to ask if the effect of ResD is direct in other words, whether ResD plays the role by directly binding to the regulatory region of these genes. We used ResD-His_{12} ChAP-qPCR to determine if ResD interacts with any of the NsrR-regulated gene promoters. The *B. subtilis* strain carrying *resD-*His_{12} at the native locus was able to activate *nasD* transcription during nitrate respiration (data not shown), confirming that the tagged ResD protein is functional in *B. subtilis*. We used the *nasD* promoter as a positive control, since our previous results showed direct binding of ResD to *nasD in vitro* (Nakano *et al.* 2000). The ResD-His_{12} strain was grown anaerobically under fermentation conditions and cultures harvested at T_1 were processed for ChAP-qPCR analysis. A 5-fold ResD-dependent enrichment of the *nasD* and *ykuN* promoters were observed (Figure 4.5A). Transcription of *nasD* and *ykuN* is repressed by

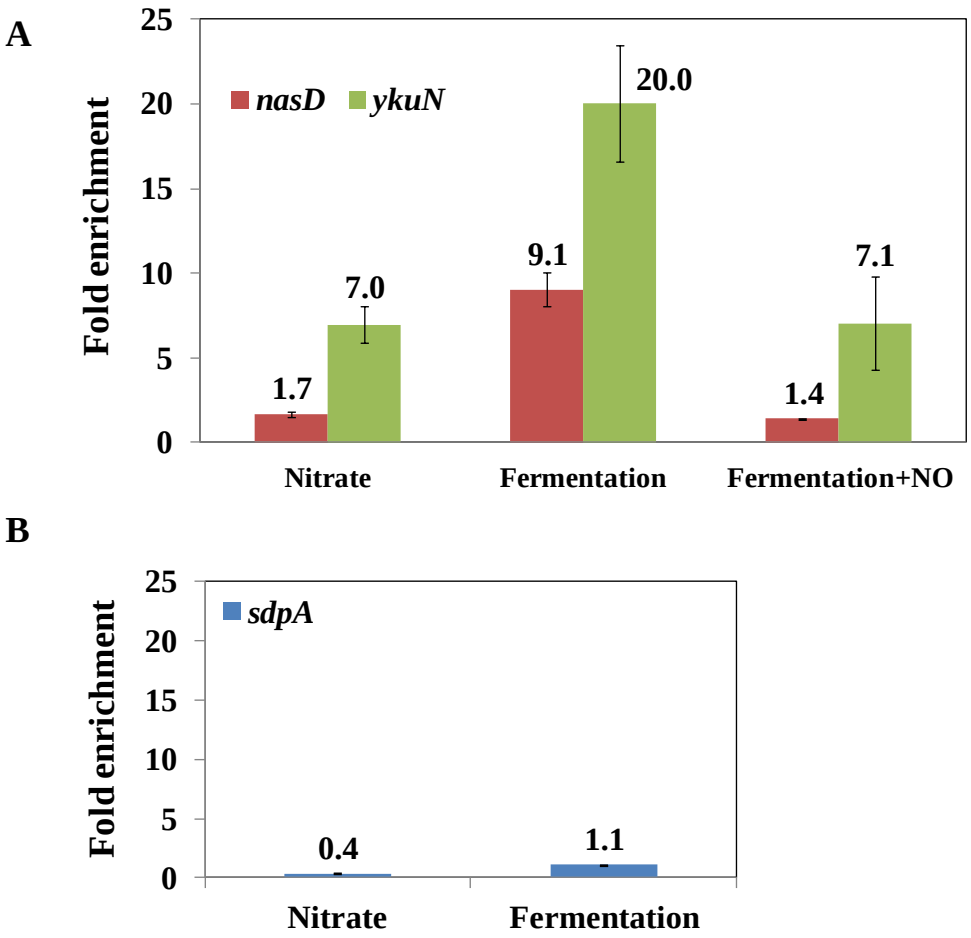


Fig. 4.4 NsrR interaction with class I (*nasD)* and class II (*ykuN)* regulon genes is NO sensitive. *B. subtilis* strains were grown anaerobically in 2×YT supplemented with 1% glucose and 0.2% nitrate (Nitrate) (A, B) or with 0.5% glucose and 0.5% pyruvate (Fermentation) (A, B), or 0.5% glucose and 0.5% pyruvate, followed by the addition of 50 µM SperNO when OD_{600} of cultures reached around 0.5 (Fermentation+NO) (A). Interaction of NsrR-His_{12} with *nasD* (A), *ykuN* (A), and *sdpA* (B) was analyzed by ChAP-qPCR. The ChAP-qPCR quantities were normalized against the negative control, *rpsD* as described in Materials and Methods. Data are averages from at least three independent cultures. Numbers above the bars represent the average of the fold enrichment obtained from three independent biological samples. Error bars indicate the standard error.

