# ResDE-dependent transcriptional control in response to oxygen limitation

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# ABSTRACT

## ResDE-dependent transcriptional control in response to oxygen limitation

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The ResD-ResE two-component signal transduction system is required for the expression of genes involved in anaerobic nitrate respiration in *Bacillus subtilis*. Response regulator ResD is phosphorylated by the cognate histidine sensor kinase ResE. Phosphorylated ResD (ResD~P) activates transcription of genes including *fnr*, *nasDEF*, and *hmp*. High-resolution hydroxyl radical footprinting assay and mutational analysis of the ResD-controlled promoter regions revealed that two monomers of ResD bind to the $TTGTAAN_3TTN_4A$ sequence. ResD binds either to a single site or tandemly to multiple sites as monomer or dimer, and it likely binds in more than one orientation, indicating the flexibility of ResD binding to DNA.

E254, V260, Y263, K267, A269 of the C-terminal domain of the α subunit (αCTD) of RNA polymerase (RNAP) are critical for transcription activation of *fnr* and *nasD*. These residues constitute a surface-exposed patch that might interact with ResD during transcription initiation. In contrast, ResD likely interacts with region 4.2 of $\sigma^A$ at the *hmp* promoter. Amino acid residues in the transactivation loop of ResD were shown to be essential for *fnr* and *nasD* expression, implying that these residues directly interact with αCTD; however, DNase I footprinting experiments showed that these residues are likely involved in DNA binding. None of the mutations in the transactivation loop

affected *hmp* expression, leaving ResD regions that interact with RNAP at these promoters unidentified.

In addition to the phosphorylation-dependent activation, unphosphorylated ResD upregulates genes of the ResDE regulon in response to oxygen limitation. Furthermore, the NsrR transcriptional regulator participates in activation of of the ResDE regulon, particularly *hmp* and *nasD* when nitrate is available. NsrR is likely to contain an iron-sulfur cluster that may sense NO that is generated during nitrate respiration. Interaction of NsrR with NO results in the altered NsrR activity as a transcription regulator.

# CHAPTER 1
# INTRODUCTION

## 1.1 BACILLUS SUBTILIS AND ANAEROBIC GROWTH

*Bacillus subtilis* has been used in the food industry for thousands of years in eastern Asia. *B. subtilis* is also widely applied to the enzyme industry due to the ability to host exogenous recombinant DNA and secrete functional proteins. Besides extensive application in industry, *B. subtilis* is used as a model organism of gram-positive bacteria because the bacterium is amenable to genetic manipulation.

*B. subtilis* had been believed to be a strictly aerobic organism for a long time. Our laboratory together with others showed that *B. subtilis* can grow under anaerobic conditions [see review (Nakano and Zuber, 1998, 2002)]. Its capability to grow under anaerobic conditions makes sense from a physiological viewpoint because the natural habitat of *B. subtilis* is soil where oxygen availability is easily changed by fluctuations in water content. In soil, when oxygen concentration is lower than 1%, bacteria have to switch to the anaerobic mode of metabolism (Paul, 1996).

Global gene expression in response to oxygen-limited conditions in *B. subtilis* was studied using microarray analysis (Ye *et al.*, 2000). Compared to gene expression profile under aerobic growth conditions, several hundreds of genes are induced or repressed when oxygen is limited. These genes are involved in carbon metabolism, electron transport, iron uptake, antibiotic production, and stress response. Certain genes required for anaerobic respiration are highly induced during anaerobic growth in the presence of nitrate, while genes involved in fermentation are induced when cells are anaerobically grown in the presence of glucose and pyruvate, indicating that *B. subtilis* can adjust anaerobic metabolism in response to the presence of nitrate. The goal of our research is to elucidate the mechanism by which *B. subtilis* controls gene regulation in response to oxygen limitation and the presence of nitrate.

When oxygen becomes limited, some facultative bacteria can carry out anaerobic respiration using alternative electron receptors including nitrate, ferric iron, Mn (IV),

sulfate, carbon dioxide and organic intermediates, however only nitrate can be utilized as an alternative electron acceptor in *B. subtilis*. If nitrate is not available under oxygen-limited conditions, *B. subtilis* still can survive and grow by mixed acid fermentation (Cruz Ramos *et al.*, 2000; Nakano *et al.*, 1997). Anaerobic fermentative growth of *B. subtilis* is enhanced when pyruvate is supplemented in addition to glucose (Nakano *et al.*, 1997). The requirement of pyvurate is not clear; however, *B. subtilis* lacks formate lyase and utilizes pyvurate dehydrogenase to convert pyvurate to acetyl-CoA. The transcriptome analysis described above showed that the pyvurate dehydrogenase genes are repressed during anaerobic fermentative growth and the repression is alleviated by the addition of pyvurate (Ye *et al.*, 2000). Therefore, pyvurate might be required for efficient fermentation to fully express the pyvurate dehydrogenase genes. Fermentation is a less energy-generating pathway than respiration that generates energy by oxidative phosphorylation, therefore the bacterium prefers respiration rather than fermentation. It was reported that the growth of *B. subtilis* under strict anaerobic conditions requires deoxyribonucleosides or DNA likely due to weak ribonucleotide reductase activity (Folmsbee *et al.*, 2004); however, another report showed that *B. subtilis* grows under strict anaerobic conditions in the absence of deoxyribonucleosides and that class I ribonucleotide reductase is essential both for aerobic and anaerobic growth (Hartig *et al.*, 2006).

One of challenges to some pathogenic bacteria is to adjust to anaerobic growth conditions during host invasion. Therefore, anaerobic growth conditions stimulate virulence genes expression and may lead to biofilm formation in certain pathogenic *Staphylococci* (Cramton *et al.*, 2001). Bacteria encased in biofilms are more resistant to host defense and chemotherapy than planktonic bacteria, which accounts for persistent infection of bacteria in biofilm (Lewis, 2005). In a biofilm community, bacteria have to undergo anaerobic growth in the deep layer where oxygen is not sufficient to support aerobic growth. Therefore, the results obtained from the research of *B. subtilis* anaerobiosis would provide an insight into our understanding of pathogenesis of low GC gram-positive bacteria, such as *Bacillus anthracis*, *Bacillus cereus*, *Staphylococcus aureus* and *Listeria monocytogenes*.

## 1.2 ANAEROBIC RESPIRATION OF ESCHERICHIA COLI

*Escherichia coli* and *B. subtilis* share a part of regulatory pathway that includes FNR which controls gene expression in response to anaerobic growth conditions, whereas some regulatory pathways such as ArcAB and ResDE are unique to either bacterium. Therefore, we will herein summarize gene regulation involved in *E. coli* anaerobic nitrate respiration. When oxygen becomes limited, *E. coli* can gain energy by either anaerobic respiration or mixed acid fermentation. *E. coli* can use various alternative electron acceptors that include nitrate, fumarate, and DMSO (Gennis and Stewart, 1996). The Arc (aerobic respiration control) two-component signal transduction system [see review (Gunsalus and Park, 1994)] and FNR (fumarate and nitrate reductase regulator) [see review (Kiley and Beinert, 1999)] are involved in the switch from aerobic to anaerobic respiration. In addition, the NarXL and NarPQ two-component regulatory proteins regulate the expression of genes involved in anaerobic respiration in response to nitrate and nitrite, respectively (Gennis and Stewart, 1996).

### 1.2.1 ArcA-ArcB system

ArcAB activates expression of genes whose products are important for anaerobic growth, while it represses expression of genes involved in aerobic respiration (Iuchi and Lin, 1988). However, a study using steady-state glucose-limited chemostat cultures indicated that the ArcAB system plays a role in sensing microaerobiosis (Alexeeva *et al.*, 2003). Furthermore, analysis of the *arc* knock-out mutation identified that ArcAB regulates genes required for functions other than respiration such as those involved in flagellar synthesis (Oshima *et al.*, 2002). The ArcAB system was also shown to control virulence in *Haemophilus influenzae* and *Vibrio cholerae* (De Souza-Hart *et al.*, 2003; Sengupta *et al.*, 2003). In addition, microarray analysis and bioinformatics approach identified that ArcA can affect expression of 9% of the open reading frames in *E. coli*, indicating that ArcA is a global regulator (Liu and Wulf, 2004).

The ArcAB two-component signal transduction system consists of ArcA, a DNA-binding response regulator, and ArcB, a membrane-bound histidine kinase. The mechanism of signal sensing and output by the ArcAB system has been elucidated in

detail. The kinase activity of ArcB is regulated by sensing the redox state of the membrane quinone pool (Georgellis *et al.*, 2001a). Under anaerobic conditions, the quinone pool remains in the reduced state due to low electron transfer and ArcB kinase domain dimerizes, which is essential for its kinase activity. When oxygen is present, the quinone pool shifts to the oxidized state. Cysteine residues in the PAS domain are oxidized by quinone, and form intermolecular disulfide bond between the two monomers, which causes a conformational change, resulting in the silencing of kinase activity of ArcB (Malpica *et al.*, 2004).

In addition to quinones, anaerobic metabolites such as D-lactate, acetate and pyvurate were shown to enhance ArcB kinase activity (Georgellis *et al.*, 1998; Georgellis *et al.*, 1999). There are putative leucine zipper and the PAS domains in the cytoplasmic region of ArcB, which may be involved in sensing the metabolic signal. Some orthologs of ArcB in other bacteria, for example *H. influenzae*, do not contain the PAS (Per-Arnt-Sim) domain. However, an *arcAB* mutation in *E. coli* can be complemented by the *arcAB* genes from *H. influenzae*, indicating that the PAS domain is not essential for redox-sensing of *H. influenzae* ArcB (Georgellis *et al.*, 2001b).

### 1.2.2 FNR

FNR was first identified in *E. coli* as a mutation that causes defective anaerobic growth in the presence of fumarate or nitrate but does not affect aerobic growth (Lambden and Guest, 1976). FNR belongs to the cyclic AMP receptor (CRP) super family which regulates transcription via binding to a specific DNA sequence. Microarray analysis showed that FNR is a global anaerobic regulator required for adaptation to anaerobic conditions in *E. coli* (Kang *et al.*, 2005; Salmon *et al.*, 2003). Recent work has shown that FNR also regulates gene expression involved in *Salmonella* pathogenesis, an quite apart anaerobic metabolism (Fink *et al.*, 2007).