NsrR and NsrR/Fur, respectively (Chapter 3, Figure 3.10). We therefore asked whether a null mutation in these genes affects ResD binding to the tested promoters. The *nsrR* mutation led to a 5-fold increase in association with the *nasD* promoter. Based on the result, we conclude that NsrR inhibits ResD binding to the *nasD* promoter. In contrast, the *abrB* and *fur* mutation did not significantly affect ResD interaction with *nasD* as expected (Figure 4.5A). ResD-dependent enrichment of the *ykuN* promoter observed in the wild-type cells was reduced by 3-fold in the *nsrR* and *fur* mutants (Figure 4.5A). The study uncovered the direct interactions of three transcriptional regulators, namely, ResD, NsrR, and Fur with the *ykuN* promoter in post-exponential fermentative growth. It also demonstrated that efficient binding of ResD to the *ykuN* promoter requires NsrR and Fur. Like NsrR, the ResD ChAP-qPCR did not enrich for the *sdpA* promoter DNA in the wild-type and the mutant strains (Figure 4.5A), leading us to conclude that ResD does not bind to the *sdpA* promoter under the conditions tested.

4.4.3 NO effect on ResD interaction with the *nasD* and *ykuN* promoters

Figure 4.4A suggests that NsrR binding to both *nasD* and *ykuN* is reduced after NO treatment, yet ResD binding to these promoters was oppositely affected by the *nsrR* mutation (Figure 4.5A). Therefore, we decided to determine the effect of NO on ResD binding to these promoters. In Chapter 3, Figure 3.9 showed that NsrR and Fur repression of *ykuN* transcription is NO-sensitive *in vivo.* NsrR, not Fur, plays a major role in NO-sensitive repression in cells at post-exponential growth phase from which the ChAP samples were prepared (Chapter 3, Figure 3.9). If NsrR interaction to the *ykuN* promoter is NO sensitive, we expect that the presence of NO in cultures would

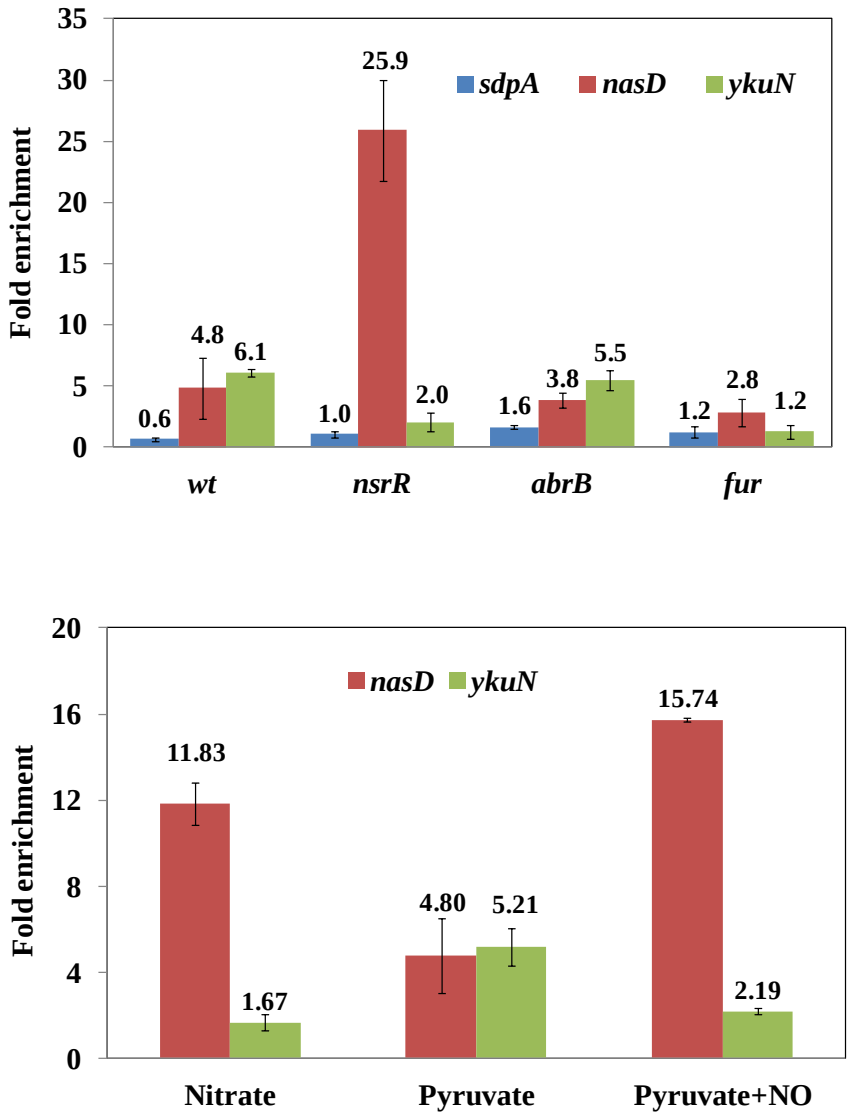


Fig. 4.5 *In vivo* association of ResD with *nasD*, *ykuN*, and *sdpA*. (A) wild type, *nsrR*, *abrB* and *fur* mutants producing ResD-His$_{12}$ were grown anaerobically in 2×YT supplemented with 0.5% glucose and 0.5% pyruvate (Fermentation). Cells were harvested at T_1 to determine ResD-dependent enrichment of *sdpA*, *nasD*, and *ykuN*. (B) NO effect on ResD binding to the class I (*nasD)* and class II (*ykuN*) promoters was determined from the cell cultures grown in 2×YT supplemented with 1% glucose and 0.2% nitrate (Nitrate) or with 0.5% glucose and 0.5% pyruvate (Fermentation) or 0.5% glucose and 0.5% pyruvate in the presence of 50 µM SperNO (Fermentation+NO). Interaction of ResD-His$_{12}$ with *nasD* (A,B), *ykuN* (A,B), and *sdpA* (A) was analyzed by ChAP-qPCR. The ChAP-qPCR quantities obtained were normalized against the negative control, *rpsD* (Materials and Methods). Data are averages from at least three independent cultures. Error bars indicate the standard error.

cause the dissociation of NsrR from *ykuN*, thus affecting ResD binding at the *ykuN* promoter. The result in Figure 4.5B showed that is the case, as NO resulted in a 3-fold and 2.5-fold reduction in ResD-*ykuN* interaction during nitrate respiration and by SperNO treatment, respectively.

As described previously, NO-treated NsrR showed substantially reduced binding to the *nasD* promoter *in vitro* (Chapter 2, Figure 2.10), resulting in the upregulation of ResD-dependent *nasD* transcription both *in vivo* (Nakano *et al.* 2006) and *in vitro* (Chapter 2, Figure 2.6). We next examined the effect of NO on ResD binding to *nasD*. After the exposure of cultures to SperNO, binding of ResD increased 3.2-fold compared to the untreated cultures (Figure 4.5B). Enhanced binding of ResD (2.4-fold) was also evident under NO-producing nitrate respiration conditions (Figure 4.5B). Taking these results together, we concluded that interaction of NO leads to NsrR dissociation from the *nasD* promoter, which in turn increases ResD binding to the promoter DNA, thus activating transcription. The NO effect on ResD binding coincides with the *nsrR* mutation effect (Figure 4.5A and B), suggesting that it is the loss of NsrR binding that affects ResD interaction positively (*nasD*) or negatively (*ykuN*) depending on the promoter.

4.4.4 *In vivo* phosphorylation of ResD is independent of NsrR

The ChAP-qPCR studies in the preceding section indicated that NsrR and ResD play direct roles in transcriptional regulation of *nasD* and *ykuN* by interacting with these promoter regions. In contrast, as NsrR and ResD do not bind the *sdpA* promoter, it is likely that these regulators indirectly affect the transcription of the *sdpA* operon as well as

other AbrB/Rok-controlled genes in the class II NsrR regulon. The derepression of *sdpA* caused by the *nsrR* mutation was abolished in the *nsrR resD* mutant (Chapter 3, Figure 3.10), indicating that the *resD* mutation is epistatic to the *nsrR* mutation. These results raised a possibility that the *nsrR* mutation either upregulates *resD* expression and/or ResD phosphorylation, which in turn activates transcription of a yet-unidentified transcription factor that is directly involved in *sdpA* transcription.