FNR of *E. coli* has an iron-sulfur cluster in the N-terminus and HTH (helix-turn-helix)-DNA binding domain in the C-terminus. Unlike *B. subtilis* FNR that will be discussed in 1.3.4, *E. coli* FNR has DNA-binding activity only in the dimerized form (Lazazzera *et al.*, 1996). Dimerization requires an intact [4Fe-4S] cluster, which is damaged via [2Fe-2S] transition state by oxygen (Kiley and Beinert, 2003). In addition to

the oxygen-dependent regulation of FNR activity, *E. coli* FNR is controlled at the transcriptional and post-translational levels. Under anaerobic conditions, *fnr* transcription is repressed by FNR itself (Mettert and Kiley, 2007) and is stimulated by integration host factor (IHF) in an oxygen-independent manner (Mettert and Kiley, 2007). Furthermore, FNR lacking the iron-sulfur cluster is degraded by ClpXP proteolysis under aerobic conditions (Mettert and Kiley, 2005). DNA microarray analysis revealed that about 100 genes are regulated by FNR and most of the genes are involved in anaerobic growth (Salmon *et al.*, 2003). There is an overlap of gene regulation between the ArcAB and FNR systems, i.e. some genes are under the dual-control, which benefits quick and accurate response of cells to oxygen-limited conditions (Liu and Wulf, 2004).

### 1.2.3 NarX-NarL and NarQ-NarP systems

In addition to the Arc system and FNR that regulate gene expression in response to oxygen-limitation conditions, the Nar system is involved in anaerobic gene regulation by sensing nitrate and nitrite. Although *E. coli* can use several alternate electron acceptors in the absence of oxygen, nitrate is an energically favorable acceptor due to its high redox potential and easy integration to nitrogen metabolism. The redox potential $E_h$ of nitrate/nitrite pair is 0.42V, whereas fumarate/succinate redox potential $E_h$ is 0.03V. The homologous dual two-component signal transduction systems NarX-NarL and NarQ-NarP are involved in anaerobic nitrate respiration in *E. coli* (Chiang *et al.*, 1992; Stewart *et al.*, 1989).

NarX and NarQ, the membrane sensor kinases, respond to nitrate and/or nitrite and phosphorylate, respectively NarL and NarP response regulators (Cavicchioli *et al.*, 1995; Schröder *et al.*, 1994; Walker and DeMoss, 1993). In the absence of nitrate and nitrite, NarX can act as a phosphatase to keep NarL in the unphosphorylated state (Darwin and Stewart, 1996). The presumed NarQ-dependent dephosphorylation of NarP has not yet been examined directly (Stewart, 2003). The anion-specific in vitro assay using full-length NarX under the *E. coli* membrane environment directly showed NarX undergoes autophosphorylation and phosphorylates NarL, which was stimulated by nitrate and nitrite although nitrite is less efficient than nitrate (Lee *et al.*, 1999). NarQ recognizes nitrate and nitrite equally (Chiang *et al.*, 1997). A 17 amino acid sequence in

the periplasmic loop is highly conserved among NarX and NarQ orthologs and was designated the P box element (Cavicchioli *et al.*, 1996; Williams and Stewart, 1997). Certain mutations in P box altered NarX and NarQ response to nitrate or nitrite in vivo, suggesting that the P box is involved in nitrate- and nitrite-sensing.

NarL and NarP control the transcription of many genes involved in anaerobic respiration and fermentation (Böck and Sawers, 1996; Gennis and Stewart, 1996; Gunsalus, 1992; Stewart, 1993). The respiratory pathway genes include those for two cellular nitrate reductases (*narGHJI* and *napA*), two nitrite reductases (*nirBDC* and *nrfABCDEFG*), a nitrite exporter (*narK*). Among genes regulated by the Nar system, some of them can be regulated by either NarL or NarP (for example *nirB*) (Browning *et al.*, 2000), whereas some of them are only regulated by NarL (for example *narK* and *narG*) (Rabin and Stewart, 1993; Stewart, 1993).

The *narX/L* and *narQ/P* paralogs were also found in *Salmonella enterica*, whereas *H. influenzae*, *Neisseria meningitidis*, *Pasteurella multocida*, *V. cholerae*, and *Yersinia pestis* have only *narQ/P*, and *Pseudomonas aeruginosa* and *Ralstonia solanacearum* have only *narX/L* (Stewart, 2003). The implication of dual systems coexistence in *E. coli* and *S. enterica* is unknown. One hypothesis is that *E. coli* and *S. enterica* employ dual interacting Nar systems to respond to the dynamic ratios of the respiratory substrates, nitrate and nitrite (Stewart, 2003).

Some of the Nar regulon genes are FNR-dependent (Constantinidou *et al.*, 2006; Rabin and Stewart, 1993) and the Nar system also affects expression of the Arc regulon probably via alerting redox state of quinone pool (Constantinidou *et al.*, 2006; Georgellis *et al.*, 2001a; Malpica *et al.*, 2004). Therefore, the Arc, FNR and Nar system integrate a network to adapt metabolism pathway to anaerobiosis of *E. coli*.

## 1.3 ANAEROBIC RESPIRATION OF *B. SUBTILIS*

As mentioned earlier, *B. subtilis*, unlike *E. coli*, can only use nitrate as a terminal electron-acceptor alternative to oxygen. Our laboratory, together with others, identified genes involving in anaerobic nitrate respiration [see review (Nakano and Zuber, 1998) (Fig. 1.1)]. In this section, I will discuss how these genes are involved in nitrate

respiration. In addition, the role of ResD and ResE in anaerobic gene regulation will be discussed separately in section 1.4.

### 1.3.1 Nitrate reductase

In *B. subtilis*, two types of nitrate reductase are encoded by separate operons – *nasBC* and *narGHJI*. These enzymes have different cellular locations and physiological functions (Glaser *et al.*, 1995; Ogawa *et al.*, 1995). The nitrate reductase required for assimilation of nitrate is a cytoplasmic protein, while nitrate reductase required for nitrate respiration under anaerobic conditions is membrane-integrated. Assimilatory nitrate reductase is encoded by the *nasBC* genes in the *nasBCDEF* operon (Ogawa *et al.*, 1995). The operon is transcribed divergently from *nasA* gene (Ogawa *et al.*, 1995). The *nasA* gene product was proposed to be a nitrate transporter (Ogawa *et al.*, 1995). The *nasDEF* genes encode nitrite reductase, which will be discussed in the next section. The respiratory nitrate reductase is encoded by *narGHJI* (Glaser *et al.*, 1995). Comparison of the amino acid sequence of *narGHJI* with that of *E. coli* indicates that the products of *narG*, *H*, and *I* are the subunits of nitrate reductase and *narJ* gene product plays a role in the assembly of the enzyme (Nakano and Zuber, 1998). The expression of two types of nitrate reductase is activated in response to different environmental conditions. The transcription of *nasBC* is activated by nitrogen limitation and the activation is dependent on TnrA (Nakano *et al.*, 1995), which is involved in the control of global nitrogen metabolism in *B. subtilis* (Wray *et al.*, 1996). In contrast, the transcription of *narGHJI* is activated by FNR under anaerobic conditions (Cruz Ramos *et al.*, 1995).

### 1.3.2 Nitrite reductase

*B. subtilis* has only one nitrite reductase with assimilatory and respiratory dual-function. The subunits of nitrite reductase are encoded by the *nasDE* genes in the *nasBCDEF* operon (Kunst *et al.*, 1997). The *nasBC* genes encode assimilatory nitrite reductase as described in section 1.3.1 and the *nasF* gene codes for an enzyme involved in the synthesis of siroheme, a cofactor of nitrite reductase (Nakano and Zuber, 1998). *nasDEF* transcription is regulated by TnrA in response to nitrogen availability and is regulated by the ResD-ResE two-component signal transduction system under anaerobic

conditions (Nakano *et al.*, 1998). NsrR, a NO-sensing regulator, is also involved in the regulation of *nasDEF* transcription (Nakano *et al.*, 2006). Elucidation of the transcriptional regulation of *nasDEF* is one of the major aims of the thesis research described herein.

### 1.3.3 NarK

The function of NarK is not well established, but *E. coli* NarK was believed to be function in extrusion of nitrite formed by nitrate reduction during anaerobic respiration (Rowe *et al.*, 1994), thus keeping optimal intracelluar concentration of nitrite for cell growth.

Transcription of the *narK-fnr* operon is autoinduced by FNR under anaerobic conditions. The role of FNR in anaerobic gene regulation in *B. subtilis* will be further discussed in the next section.

### 1.3.4 FNR

As described earlier, FNR plays an important role in the response to oxygen limitation in *E. coli*. The FNR ortholog of *B. subtilis* was first identified during the *B. subtilis* genome sequencing project (Sorokin *et al.*, 1993) and was shown to be required for anaerobiosis in *B. subtilis* (Cruz Ramos *et al.*, 1995). Although the structure and function of FNR from *B. subtilis* ($FNR_{bs}$) and *E. coli* ($FNR_{ec}$) are similar, there are certain differences between these orthologs: (1) $FNR_{bs}$ has a characteristic HTH motif in the N-terminus and an iron-sulfur cluster in the C-terminus, which is opposite to $FNR_{ec}$ (Cruz Ramos *et al.*, 1995). (2) There are six cysteine residues in $FNR_{bs}$ (C72, C185, C227, C230 C233, and C235). In vivo study showed that only three cysteines (C227, C230 and C235) are essential for $FNR_{bs}$ activity (Reents *et al.*, 2006a). Furthermore, UV/VIS spectroscopy analysis of purified FNR mutant proteins carrying single serine substitution of the six cysteine residues demonstrated that only these three cysteines are involved in the formation of the 4Fe-4S cluster in $FNR_{bs}$ (Reents *et al.*, 2006a). Other acidic amino acids may serve as the fourth coordinated residue. In contrast to $FNR_{bs}$, four cysteines (C20, C23, C29 and C122) coordinate the 4Fe-4S cluster in $FNR_{ec}$ (Green *et al.*, 1996). (3) Intact 4Fe-4S is also required for $FNR_{bs}$ activity as a transcription regulator. Although the

4Fe-4S cluster is damaged by oxygen, the $FNR_{bs}$ oligomeric state is not affected by the presence of the 4Fe-4S cluster (Reents *et al.*, 2006a). In contrast, the 4Fe-4S cluster promotes dimerization which is required for active $FNR_{ec}$. (4) *fnr* transcription is activated by the ResD-ResE two-component signal transduction system in *B. subtilis* (section 1.4), whereas *E. coli fnr* transcription is negatively auto-regulated by $FNR_{ec}$ under oxygen-limited conditions (Mettert and Kiley, 2007). (5) The number of genes that are directly regulated by $FNR_{bs}$ is much fewer compared to *E. coli*. Only the *arfM* gene, the *narK-fnr* and *narGHJI* operons were identified as direct targets of $FNR_{bs}$ (Cruz Ramos *et al.*, 1995; Marino *et al.*, 2001; Nakano *et al.*, 1996). Transcriptome analysis revealed that $FNR_{bs}$ indirectly affects expression of other genes including the *acoABCL* operon under anaerobic conditions. Genes of the *acoABCL* operon encode components of acetoin dehydrogenase complex that catalyzes acetoin degradation (Reents *et al.*, 2006b). *fnr* is transcribed from the FNR-dependent *narK* promoter (Cruz Ramos *et al.*, 1995) and the ResDE-dependent *fnr*-specific promoter (Nakano *et al.*, 1996).