First, we examined if the *nsrR* mutation leads to an increase in intracellular ResD concentrations by western blot analysis. Cell lysates were prepared from cells at T_1 where *sdpA* expression in the *nsrR* mutant reaches a maximum. The wild-type strain and *nsrR* mutant showed a similar level of ResD amount under the three conditions tested (Figure 4.6A). Second, *in vivo* levels of ResD~P were determined by Mn^{2+}-Phos-tag SDS-PAGE, followed by western blot analysis. Because phosphoproteins run slower than unphosphorylated proteins during electrophoresis in Phos-tag gels, this technique has been used to detect phosphoproteins (Kinoshita *et al.* 2006; Barbieri *et al.* 2008). As shown in Figure 4.6B and C, we compared the ratio of ResD~P in the wild-type and *nsrR* mutant strains. As a qualitative control, purified ResD protein phosphorylated by acetyl phosphate *in vitro* was used to determine the mobility of the unphosphorylated and phosphorylated forms of ResD (Figure 4.6B). Western blot analysis of ResD in the cell lysates often revealed two bands specific to ResD~P (Figure 4.6B). The reason for and nature of the two bands are currently unknown. However, as both bands are absent in the *resD* mutant, the two bands were considered as ResD~P for the quantitation shown in Figure 4.6C. The results showed that ResD phosphorylation is enhanced under fermentation conditions compared to nitrate respiration conditions (Figure 4.6C). Even

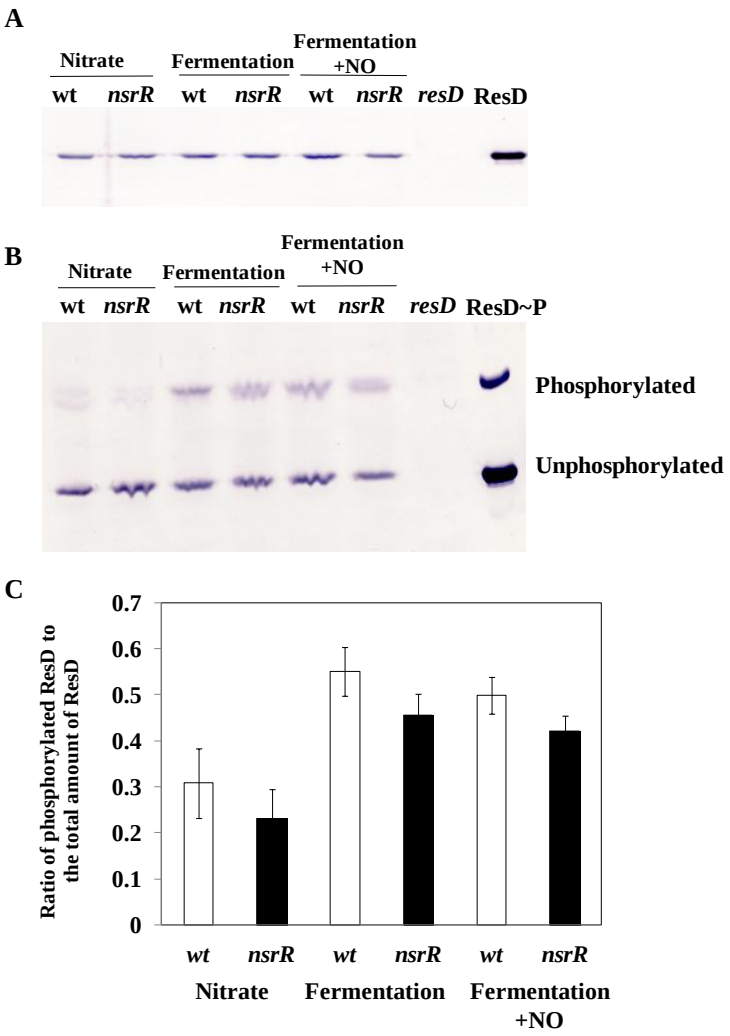


Fig. 4.6. Effect of *nsrR* on ResD concentrations and phosphorylation during anaerobiosis. The wild-type (JH642) and *nsrR* mutant (ORB 6179) strains were grown anaerobically in 2×YT supplemented with 1% glucose and 0.2% nitrate (Nitrate) or with 0.5% glucose and 0.5% pyruvate (Fermentation), or 0.5% glucose and 0.5% pyruvate followed by the addition of 50 µM SperNO when OD_{600} of the cultures reached around 0.3-0.4 (Fermentation+NO). Cells were harvested at post-exponential phase (T_1) or one hour after the addition of SperNO. As a negative control, the *resD* mutant (LAB 2511) cell lysate was used (A and B) and ResD protein phosphorylated with acetyl phosphate was used as a positive control (A and B). (A) Western blot analysis of cell lysates to detect total ResD amount. (B) Western blot of cell lysates separated on a 25 µM Phos-tag acrylamide gel to detect ResD-P. (C) ImageJ software was used to quantify the ratio of shifted band caused by the phosphorylation of ResD to the total amount of ResD. The amount of ResD in each sample was calculated from the sum of the intensities of all bands in each lane and subtracted from the background obtained in the *resD* mutant. Each value is the average of three independent experiments derived from the western blots similar to that in A. Error bars indicate standard deviation. Open boxes indicate wild type and closed boxes indicate *nsrR* mutant.

when exogenous NO was supplied in fermentation cultures, the phosphorylation of ResD was not affected. The *nsrR* mutation did not affect the phosphorylation of ResD under all three conditions tested (Figure 4.6C). Therefore, these results ruled out the possibility that the *nsrR* mutation either upregulates *resD* expression or ResD phosphorylation. Additionally our results might indicate the role of pyruvate in phosphorylation of ResD, which will be discussed later in Chapter 5.

4.5 DISCUSSION

Transcriptome studies revealed that NsrR functions more widely in transcriptional control than originally thought (Chapter 3). In this study, we investigated how multiple transcriptional regulators control expression of the class I gene (*nasD*) and class II genes (*sdpA* and *ykuN*). Specifically, we examined whether some or all of the regulators interact with each promoter, thus affecting binding and/or activity of other regulators. We presented evidence using ChAP-qPCR experiments that both NsrR and ResD bind to the *nasD* and *ykuN* promoters and NsrR interaction is NO sensitive (Figure 4.4A and 4.5A). This result finally settled the question as to the NO-sensitivity issue of NsrR binding to *ykuN* and likely other Fur regulon genes. Accordingly, we now conclude that [4Fe-4S]-NsrR participates in repression of the Fur regulon genes.

The study also uncovered that NsrR has the opposite effect on ResD interaction with the *ykuN* promoter than observed with the *nasD* promoter (Figure 4.5A). NsrR inhibits ResD binding to the *nasD* promoter (Figure 4.5A), and in the presence of NO, NsrR dissociates from the promoter (Figure 4.4A), leading to the enriched ResD interaction with DNA to activate transcription (Figure 4.5B). This study further

confirmed the previously identified inhibitory role of NsrR on the ResD-RNAP-*nasD* ternary complex (Chapter 2, Figure 2.8).

In contrast to the effect of NsrR on the *nasD* promoter, efficient binding of ResD to the *ykuN* promoter requires the association of Fur and NsrR with the promoter region (Figure 4.5A). We proposed earlier that ResD likely antagonizes Fur repressor activity, while it enhanced NsrR repressor activity, in transcription of *ykuN* during postexponential growth. According to the ChAP-qPCR results, we further speculate that binding of NsrR or Fur likely brings about changes in the DNA topology nearby, which facilitates ResD binding. The binding of ResD changes the DNA conformation, resulting in decreased Fur binding and increased NsrR binding. However, it is possible that the effect of ResD on the repression by Fur does not involve the dissociation of Fur. A previous study demonstrated that ComK functions as an antirepressor of Rok and CodY without preventing binding of the repressors to DNA (Smits *et al.* 2007). To test these possibilities, we will examine the effect of ResD on binding of NsrR and Fur to the *ykuN* promoter. To this end, we will construct a strain carrying *fur-his*$_{12}$ at its own locus in a similar way used for the *resD-his*$_{12}$ construct.

The work in this chapter only focused on cells at T_1 where the effect of NsrR on *ykuN* transcription is prominent. As shown in Chapter 3 (Figure 3.9), Fur represses *ykuN* transcription during exponential growth (at T_{-2} to T_{-1}) and ResD is required for upregulation of *ykuN* in the absence of Fur, which is reminiscent of the *resD* effect on *sdpA* expression. In order to determine whether the role of ResD in exponential growth is through binding to *sdpA*, ChAP-qPCR of exponential cultures (at T_{-2}) will be carried out in the future.