### 1.3.5 ArfM

ArfM is an anaerobic modulator, which stimulates expression of *lctEP* (lactate fermentation), *alsSD* (acetoin formation) (Marino *et al.*, 2001), and heme biosynthetic genes (Homuth *et al.*, 1999) under anaerobic conditions. The mutation of *arfM* also reduced transcription of ResDE-dependent genes such as *nasDEF* and *hmp* (Marino *et al.*, 2001). However how ArfM regulates transcription of these genes remains unknown. *arfM* transcription is activated by FNR under oxygen-limited conditions as described above (Marino *et al.*, 2001).

### 1.3.6. Flavohemoglobin

NO and NO donors induce expression of the flavohemoglobin gene (*hmp*) from various microorganisms and the induction requires transcriptional regulators specific to each microorganism (Arai *et al.*, 2005; Crawford and Goldberg, 1998b; Membrillo-Hernández *et al.*, 1998; Nakano, 2002; Poole *et al.*, 1996). The up-regulation of *hmp* by NO suggested a possible role of Hmp in NO metabolism, which was confirmed by the findings that Hmp has the oxygenase activity to convert NO to nitrate (Gardner *et al.*,

1998; Gardner *et al.*, 2000; Hausladen *et al.*, 1998; Hausladen *et al.*, 2001). However recent study showed that Hmp is denitrosylase that catalyzes $NO^-$/O2 reaction (Gardner *et al.*, 1998; Gardner *et al.*, 2000; Hausladen *et al.*, 1998; Hausladen *et al.*, 2001). Therefore Hmp is involved in NO detoxification (Crawford and Goldberg, 1998a; Gardner *et al.*, 2000; Hausladen *et al.*, 1998; Liu *et al.*, 2000; Membrillo-Hernández *et al.*, 1999).

The *Salmonella enterica* Serovar Typhimurium *hmp* mutant showed lower infection frequency and higher susceptibility to NO-related killing by human macrophages, indicating the important role of flavohemoglobin in bypassing host cells defense during bacteria invasions (Stevanin et al., 2002). Similarly, *S. aureus* mutants lacking Hmp are avirulent in wild-type mice but cause lethality to mice that do not produce inducible nitric oxide synthase, indicating that flavohemoglobin is involved in detoxification of NO in the infected host (Richardson et al., 2006). *B. subtilis* flavohemoglobin is also involved in protecting cells from nitrosative stress under aerobic conditions (Rogstam et al., 2007). In addition, the essential role of flavohemoglobin for long-term anaerobic survival was reported in *B. subtilis* cultured in the presence of nitrate (Nakano, 2006).

## 1.4 REGULATION OF NITRATE RESPIRATION BY THE RESD-RESE SIGNAL TRANSDUCTION SYSTEM

### 1.4.1 ResD and ResE are required for anaerobic respiration

ResD-ResE plays an important role in the adaptation of *B. subtilis* to oxygen-limited environments (Sun *et al.*, 1996b). The ResD-ResE system also regulates gene expression involved in aerobic respiration (Sun *et al.*, 1996b). In addition, the *resDE* mutant shows a minor defect in sporulation and fermentation (Nakano *et al.*, 1997), indicating that ResDE are important in diverse physiological funstions in *B. subtilis*.

*resD* and its downstream gene *resE* were identified by *B. subtilis* genome sequencing project (Sorokin *et al.*, 1993). *resD* and *resE* form an operon with the upstream *resA*, *resB* and *resC* genes. ResA, a thiol-disulfide oxidoreductase, specifically recognizes cytochrome *c* and functions during cytochrome *c* maturation, which is ResA redox state-dependent (Colbert *et al.*, 2006; Crow *et al.*, 2004; Erlendsson *et al.*, 2003).

The roles of ResB and ResC in cytochrome *c* synthesis were also demonstrated by the loss of cytochrome *c* in *resB* or *resC* strains (Le Brun *et al.*, 2000).

*resD* and *resE* are transcribed from *resA* promoter and a promoter located upstream of the *resD* gene. During anaerobic growth or at the end of aerobic exponential growth, the transcription of *resDE* from the *resA* promoter is activated by phosphorylated ResD (ResD~P), while transcription from the promoter upstream of *resD* is ResD-independent (Sun *et al.*, 1996b).

Under phosphate-limited conditions, ResD protein level is controlled by the PhoPR system, a two-component signal transduction system that is involved in inorganic phosphate metabolism (Birkey *et al.*, 1998). The effect of PhoPR on ResD is direct as PhoP~P activates *resD* transcription from the *resA* promoter. On the other hand, *resD* transcription from the *resD* upstream promoter, which is much weaker than that from the *resA* promoter, is repressed by PhoP~P (Sun *et al.*, 1996a). The induction of *resDE* under different growth conditions suggests that the ResDE regulatory system is involved in multiple cellular responses to the diverse environmental signals.

Genomes of other low GC gram-positive bacteria were sequenced, such as *Bacilli*, *Listeria*, *Staphylococci*, *Streptococci*, and *Clostridia*, among which ResDE orthologs were found in *Bacilli*, *Listeria* and *Staphylococci* (http://genolist.pasteur.fr/). The ResDE orthologs of gram-positive pathogenic bacteria are essential for anaerobiosis and virulence gene expression as seen in *Bacillus cereus*, *Listeria monocytogenes* and *Staphylococcus aureus*. Similarly to the *B. subtilis* orthologs, *B. cereus resDE* genes reside in the *resABCDE* operon and are transcribed from both the *resA* and *resD* promoters. The ResDE system plays an important role in both fermentative growth and enterotoxin expression in *B. cereus* F4430/73 (Duport *et al.*, 2006). In contrast, *Listeria* and *Staphylococci* do not have the *resABC* genes, instead, *resD* and *resE* constitute a dicistronic operon. Unlike other *resDE* orthologs, *resE* from *L. monocytogenes* is transcribed from the *resE* promoter in addition to the *resD* operon promoter. ResD modulates virulence gene expression in response to carbohydrates in *L. monocytogenes* (Larsen *et al.*, 2006). SrrAB, the ResDE orthologs of *S. aureus*, regulate virulence factor expression (Pragman *et al.*, 2004).

How ResDE regulates gene expression relating to anaerobic nitrate respiration is the focus of this thesis. ResD and ResE constitute a typical two-component signal transduction system. In bacteria, two-component signal transduction systems are evolutionally selected to sense, respond and adapt to physical and chemical stimuli [see review (Stock *et al.*, 2000)]. Thus, the knowledge of typical two-component signal transduction system will be summarized before the ResDE system is introduced in detail.

### 1.4.2 Two-component signal transduction system and response regulators

Two-component signal transduction systems exist widely in prokaryotes, archaea and lower eukaryotes such as yeast and some plants. Two-component signal transduction system can trigger diverse responses to environmental changes, ranging from adjusting metabolic pathway by responding to such changes, to engaging more complex physiological events such as formation of spores, biofilms and fruiting bodies. Two-component signal transduction systems are predominant signal pathways in bacteria, but have not been identified in animals, although histidine residues in vertebrate proteins can be reversibly phosphorylated (Besant *et al.*, 2003). Therefore, two-component systems have been considered as potential targets for development of new sets of therapeutic drugs (Barrett *et al.*, 1998).

There are dozens of two-component signal transduction systems in *E. coli* (Mizuno, 1997) and *B. subtilis* (Fabret *et al.*, 1999), which have been extensively studied. Figure 1.2 shows a schematic view of how a signal is transmitted in two-component systems. The simplest form of the system consists of a histidine kinase (HK) and a response regulator (RR) (Fig. 1.2A). HK senses either an exogenous or endogenous signal and undergoes autophosphorylation at a conserved histidine residue using ATP as a substrate. The high-energy phosphoryl group is then transferred to an invariant aspartate residue of the RR. Generally only the phosphorylated RR triggers the cell response, in most cases by binding promoter DNA and regulating gene expression, thus resulting in adaptation to new environment. There are more complex forms of two-component signal transduction systems which include multiple phosphorelay steps (Fig. 1.2B). The signal transduction system required for the initiation of sporulation of *B. subtilis* contains four proteins, KinA (B, C or D), Spo0F, Spo0B, and Spo0A (Grimshaw

*et al.*, 1998; Quisel *et al.*, 2001). The high-energy phosphoryl group is transferred from histidine in KinA (B, C or D) to aspartate in Spo0F, a receiver domain-like protein, then to a histidine residue of Spo0B, and finally to Spo0A. Phosphorylated Spo0A is required for the entry into sporulation. This phosphorelay is referred to as intermolecular phosphorelay because phosphotransfer occurs between separate proteins. Bacteria and especially lower eukaryotes have another way of phosphoryl group transfer, the so-called intramolecular phosphorelay, in which a phosphoryl group is transferred to sites within the same protein. Moreover, a protein carrying a histidine-containing phosphotransfer domain (Hpt) could serve as an intermediate in the intramolecular phosphorelay. The *Saccharomyces cerevisiae* osmosensor system is an example of the intramolecular phosphorelay that contains a phosphorylated intermediate (Fig. 1.2C) (Ota and Varshavsky, 1993).

HKs can be divided into two functional domains, a signal input domain and a kinase domain. The kinase domain consists of a phosphotransferase subdomain and an ATP-binding subdomain. Amino acid sequence of the signal input domains is less conserved, whereas the sequence of kinase domains is more similar among HKs, reflecting a fact that HKs sense various signals and all use autophosphorylation to respond. The sequence homology of kinase domains was used to classify HKs into five major groups (Fabret *et al.*, 1999). HKs were also classified into three major groups by another method based on their domain architecture and membrane topology (Mascher *et al.*, 2006). The advantage of the latter method is that the signal-sensing functions of HK are related to their higher order structure.