Finally, this study showed that both ResD and NsrR play indirect roles in transcription of another member of class II genes, *sdpA* that belongs to the AbrB/Rok regulon. Because the *resD* mutation is epistatic to the *nsrR* mutation in the transcriptional control of *sdpA* (Figure 3.10A), we thought that NsrR might play an indirect role to repress *resD* transcription and/or ResD phosphorylation, which indirectly affects *sdpA* transcription through another transcription factor. Figure 4.6 revealed that the *nsrR* mutation does not affect either ResD amount or its phosphorylation and the nature of indirect role of ResD and NsrR remains to be unveiled. We are also aware that the *ykuN* and *sdpA* regions amplified by qPCR are rather small (130bp to 180bp) and might not fully cover NsrR- and ResD-binding regions. The apparent lack of *sdpA* enrichment by NsrR and ResD could be caused by this technical limitation. We will address the questions by using different and overlapping PCR primers in ChAP-qPCR. Furthermore, we extend our collaboration with Dr. Shu Ishikawa's group (NAIST, Japan) to identify genome-wide NsrR and ResD-binding profiles using ChAP-chip analysis. This approach will be more effective in identifying interaction with ResD and NsrR anywhere in or in the vicinity of the *sdpA* gene. Binding of AbrB to *sdpA* was clearly detected by a similar method (Chumsakul *et al.* 2011). If we find that ResD and/or NsrR do not interact with the *sdpA* promoter, we will search in the NsrR regulon for a gene that encode a possible transcription factor directly involved in *sdpA* transcription.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

5.1 SUMMARY

5.1.1 Mechanism of NsrR-dependent transcription regulation at the *nasD* promoter in response to NO

Due to the existing controversy about the nature of the Fe-S cluster, we confirmed that *B. subtilis* NsrR carries a [4Fe-4S] cluster in collaboration with Dr. Pierre Moënne-Loccoz laboratory. We concluded from the results that anaerobically purified BsNsrR, purified either from *E. coli* or *B. subtilis* cultures, contains a [4Fe-4S] cluster. We also investigated the hypothesis that NsrR activity is regulated by NO-dependent modification of the [4Fe-4S] cluster. EMSA using a *nasD* -35 region probe containing the putative NsrR binding site showed that the [4Fe-4S] cluster is essential for NsrR to bind to the target DNA with high affinity. A NO donor, SperNO, inhibits binding of the holo-NsrR to the target DNA, whereas binding of apo-NsrR is not affected by SperNO. The ternary complex formed by ResD-RNAP-*nasD* promoter DNA was dissociated by the addition of [4Fe-4S]-NsrR. This is partly through a disruption of the RNAP-*nasD* binary complex as shown by the EMSA studies. This complex was restored by the addition of SperNO, which explains the upregulation of *nasD in vivo* and ResD-dependent transcriptional activation *in vitro* by NO. These findings were further strengthened by the *in vivo* binding studies with ResD and NsrR. ResD interaction with the *nasD* promoter was enhanced in the absence of NsrR or in the presence of NO. It is speculated that NsrR that displaces RNAP-*nasD* binary complex can also inhibit ResD binding by two mechanisms: (1) NsrR either directly inhibits ResD binding by occupying the class II

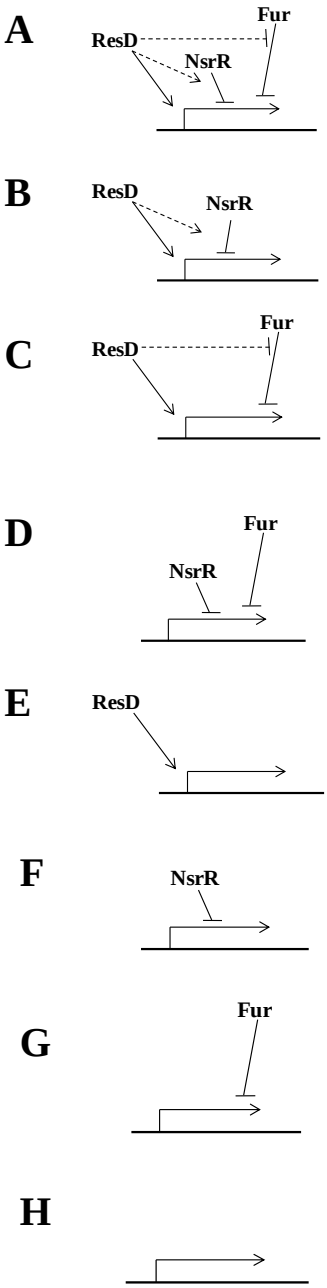
binding site or/and (2) exerts its inhibitory role through displacing RNAP, as efficient ResD interaction with DNA requires RNAP.