Two functional domains, a receiver domain and a signal output domain, are present in most of the response regulators with a few exceptions such as CheY in which phosphorylation and signal output are carried out in the same domain. In *E. coli*, CheY is phosphorylated by CheA, a membrane kinase that senses a chemical gradient. CheY~P interacts with a flagellar motor complex, which favors swimming behavior of *E. coli* (Falke *et al.*, 1997). Most RRs bind DNA and act as transcriptional regulators. There are RRs that do not have DNA-binding activity, which include CheB with methylesterase activity (Falke *et al.*, 1997), ANTAR (AmiR and NasR transcription antitermination regulators) with RNA binding ability (Shu and Zhulin, 2002), and RssB interacting with

pro-$\sigma^s$ (Becker *et al.*, 2000). RRs with the DNA binding output domain fall into three major subfamilies, OmpR/PhoB, NarL/FixJ and NtrC according to their DNA binding domain architecture and domain combinations (Galperin, 2006). Figure 1.3 shows the output domain structure in the three subfamilies. Both OmpR/PhoB and NarL/FixJ have HTH DNA-binding motif. There is a signature wing motif before HTH in the OmpR/PhoB subfamily (Brennan, 1993).

The Fis (factor for inversion stimulation) DNA-binding domain is characteristic to RRs of the NtrC subfamily (Kostrewa *et al.*, 1991). There is an additional $AAA^+$-type ATPase subdomain in RRs of NtrC subfamily not present in the other two subfamilies. Phosphorylation facilitates ring assembly of the $AAA^+$-type ATPase subdomain that is required for activation of NtrC subfamily RRs (De Carlo *et al.*, 2006).

ResD belongs to the OmpR/PhoB response regulator subfamily, the largest subfamily of RRs (Fig. 1.4). Among members of this subfamily, OmpR and PhoB in *E. coli* and PhoP in *B. subtilis* are best studied. PhoB and the cognate histidine kinase PhoR are required for gene regulation under phosphate depleted conditions (Makino *et al.*, 1988). OmpR, together with the histidine kinase EnvZ, regulates the porin genes in response to osmolarity (Aiba *et al.*, 1989a). PhoP with a histidine kinase PhoR regulates expression of the Pho regulon expression to cope with phosphate starvation in *B. subtilis* (Seki *et al.*, 1987).

In RRs of this subfamily, the N-terminal receiver domain and C-terminal DNA binding domain are connected by a flexible linker of different length. The OmpR linker (15 amino acid length) is longer than the PhoB linker (9 amino acid length). The mutations of the OmpR linker affected the phenotype of OmpR, indicating that the linker is involved in communication between two domains (Mattison *et al.*, 2002). In addition, chimeras between OmpR and PhoB containing either linker indicate that DNA-binding domain regulation by either receiver domain requires its cognate linker (Walthers *et al.*, 2003) consistent with the different activation mechanisms for OmpR and PhoB (see 1.4.7).

The structures of each domain were solved for $OmpR_C$ (C-terminal domain) (Martinez-Hackert and Stock, 1997b), $PhoB_C$ (Blanco *et al.*, 2002), and $PhoB_N$ (Bachhawat *et al.*, 2005) in *E. coli* and $PhoP_N$ (N-terminal domain) (Birck *et al.*, 2003) in

*B*. *subtilis*. However, their full-length crystal structures are not available, perhaps due to flexibility of the linker. DrrD and DrrB, RRs of this subfamily from *Thermotoga maritima*, were successfully crystallized thanks to their thermo stability and short linkers (Buckler *et al.*, 2002; Robinson *et al.*, 2000). In addition, the structure of a full-length PrrA from *Mycobacterium tuberculosis* was also obtained (Nowak *et al.*, 2006). The response regulator PrrA is involved in the early intracellular multiplication of *M. tuberculosis* (Ewann *et al.*, 2002; Ewann *et al.*, 2004). The crystal structure of DrrD reveals only a small interdomain interface (Buckler *et al.*, 2002). In contrast, DrrB (Robinson *et al.*, 2003) and PrrA (Nowak *et al.*, 2006) have extensive interdomain interface. The same α4-β5-α5 surface of DrrB and PrrA receiver domain is involved in the interface, although the DNA-binding domains involved in the interface are different. In DrrB, a β-sheet platform contributes to the interface (Robinson *et al.*, 2003), whereas an α helix contacts the receiver domain in PrrA (Nowak *et al.*, 2006). The differences in the interface structures of DrrD, DrrB and PrrA suggest that different signal transduction mechanisms from the N-terminus to the C-terminus in RRs may exist in individual members of the OmpR/PhoB subfamily.

Based on resolved structures, each domain of the response regulators in this subfamily has an overall similar three-dimensional structure (Fig. 1.5). There are five β sheets surrounded by five α helices in the receiver domain. The invariant Asp that receives the phosphoryl group from HK resides in the end of β3. In the DNA-binding domain, four-stranded antiparallel β sheets are followed by three α helices and β hairpin in the C-terminus. The small β10 between α6 and α7 forms a three-stranded antiparallel β sheet with β6 and β7. The topology of the DNA binding domain is β6-β7-β8-β9-α6-β10-α7-α8-β11-β12. α8 is believed to recognize the specific DNA sequence. The loop between α7 and α8 is often important for transcriptional activation, and is thus named the transactivation loop (Chen *et al.*, 2003a; Makino *et al.*, 1996; Pratt and Silhavy, 1994). In Chapter 4, I will examine the role of the transactivation loop of ResD in the transcription of *nasD*, *hmp* and *fnr*.

### 1.4.3 ResD

ResD is a monomer in solution regardless of its phosphorylation state (Zhang and Hulett, 2000). Previous *in vitro* studies showed that ResD can bind promoter regions of *ctaA* (Zhang and Hulett, 2000), *hmp* (Nakano *et al.*, 2000b), *nasD* (Nakano et al., 2000b), *fnr* (Nakano *et al.*, 2000b), and *yclJK* (Härting *et al.*, 2004). Phosphorylation of ResD significantly stimulates the binding of ResD to the *hmp* and *nasD* promoters (Nakano *et al.*, 2000b). High-resolution DNA footprinting analysis demonstrated that ResD tandemly binds the *hmp* and *nasD* promoters (Geng *et al.*, 2004). In vitro run-off transcription experiments showed that ResD is necessary and sufficient to activate transcription of *hmp*, *nasD*, *fnr*, and *ctaA*, and phosphorylation of ResD enhances transcription of these genes (Geng *et al.*, 2004; Zhang and Hulett, 2000). The transcriptional activation of the ResDE regulon by ResD is discussed in Chapter 2 and 4.

### 1.4.4 ResE

ResE is a bifunctional kinase, which mainly acts as a phosphatase under aerobic growth conditions, while the kinase activity is dominant when oxygen is limited, thus leading to increased ResD phosphorylation under anaerobic conditions (Nakano and Zhu, 2001). Two transmembrane regions are connected by a relatively long extracellular loop. The second transmembrane helix is followed by a HAMP (histidine kinase, adenylyl cyclase, methyl-accepting chemotaxis proteins, and phosphatase) linker and a PAS domain. There is an ATPase subdomain at the C-terminus. An extracellular loop is a classical signal sensor domain in membrane-bound HKs. The simplest mechanism for signal sensing by the loop is direct interaction between the loop and small signal molecules; such examples include nitrate sensed by NarX [see 1.2.3 and (Stewart, 2003)] and citrate by CitA (Janausch *et al.*, 2002).

The HAMP linker that connects the signal input domain and the kinase domain widely exists in membrane-bound sensor kinases as a signal-transducing element (Williams and Stewart, 1999). The role of the HAMP linker in signal transmission was demonstrated by analysis of HKs carrying mutant HAMP linkers (Appleman and Stewart, 2003). Some hybrid kinases with a heterologous HAMP linker retain the ability of responding to the signal, indicating that HAMP is a commonly used signal-transducing

structure in HK (Appleman *et al.*, 2003). PAS domains are known to sense various signals including light, oxygen, small ligands, internal energy level and the redox state of cells [see review (Taylor and Zhulin, 1999)].

The signal-sensing subdomains of ResE were identified by deletion and mutational analysis (Baruah *et al.*, 2004). Cytoplasmic ResE without membrane anchor regions retains the ability to sense oxygen-limited signal(s). The deletion of PAS domain results in the loss of activity, indicating that the PAS domain is a major signal sensing module in ResE (Baruah *et al.*, 2004). FixL, a HK involving in nitrogen fixation in *Rhizobium meliloti*, has a heme cofactor bound to the PAS domain (Gilles-Gonzalez *et al.*, 1991). The iron coordinated by heme directly senses oxygen. Cysteine residues in the PAS domain also participate in ArcB signal-sensing as described in 1.2.1. However a similar mechanism of signal sensing by the PAS domain is not applicable to ResE because there is no heme or cysteine in ResE. The function of the long extracytoplasmic loop in signal sensing of ResE was proposed to amplify the signal sensed by the cytoplasmic PAS domain (Baruah *et al.*, 2004). Although what signal(s) ResE senses and how ResE senses signal(s) are unknown, the role of the ResDE system in adaptation to anaerobic conditions (section 1.4.1) and how ResDE system activate gene transcription (section 1.4.9) have been significantly uncovered.

### 1.4.5 ResDE regulon

As described earlier, ResDE-target genes include *nasDEF* (Geng *et al.*, 2004), *fnr* (Nakano *et al.*, 1996), and *hmp* (LaCelle *et al.*, 1996) that are involved in anaerobic nitrate respiration and are studied in this thesis research (Fig. 1.1).

Apart from its involvement in anaerobic respiration, ResDE also plays an important role in aerobic respiration. The expression of *ctaABCDEFG* (Liu and Taber, 1998; Sun *et al.*, 1996b), *resABCDE* (Sun *et al.*, 1996b), *qcrABC* (Sun *et al.*, 1996b) and *cydABCD* (Puri-Taneja *et al.*, 2007) is controlled by ResDE under both anaerobic and aerobic conditions. These genes can be divided into two groups. Group I genes are involved in heme synthesis, for example *ctaA* and *ctaB* encode heme A and heme O synthase, respectively (Liu and Taber, 1998; Zhang and Hulett, 2000). Group II genes include *ctaCD*, *resABC*, *qcrABC* and *cydABCD*, which function in cytochrome synthesis. *ctaCDEF* encodes

subunits of cytochrome *caa*$_3$ (Liu and Taber, 1998); *resABC* are required for cytochrome *c* biogenesis as described in section 1.4.1 ; *qcrABC* encode subunits of the cytochrome *bf* complex (Sun *et al*., 1996; Yu and LeBrun, 1998); *cydAB* encode subunits of cytochrome *bd* oxidase, and *cydCD* encode ABC transporter required for expression of cytochrome *bd* oxidase (Puri-Taneja *et al.*, 2007). ResD binds to similar DNA sequences in aerobic and anaerobic respiratory genes. In addition, the transcription of ResDE-controlled genes can be activated by ResD and phosphorylation of ResD further enhances ResD activation. How ResD-ResE system activate aerobic or anaerobic respiration genes in response to the oxygen availability is not very clear. In my Ph. D study, we identified that two new mechanisms, unphosphorylated ResD (Chapter 3) and NsrR transcriptional regulator (Chapter 5), which are involved in the up-regulation of ResDE-controlled genes such as *nasD* and *hmp* involved in the anaerobic respiration.

ResDE also activates the *sbo-alb* operon (Nakano *et al.*, 2000a) and *yclJK* operon (Härting *et al.*, 2004). Products of *sbo-alb* operon are involved in biosynthesis of the bacteriocin subtilosin (Zheng *et al.*, 1999; Zheng *et al.*, 2000). *yclJK* encodes a two-component signal transduction system whose function is unknown (Härting *et al.*, 2004). The biological implication why these genes are activated by ResDE is still not clear at the moment.

How ResD activates genes involved in anaerobic respiration has been investigated in our laboratory. Before discussing transcription activation controlled by ResD, I will introduce the general knowledge about the transcription initiation mechanism in bacteria.

### 1.4.6 Transcription in prokaryotes

Actions and properties of cells are determined by the proteins they produce. One principle of life is gene expression and regulation, which determines the types and amounts of proteins produced in different developmental stages, different cell types or in response to the environmental changes. Gene expression generally refers to an entire process whereby genetic information encoded in DNA is decoded and flows into the corresponding protein product, i.e. RNA polymerase (RNAP) synthesize mRNA using the DNA template strand and then mRNA is translated into protein by ribosomes. To acquire proper activity, certain proteins need to be modified. Although gene regulation

can occur at each step in gene expression, the control of transcription initiation is one of the most important mechanisms because transcription initiation is the first step that decides whether or how much genes are expressed. Transcriptional regulation has been intensively investigated in prokaryotes, especially in *E. coli*, a model organism of bacteria. Unlike eukaryotes that have three types of RNAP with different functions, prokaryotes have only one type of RNAP responsible for all transcription events (Ebright, 2000). Bacterial multi-subunit RNAP consists of αββ′ω designated as core RNAP. Core RNAP of bacteria has a structure similar to RNAP II in yeast (Fu *et al.*, 1999; Zhang, 1999). In eukaryotes, RNAP II transcribes messenger RNAs, several small nuclear RNAs and microRNA. The large β and β′ subunits are in charge of DNA-dependent RNAP synthesis (Korzheva, 2000). Each monomer of the α dimer consists of two independent functional domains connected by a flexible linker. αNTD (N-terminal domain of α) can dimerize and is involved in the assembly of β and β′ subunits. αCTD (C-terminal domain of α) can facilitate RNAP binding to a specific promoter region by recognizing the up-element or interacting with transcription regulators (Gourse *et al.*, 2000). I will discuss the role of αCTD in transcription initiation in Chapter 4. The ω subunit seems to be a chaperon that helps correct β′ subunit folding (Hampsey, 2001). Core RNAP itself cannot initiate promoter-specific transcription unless core RNAP and the σ subunit form the holo RNAP (Browning and Busby, 2004). The σ subunit localizes holo RNAP to a specific promoter by recognizing the promoter sequence and is also involved in unwinding of DNA duplex (Gross *et al.*, 1998; Wosten, 1998). The σ subunit consists of four functional domain joined by linkers (Campbell *et al.*, 2002). Domain 2, 3 and 4 mediate binding to core RNAP and to promoter DNA (Gross *et al.*, 1998). Domain 2 recognizes the -10 element and is important for melting and recognition of the non-template strand of the -10 element. Domain 3 recognizes two conserved bases located upstream of the -10 region. Domain 4 can bind to the -35 element. Domain 4 is also a target of positive transcription regulators. The role of domain 1 is not clear. Most bacteria contain multiple σ subunits that recognize and transcribe different sets of promoter in response to environmental changes, which is an important mechanism used for gene regulation in bacteria (Wosten, 1998). The essential $\sigma^{70}$ and $\sigma^{A}$ subunits participate in the transcription of housekeeping genes in *E. coli* and in *B. subtilis*, respectively.

Transcriptional processes include initiation, elongation and termination. During transcription initiation, RNAP first binds promoter DNA to yield a closed complex. Then the closed complex transits to an open complex in which RNAP melts the DNA around the transcription start site (deHaseth *et al.*, 1998). De novo RNA synthesis begins. Short RNA transcripts (~2 to 9 nucleotides in length) are released from RNAP called abortive initiation. When the transcript reaches a length of about 10 nucleotides, RNAP loses the contact with promoter region and starts moving along the DNA, which is referred to as promoter clearance. The synthesis of RNA chain begins as an elongation complex form. Transcription initiation can be activated or repressed by transcriptional regulators in response to environmental signals [reviewed in (Busby and Ebright, 1994)]. This thesis is focused on transcription activation by ResD that belongs to OmpR/PhoB subfamily, therefore, I will summarize the mechanisms of transcription activation exerted by OmpR/PhoB.

**1.4.7 Mechanisms of transcription activation by OmpR/PhoB subfamily RRs**

How transcriptional activators are involved in transcription initiation has been well studied in *E. coli* [reviewed in (Rhodius and Busby, 1998)]. Most activators including RRs stimulate transcription by interacting with a subunit (or subunits) of RNAP. The activators that contact αCTD of RNAP were shown to recruit RNAP at promoters (Ebright, 1993), whereas the interaction between activators and the σ subunit may facilitate isomerization of closed complexes to open complexes (Dove *et al.*, 2003). Sigma region 4.2 is known to interact with activators (Lonetto *et al.*, 1998). The third mechanism of how transcriptional activators enhance transcription initiation is to change a conformation of promoter DNA so that RNAP can interact with DNA -35 and/or -10 elements (Sheridan *et al.*, 1998; Sheridan *et al.*, 2001).

PhoB is a dimer in solution and phosphorylation does not affect its oligomeric state (Fiedler and Weiss, 1995). PhoB binds the Pho-box tandemly via a head-to-tail dimer (Blanco *et al.*, 2002). A truncated PhoB containing only the C-terminus domain (CTD) can bind Pho-box and stimulates the transcription of genes in the Pho regulon (Ellison and McCleary, 2000; Makino *et al.*, 1996). In contrast to PhoB, OmpR is a monomer in solution before and after phosphorylation (Aiba *et al.*, 1989b). The isolated

C-terminal domain of OmpR has a much lower binding affinity than full OmpR (Tsuzuki *et al.*, 1994; Walthers *et al.*, 2003). Unlike PhoB, the CTD of OmpR cannot stimulate transcription, unless the stable artificial dimer was generated by cross-linking between cysteines that are introduced by amino acid substitutions (Walthers *et al.*, 2003). The observed difference in the activity of the isolated CTD of PhoB and OmpR may result from the different effects of phosphorylation on the PhoB and OmpR CTD activity (see 1.4.8).

Two types of OmpR arrangement in DNA binding have been reported. The head-to-tail orientation model of OmpR dimer was proposed based on DNA affinity cleavage analysis of OmpR-DNA complex (Harrison-McMonagle *et al.*, 1999). However cross-linking experiments using mutant OmpR with introduction of cysteine revealed the head-to-head orientation in OmpR-binding (Harrison-McMonagle *et al.*, 1999). The conflicted results may indicate that OmpR can adopt more than one conformation to bind DNA.

The mechanisms of transcriptional activation used for PhoB and OmpR are also different. Mutations of αCTD affect OmpR-dependent gene expression (Kato *et al.*, 1996; Kondo *et al.*, 1997; Slauch *et al.*, 1991), while PhoB-dependent gene expression is not affected by these αCTD mutations (Igarashi *et al.*, 1991). Mutations of $\sigma^{70}$ specifically affect expression of PhoB-dependent genes, suggesting PhoB interacts with $\sigma^{70}$ (Makino *et al.*, 1993). The transactivation loop between α7 and α8 helix of the PhoB effector domain (Fig. 1.4) interacts with the $\sigma^{70}$ subunit of RNAP to activate transcription. As mentioned before, RRs of OmpR/PhoB subfamily have similar secondary structures (Fig. 1.4). Mutational analysis showed that the transctivation loop of OmpR might interact with αCTD (Pratt and Silhavy, 1994). However, suppressor analysis of the V264G mutation in αCTD showed that αCTD interacts with two residues – Pro179 and Ser181 – that reside near the end of α2 (Fig. 1.4)(Kato *et al.*, 1996). These two residues were speculated to interact with αCTD due to the notion that they are well positioned on the outside of the head-to-head OmpR dimer (Maris *et al.*, 2005). In conclusion, different mechanisms are used for the response regulator OmpR and PhoB to activate transcription initiation.

### 1.4.8 Effect of phosphorylation of RRs on their activity

How phosphorylation of aspartate in the N-terminal phosphoacceptor domain affects the activity of the C-terminal output domain in RRs is a key factor in signal transduction and responses of cells to environmental changes. Structure analysis of full-length unphosphorylated NarL indicated that the phosphoacceptor domain negatively regulates the activity of the output domain (Baikalov *et al.*, 1996). The NarL phosphoacceptor domain makes extensive contact with a functional region of the output domains, thereby, blocking the DNA binding region of NarL, which can be relieved by phosphorylation (Baikalov *et al.*, 1996). On the other hand, phosphorylation of RRs can be exert effect, for example phosphorylation of NtrC stimulates oligomerization to enhance its activity as a transcriptional activator (De Carlo *et al.*, 2006).