5.1.2 Classification of the NsrR regulon into class I and class II

Identification of additional NsrR-binding sites at the *nasD* promoter with unique binding requirements suggested another type of transcriptional control governed by NsrR. This led us to classify NsrR binding into class I and class II based on the nucleotide sequence specificity for its binding and affinity requirement for [4Fe-4S]. The *in vitro* and *in vivo* results of mutational analysis indicated that a partial dyad symmetry sequence is required for efficient [4Fe-4S]-NsrR binding to the class I site. On the other hand, class II binding of NsrR involves relaxed DNA sequence specificity involving an A+T-rich target sequence. We extended our study on identifying the class II regulon to further understand NsrR regulation. Transcriptome analysis suggested a significant number of genes are derepressed in the *nsrR* mutant and most of these were previously identified to be regulated by other transcription factors, like AbrB, Rok and Fur. *In vivo* transcription experiments, followed by *in vitro* binding studies, suggested that NsrR repression of some of AbrB/Rok and Fur regulon genes is likely direct. NsrR repression has a stronger effect under fermentative conditions than in nitrate respiration. Expression of class II genes is moderately induced by NO in an NsrR-dependent manner, suggesting that class II regulation is NO sensitive *in vivo*. *In vivo* binding experiments further confirmed that NsrR interaction to the *ykuN* promoter is direct and NO sensitive. However, NsrR interaction *in vivo* with the *sdpA* promoter was not observed under any of the conditions tested, suggesting the influence of NsrR on *sdpA* expression is indirect.

5.1.3 Role of ResD in controlling class II regulon of NsrR

During our study on the NsrR regulon, we identified that ResD controls most (if not all) of class II NsrR genes tested. ChAP-qPCR analysis showed that ResD interacts with the *nasD* and *ykuN* promoter. We propose that ResD antagonizes Fur-mediated repression, while it enhances NsrR repression at the *ykuN* promoter. The hypothesis is based on *ykuN-lacZ* expression in different mutant backgrounds (Figure 5.1). Comparison of Figure 5.1B and 5.1F shows that NsrR-dependent repression is 2-fold stronger in the presence of ResD. Conversely, Fur repression is relieved 4-fold when ResD is present (compare Figure 5.1C and G). In the presence of ResD, repression is primarily exerted by NsrR (Figure 5.1A and B), whereas in the absence of ResD, Fur repression plays a major role (Figure 5.1D, F, and H). The effect of ResD is mainly through the repressors, as in the absence of NsrR and Fur, the *resD* mutation has no effect on *ykuN* expression (compare Figure 5.1E and H). In sum, ResD plays three different roles in controlling NsrR-repressed *nasD* and *ykuN* gene expression. As ResD is absolutely needed for *nasD* transcription both in *nsrR*$^+$ and *nsrR* strains, ResD functions as an activator of *nasD*. At the *ykuN* promoter, ResD functions as an antirepressor of Fur and it enhances NsrR repression. The current study presented evidence of a complex regulation controlled by multiple regulators that recognize A+T-rich DNA sequence in the promoter regions.



β-Galactosidase activity
(Miller units ± standard deviation)

335 ± 66

354 ± 15

829 ± 82

233 ± 47

1031 ± 15

774 ± 134

211 ± 84

1082 ± 25

Fig. 5.1. A model of transcriptional regulation at the *ykuN* promoter during the post-exponential phase of growth (T_1) under fermentation conditions. The schematic view represents the following: (A) wild type; (B) *fur;* (C) *nsrR*; (D) *resD;* (E) *nsrR fur*; (F) *fur resD*; (G) *nsrR resD* and; (H) *nsrR fur resD.* Miller units were obtained from the results shown in Figure 3.10 C/D and unpublished data. Positive regulation is labeled with an arrow, while repression is labeled with a ⊥. Dotted lines represent the regulation yet to be determined.