Both negative and positive effects of receiver domain phosphorylation on DNA-binding domain activity were found in the OmpR/PhoB subfamily. Phosphorylation of PhoB causes a conformational change that redefines the dimerization of PhoB and releases the inhibition of the receiver domain on the DNA binding domain (Ellison and McCleary, 2000; McCleary, 1996), which explains how the PhoB CTD acquires DNA-binding activity and can stimulate gene transcription. In contrast, phosphorylation stimulates dimerization of OmpR and enhances DNA binding affinity (Huang and Igo, 1996). OmpR CTD has a poor DNA-binding affinity and is unable to stimulate transcription. In addition, it is known that DNA-binding inversely stimulates phosphorylation (Maris *et al.*, 2005) or inhibits dephosphorylation of OmpR (Qin *et al.*, 2001). The contribution of phosphorylation to *B*. *subtilis* PhoP activity is distinct from PhoB and OmpR. PhoP forms a phosphorylation-independent dimer in solution, and phosphorylation of PhoP moderately enhances DNA binding affinity (Liu and Hulett, 1997). Phosphorylation regulates PhoP activity probably by enhancing cooperative DNA binding or interacting with other factors (Birck *et al.*, 2003; Liu and Hulett, 1997).

To determine the effect of phosphorylation at the structural level has been hampered by the instability of phosphoaspartate. One method to overcome the difficulty is to use a non-covalent phosphoryl analog, beryllofluoride ($BF_3^-$) (Lee *et al.*, 2001). $BF_3^-$ can coordinate aspartate residues and form a stable complex. Thus $BF_3^-$ was added to crystallizing reaction of the receiver domain of the RR to mimic its phosphorylated form

(Wemmer and Kern, 2005). Another way to gain structural information of phosphorylated RR is using constitutively active RR mutants that do not require phosphorylation to activate transcription (Arribas-Bosacoma *et al.*, 2007). The active state of the receiver domain can be mimicked by the receiver domain of the constitutive mutants. Studies using both approaches suggested that phosphorylation of the aspartate residue does not cause significant changes in the secondary structure, but results in a subtle displacement of the backbone and reorientation of residues on the α4-β5-α5 surface (Arribas-Bosacoma *et al.*, 2007; Gao *et al.*, 2007; Lee *et al.*, 2001).

### 1.4.9 Phosphorylation-dependent and -independent transcriptional activation of ResD

How phosphorylation affects ResD activity is not clear. Phosphorylation does not change the monomeric state of ResD in solution (Zhang and Hulett, 2000). However, phosphorylation moderately stimulates ResD DNA-binding affinity to the *ctaA* (Zhang and Hulett, 2000), *hmp* and *nasD* promoters (Geng *et al.*, 2004; Nakano *et al.*, 2000b). Phosphorylation also stimulates transcription of the ResDE regulon in vitro (Geng *et al.*, 2004; Paul *et al.*, 2001). How ResD~P activates *fnr*, *hmp* and *nasD* transcription will be discussed in Chapters 2 and 4.

Unlike OmpR, which cannot function without phosphorylation (Hsing *et al.*, 1998), ResD has phosphorylation-independent activity. We showed that mutant ResD with an alanine substitution of aspartate at the residue 57, the phosphorylation site, partly retains activity in vivo and vitro, indicating that unphosphorylated ResD itself or with another factor may sense anaerobic signal(s) (Geng *et al.*, 2004). Phosphorylation mutant ResD performs differently from other RRs such as DegU or UhpA that also have phosphorylation-independent activity. Unphosphorylated and phosphorylated DegU have different target genes to regulate depending on its phosphorylated state (Dahl *et al.*, 1992), and phosphorylation mutant UhpA has a constitutive activity only when it is overexpressed (Webber and Kadner, 1997). The study of the phosphorylation-independent ResD activation will be presented in Chapter 3.

## 1.5 INTERFACE OF RESDE REGULATION WITH OTHER REGULATORY PATHWAYS

### 1.5.1 Cross regulation between ResDE and other two-component signal transduction systems

PhoPR, a two-component signal transduction system, controls gene expression in response to phosphate starvation (Seki *et al.*, 1987). As described in 1.4.1, The PhoPR system participates in the ResDE regulatory pathway by affecting ResDE protein levels when phosphate is limited (Birkey *et al.*, 1998). PhoP directly binds to the *resA* promoter and activates *resDE* transcription, whereas *resDE* transcription from the internal *resD* promoter is repressed by PhoP.

Inversely, the ResDE system is responsible for 80% of the Pho response to phosphate starvation (Birkey *et al.*, 1998). Under aerobic conditions, ResD activates expression of *ctaA* and *ctaB* that are required for heme A synthesis and increases the cellular concentration of *a*-type terminal oxidases $aa_3$ and $caa_3$ containing heme A. These terminal oxidases oxidize the reduced quinones to relieve the inhibition of PhoR autophosphorylation to promote full Pho induction (Schau *et al.*, 2004).

Our study showed that ResDE regulates anaerobic expression of *yclJK*, which encodes the RR and HK of another two-component signal transduction system (Härting *et al.*, 2004). This result will be discussed in Chapter 6. Therefore, the ResDE system is interwoven with the PhoPR and YclJK regulatory systems, although the physiological function of the latter remains to be uncovered.

### 1.5.2 Regulation of the ResDE regulon by NsrR

The full induction of the ResDE regulon requires nitrate under anaerobic conditions (Nakano *et al.*, 1999). *B. subtilis*, like *E. coli*, is not a denitrifying bacterium since the final fate of nitrate is not gaseous nitrogen but ammonium. The generation of NO in these bacteria is still a mystery, although NO is apparently generated during nitrate respiration. NO was demonstrated as a signal which triggers full expression of the ResDE regulon under anaerobic growth conditions in *B. subtilis* (Moore *et al.*, 2004; Nakano, 2002). NO is an important signal molecule in all kingdoms of life, because NO is very

active as it can react with transit metal and cysteine. We will further discuss various NO-response transcription regulators in Chapter 5.

We found NsrR plays an important role in NO-dependent upregulation of expression of ResDE-controlled genes, *hmp* and *nasD* in particular (Nakano *et al.*, 2006). NsrR represses *hmp* and *nasD* expression under aerobic growth conditions. When the signal molecule NO is present, which is exogenously supplied or during nitrate metabolism under anaerobic growth conditions, the repressor activity of NsrR is relieved probably by interaction of NO with the Fe-S cluster present in NsrR. The putative NsrR-binding site was proposed by bioinformatics analysis (Rodionov *et al.*, 2005). NsrR protein purified under aerobic conditions directly binds *hmp* and *nasD* promoters and represses *hmp* and *nasD* transcription in vitro. Further studies are presented in Chapter 5.

My research focuses on how ResD activates ResDE regulon transcription. This thesis is organized as follows. In Chapter 1, the background and literature review are included. The transcriptional activation of *fnr* is investigated in Chapter 2. Chapter 3 demonstrates how ResD binds to promoters and exerts phosphorylation-dependent and -independent transcriptional activation. Chapter 4 describes how ResD activates transcription by interacting with RNAP. Chapter 5 reports NO-dependent NsrR regulation of the ResDE regulon. Chapter 6 describes another two-component regulatory system (YclJK) regulated by the ResDE system. The last chapter summarizes the research presented in this thesis and the guide to future work is discussed.

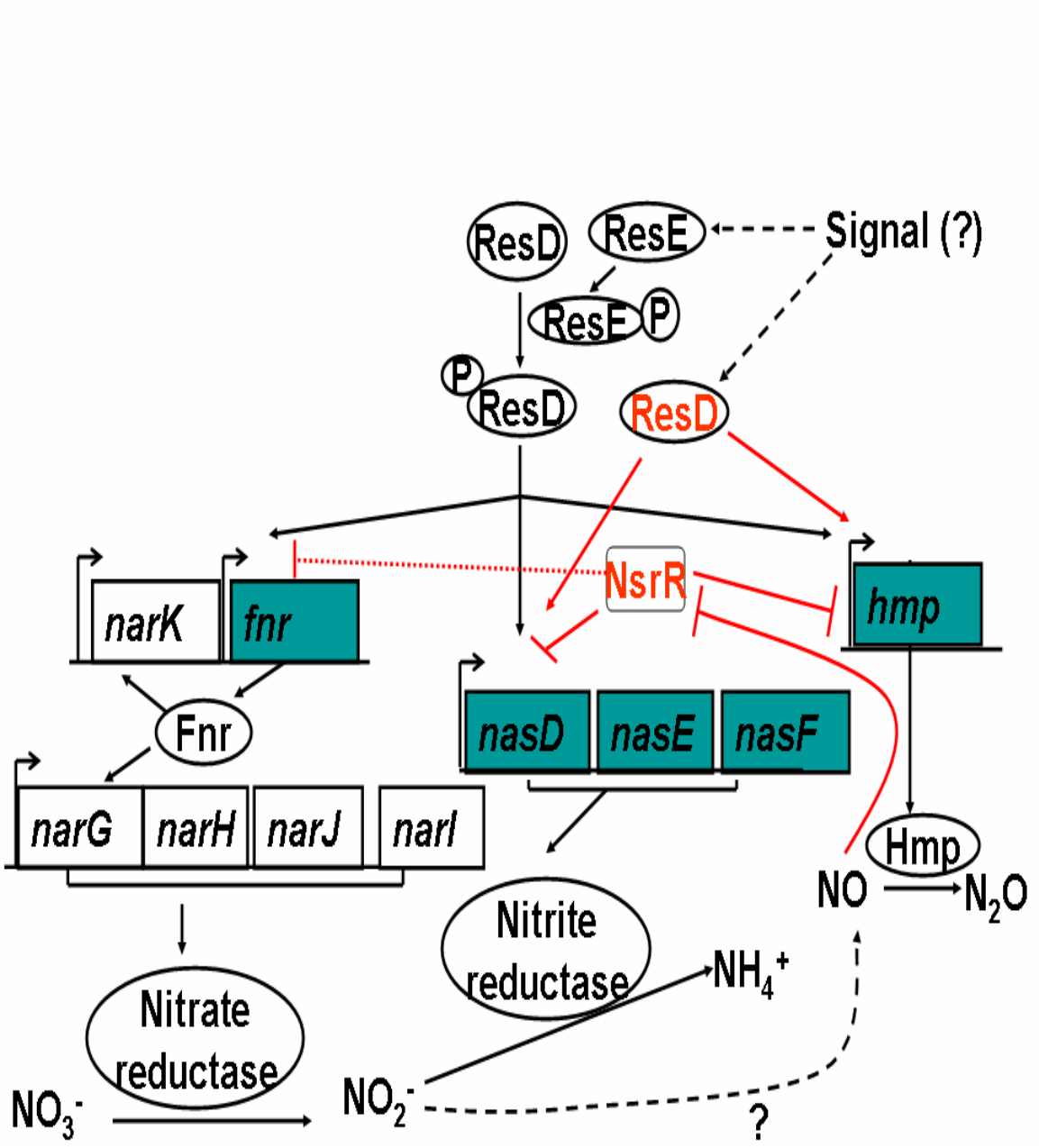


FIG. 1.1. The pathway of anaerobic nitrate respiration in *B. subtilis*. Solid lines indicate direct interaction that has been demonstrated. Positive regulation is labeled with an arrow, while repression is labeled with a ⊥. The genes under ResDE-control are highlighted in green. The regulation determined in this thesis is marked in red.

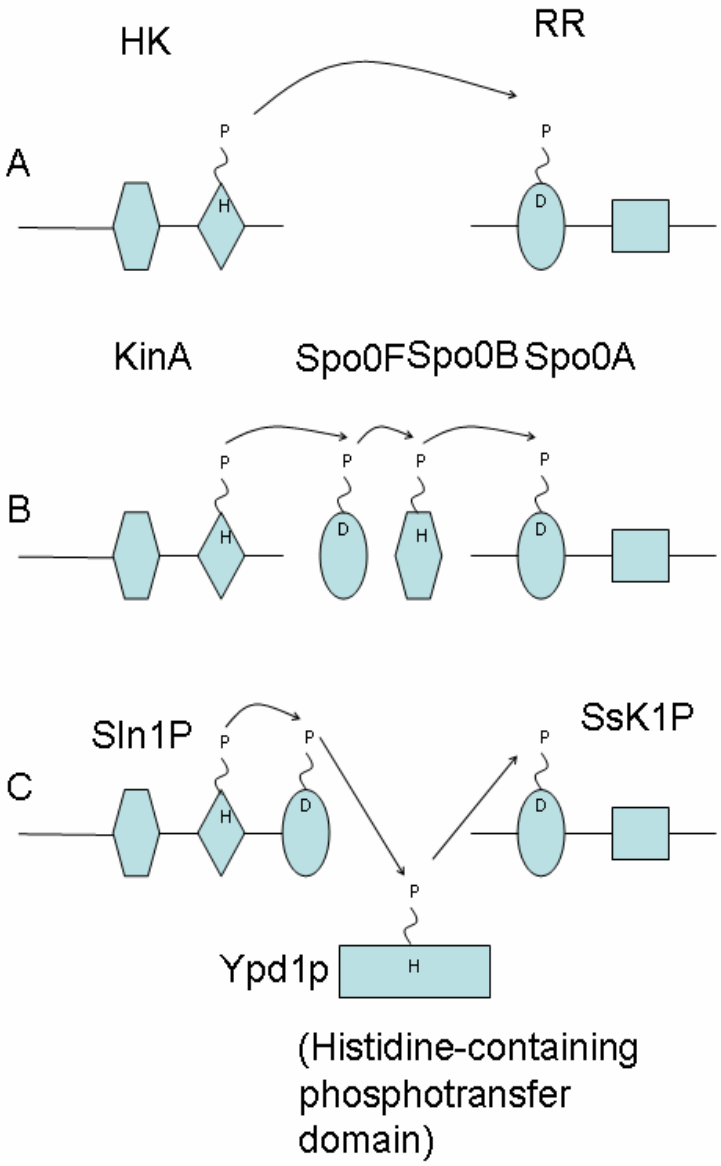


FIG. 1.2. Examples of the two-component signal transduction systems. (A) The simplest form of the system consists of a histidine kinase (HK) and a response regulator (RR). (B) The sporulation phosphorelay of *B. subtilis* has multiple members. (C) The *Saccharomyces cerevisiae* osmosensor system is an example of the intramolecular phosphorelay that contains a phosphorylated intermediate. H, histidine; D, aspartate; ~P, phosphoryl group. Arrows represent the direction of phosphotransfer.

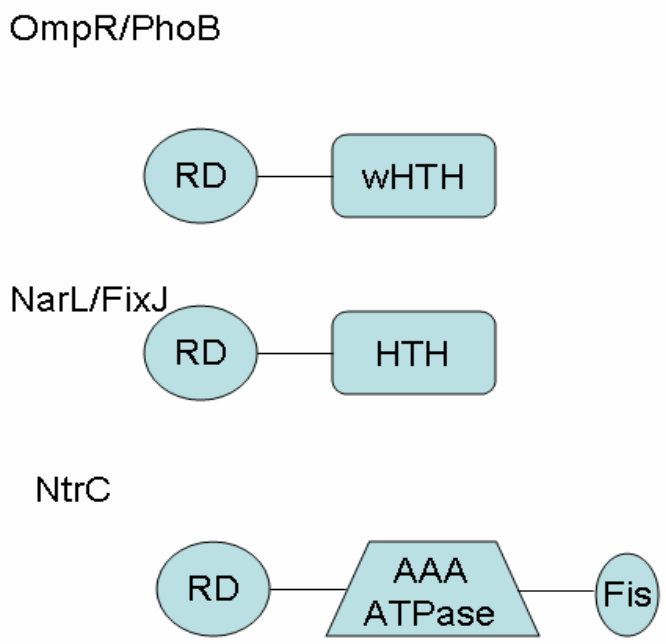


FIG. 1.3. The schematic structures of typical response regulators. RD, receiver domain, wHTH, winged-helix-turn-helix; HTH, helix-turn-helix.

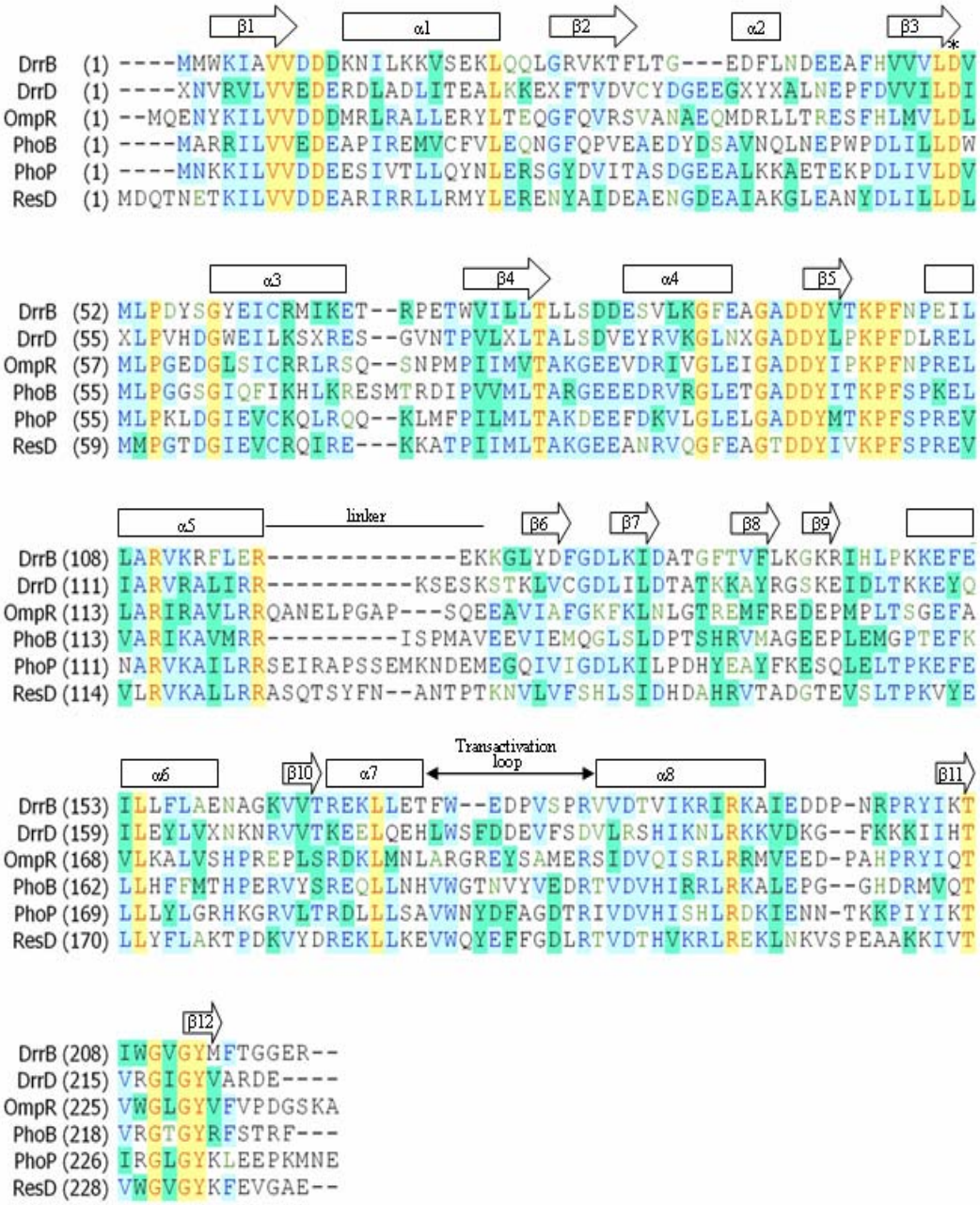


FIG. 1.4. Alignments of ResD with DrrB and DrrD from *Thermatoga maritima*, PhoB and OmpR from *E. coli* and PhoP from *B. subtilis*. The amino acid numbers are shown in parenthesis. The predicted secondary structure is labeled above sequence. The invariant phosphorylation site Asp is marked by *. The identical residues are highlighted in yellow; the conserved residues are highlighted in green; the similar residues are highlighted in blue.

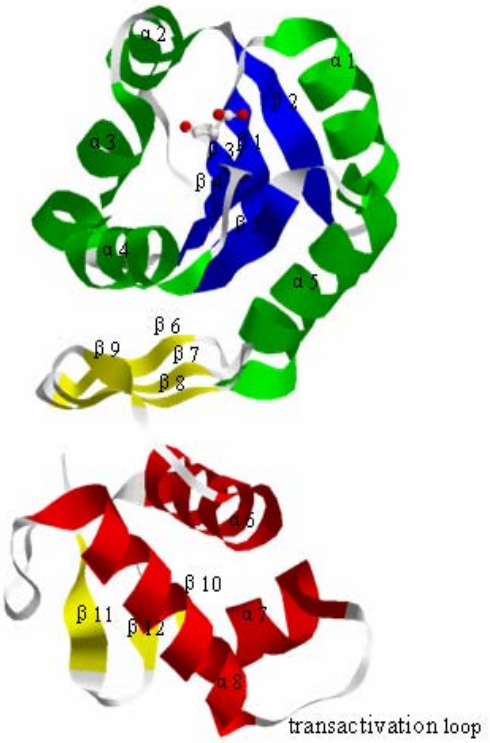


FIG. 1.5. Ribbon representation of *Thermatoga maritime* DrrB (PDB code: 1P2F). The site of phosphorylation (Asp) is shown in ball-and-stick mode. The labeling of the β-strands and α-helices is indicated. Strands of receiver domain are shown in blue, and helices are shown in green, whereas for DNA binding domain they are shown in yellow and red, respectively. The loop between α7 and α8 was named transactivation loop.