5.1.4 Effect of pyruvate on ResD phosphorylation

NsrR and NO have no effect on ResD phosphorylation levels. However, the study indicated that pyruvate but not glucose in rich medium stimulates phosphorylation. We also observed that *in vivo* ResD~P levels are around 2-fold higher in pyruvate fermentation than nitrate respiration conditions. Despite the fact that the ResD-ResE signal transduction system has been studied over the past 15 years mainly by Marion Hulett's and our laboratories, what is the signal for the ResE kinase remains uncovered. The increased phosphorylation during fermentative growth could be due to NADH accumulation or higher NADH/NAD^+ ratio, which might change ResE to kinase dominant over phosphatase. This hypothesis was previously tested and rejected in our laboratory (unpublished results). An alternative possibility is that a metabolite derived from pyruvate is a signal molecule for the ResE kinase. The use of Phos-tag acrylamide gels likely provides us with a useful tool for examining the possibility.

5.2 FUTURE DIRECTIONS

We will test the hypothesis proposed in Figure 5.1. We speculate that binding of ResD causes a change in topology around the *ykuN* promoter region, thus affecting the binding affinity of NsrR and Fur in the opposite manner. We will investigate the ResD effect on NsrR and Fur binding to *ykuN* by ChAP-qPCR analysis in wild type and the *resD* mutant. This would reveal if ResD presence is required for NsrR to bind and exert its repressor function at the *ykuN* promoter. Using a similar approach the ResD effect on Fur binding will be analyzed. We assume from the model of Figure 5.1 that Fur- and NsrR-bindings to *ykuN* do not affect each other directly, but this possibility will be

determined similarly by using the *nsrR* or *fur* mutant. We expect that *ykuN* is more enriched in Fur ChAP in the absence of ResD and reduced in NsrR ChAP.

Though all three regulators seem to bind to the *ykuN* regulatory region, it is unknown whether each binding site overlaps. To localize binding regions occupied by the regulators, we will undertake an *in vivo* and *in vitro* approach. First, deletion and mutational analysis of the promoter region will be carried out and transcriptional *lacZ* fusions to the resultant promoter fragments will be constructed. The effect of null mutations on *ykuN-lacZ* expression in the *resD, fur*, and, *nsrR* mutants will be determined and compared with expression in the wild-type strain. The effect of promoter deletions/mutations on NsrR and Fur repression could be caused by the loss of binding sites of either of these repressors or ResD. Final conclusions will be drawn in comparison with the *in vivo* binding data using ChAP-qPCR on mutant promoters. In order to localize the *cis* region targeted in each promoter, we also plan to employ *in vivo* ChAP-DNase I footprinting that involves ligation-mediated PCR. This approach, if it does work, could be a powerful alternative to *in vitro* DNase I footprinting, as the latter method is not suitable for proteins that require a distinct DNA topology for binding.

Another interesting question to be addressed is the role of iron coordination to Fur in the observed *ykuN* transcriptional regulation. Previous results on *ykuN-lacZ* expression suggested iron limitation in cells at T_1 when cells are grown in 2xYT medium under fermentation conditions. This speculation about iron limitation is based on the notion that Fur does not repress *ykuN* transcription at T_1 (Figure 3.9A) nor responds to NO (Figure 3.9C). If the speculation is correct, Fur repression observed in Figure 5.1G could be exerted by apo-Fur. If ChAP-qPCR described above shows that Fur binds to

ykuN, the questions are: (1) does apo-Fur bind to the previously identified Fur box? (2) is ResD capable of antagonizing apo-Fur, but not holo-Fur? An alternative possibility is that a small fraction of the Fur population in cells is associated with iron. We will investigate whether NO affects Fur ChAP-qPCR to answer these questions.

Finally, we will investigate roles of ResD and NsrR in transcription of *sdpA*. The possibility remains that ResD affects *sdpA* expression by antagonizing AbrB-mediated repression. If it is the case, ResD should affect AbrB repression without binding to *sdpA*, which is in sharp contrast to the effect of ResD on Fur at the *ykuN* promoter. As the ChAP-qPCR showed that ResD does not bind to the *abrB* promoter, it is unlikely that ResD directly affects *abrB* transcription. We will identify genome-wide ResD- and NsrR-binding profiles using ChAP-chip analysis in collaboration with Dr. Shu Ishikawa's group (NAIST, Japan) to identify candidate from ResD or NsrR targetted genes that might function in *sdpA* control. The studies might provide a clue to the indirect roles of ResD and NsrR in transcriptional regulation of the AbrB/Rok regulon.

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BIOGRAPHICAL SKETCH

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