# CHAPTER 2

# CHARACTERIZATION OF RESDE-DEPENDENT *FNR* TRANSCRIPTION IN *BACILLUS SUBTILIS*[1]

## 2.1 INTRODUCTION

The ResD-ResE signal transduction system is required for aerobic and anaerobic respiration in *Bacillus subtilis* (Nakano *et al.*, 1996; Sun *et al.*, 1996b). ResE is a membrane-bound sensor histidine kinase that, upon autophosphorylation, donates a high-energy phosphate to its cognate response regulator, ResD. The *resD* and *resE* genes constitute an operon with the three upstream genes, *resABC* (Sun *et al.*, 1996b). ResA is a thiol-disulfide oxidoreductase involved in cytochrome *c* maturation (Colbert *et al.*, 2006; Crow *et al.*, 2004; Erlendsson *et al.*, 2003), and ResB and ResC were also shown to play an essential role in cytochrome *c* synthesis (LaCelle *et al.*, 1996). *resD* and *resE* are transcribed from a *resDE*-specific promoter and the *resA* operon promoter, the latter of which is dependent on ResD and ResE (Sun *et al.*, 1996b).

ResDE-controlled genes that are involved in anaerobic respiration include *fnr* encoding an anaerobic transcriptional regulator and *nasDEF*, which constitute an operon encoding subunits of nitrite reductase (Nakano *et al.*, 1996; Nakano *et al.*, 1998). Fnr is essential for nitrate respiration, as expression of the respiratory nitrate reductase operon, *narGHJI*, is dependent on Fnr (Cruz Ramos *et al.*, 1995). In addition, expression of the flavohemoglobin gene *hmp* is highly induced by ResDE upon oxygen limitation (LaCelle

[1] This material has been published in this or similar form in *Journal of Bacteriology* and is used here with permission of the American Society for Microbiology.

Geng, H., Zhu, Y., Mullen, K., Zuber, C.S., and Nakano, M.M. (2007) Characterization of ResDE-dependent *fnr* transcription in *Bacillus subtilis*. *J. Bacteriol. 189*: 1745-1755.

*et al.*, 1996). Transcription of *fnr*, *nasD*, and *hmp* is activated in vitro by ResD when the protein is phosphorylated by incubation with ResE and ATP (Geng *et al.*, 2004), indicating that phosphorylated ResD (ResD~P) directly interacts with the regulatory region of these genes.

Direct interaction of ResD~P with ResD-controlled promoters, which was confirmed by DNase I or hydroxyl radical footprinting, in *ctaA* encoding heme A synthase (Zhang and Hulett, 2000), *nasD* (Geng *et al.*, 2004; Nakano *et al.*, 2000b), *hmp* (Nakano *et al.*, 2000b), *fnr* (Nakano *et al.*, 2000b), and *yclJK* encoding a two-component regulatory protein pair (Härting *et al.*, 2004) has been reported. Binding of ResD~P to *fnr* was observed either at a higher concentration of ResD~P (Nakano *et al.*, 2000b) or was not detected (Geng *et al.*, 2004). ResD~P bound to three distinct regions of *ctaA* (A1, positions –209 to –179 relative to the transcription start site; A2, –108 to –55; A3, –2 to +43) (Zhang and Hulett, 2000). The A1 site may be involved in ResD-dependent activation of the divergently transcribed *ctaBCDEF* operon encoding heme O synthase and the subunits of cytochrome $caa_3$ (Liu and Taber, 1998). A consensus ResD-binding site was proposed as TTTGTGAAT (consensus sequence a in Fig. 2.1A) by a sequence alignment of *nasD*, *hmp*, *fnr*, and *ctaA* (Nakano *et al.*, 2000b; Zhang and Hulett, 2000). Subsequently, a bioinformatics approach was used to compare ResD-binding sites in the *nasD*, *hmp*, *fnr*, *yclJ*, and *ctaA* promoters and $TTGTN_6TTTNTN_2A$ (consensus sequence b in Fig. 2.1B) was proposed as a revised consensus ResD-binding sequence (Härting *et al.*, 2004). The validity of the two proposed consensus sequences has pros and cons (Fig. 2.1A and B). For example, only a weak similarity (five of nine matches) to consensus sequence a was found in the ResD-binding region (–92 to –68) of *yclJ* (Härting *et al.*, 2004), whereas the binding region contains a sequence with a perfect match to consensus sequence b. On the other hand, consensus sequence a, but not consensus sequence b, was identified in the *ctaA3* region. We had previously shown that a region including –61 to –58 of the *fnr* promoter is indispensable for ResD-dependent activation (Nakano *et al.*, 2000b); however, consensus sequence b in the *fnr* promoter does not include the sequence. Finally, although deletion analysis showed that a *nasD* region between –87 and –76 is critical for ResD-dependent activation, this region is not included either in consensus sequence a or consensus sequence b. Both consensus sequences share 5' TTGT, and the

major difference between these sequences is GAA adjacent to TTGT that was proposed only for consensus sequence a.

Meanwhile, we noticed that there is a strong similarity among the promoter regions of ResD-controlled genes, namely, *resA*, *fnr*, and *sboA* encoding subtilosin (Nakano *et al.*, 1988; Zheng *et al.*, 1999). As shown in Fig. 2.2A, a direct repeat (TTCA $N_7$ TTCA) is present in *resA* and *sboA*, and a single TTCA sequence is present in *fnr*. Interestingly, TGAA, which is complementary to TTCA, is the sequence present in consensus sequence a but not in consensus sequence b of *ctaA1*, *ctaA2*, *ctaA3*, and *yclJ* (Fig. 2.1A). As shown in Fig. 2.1A, *ctaA1* carries the exact match to the TTCA $N_7$ TTCA sequence on the complementary strand, which is likely used as a ResD-binding sequence of the divergently transcribed *ctaBCDEF*. The direct repeat is also present in the ResD-binding consensus sequence a of *yclJK* identified by DNase I footprinting experiments (Härting *et al.*, 2004) but on the noncoding strand (Fig. 2.1A), suggesting that the *yclIH* gene transcribed divergently from *yclJK* might be regulated by ResDE as well. Expression of *lacZ* fused to the *yclI* promoter region was activated upon oxygen limitation, and this activation required ResDE (C. S. Zuber and M. M. Nakano, unpublished results), indicating that *yclIH* also belongs to the ResDE regulon. These results suggested that TGAA (and TTCA) proposed as a part of consensus sequence a is indeed used as a ResD-binding site. In this study, we generated base substitutions in the *fnr* and *resA* promoters to determine whether the TTCA sequence is required for ResD-dependent control. Furthermore, we investigated how binding of ResD~P to the *fnr* promoter facilitates transcription initiation. DNase I footprinting analysis and in vivo studies using the C-terminal domain of the α subunit (αCTD) alanine-scanning mutants indicated that interaction of ResD~P with αCTD is required for activation of *fnr* transcription.

## 2.2 RESULTS

### 2.2.1 Mutational analysis of the *fnr* promoter

As described in the introduction, a high similarity was detected in a region between positions –61 and –45 of the *resA*, *sboA*, and *fnr* promoters (Fig. 2.2A). A direct repeat (TTCA $N_7$ TTCA) is present between –47 and –61 of the *resA* and *sboA* promoters. However, the sequence corresponding to the promoter-proximal TTCA repeat present in *resA* and *sboA* is GTTA in the *fnr* promoter. We speculated that a region of the direct repeat accommodates two ResD~P monomers (or one dimer). Our previous deletion analysis of the *fnr* promoter indicated that the *cis*-acting sequence required for ResD-dependent *fnr* expression is located within a region downstream from –61 and a deletion to –58 severely reduced ResD-dependent transcription activation (Nakano *et al.*, 2000b), which is in good agreement with the hypothesis that ResD~P binds to the sequence between –60 and –46. In addition, the previous observation that ResD~P weakly binds to the *fnr* promoter (Geng *et al.*, 2004) might be explained by the less conserved promoter-proximal half-site (GTTA instead of TTCA).

In order to examine this possibility, we carried out mutational analysis of the *fnr* promoter. The result showed that any single base substitution in the TTCA sequence between –60 and –57 resulted in sharply reduced transcription, indicating that the TTCA sequence is important for ResD-dependent *fnr* transcription (Fig. 2.2B). The substitution of C at –56, at a position adjacent to the TTCA motif, moderately affected transcription. As described above, the *resA* and *sboA* promoters carry a downstream TTCA sequence. Therefore, we next determined whether generating a promoter-proximal TTCA sequence at the corresponding site of the *fnr* promoter increases transcription. *fnr* expression was slightly increased by the change of T at –47 to C that brings the sequence (GTCA) closer to the TTCA consensus sequence. The substitution of –48T to G (resulting in GGTA) nearly abolished *fnr* expression, which was also expected if the TTCA sequence is important for ResD binding. However, the change of G at –49 to the consensus T (resulting in TTTA) led to a decrease in transcription, and the substitution of –46A to G (resulting in GTTG) did not impair *fnr* expression, suggesting that generating the direct

repeat does not increase *fnr* transcription. In fact, simultaneous substitutions of three nucleotides (–49G to T, –47T to C, and –45G to A), which create the consensus TTCA sequence, resulted in transcription slightly lower than the wild-type promoter, although the adverse effect of the –49G to T change appeared to be compensated by the changes in –47T and –45G (see Discussion). β-Galactosidase activity of the mutant promoters in the *resDE* mutant is similar (around 30 Miller units), indicating that the mutations do not affect basal level of transcription, which confirmed that the affected bases are important for ResD-dependent activation.

### 2.2.2 Mutational analysis of the *resA* promoter

The results of base substitutions in the *fnr* promoter indicated that the single TTCA site, not TTCA repeats, is essential for ResD-dependent *fnr* transcription. As shown in Fig. 2.2A, the *resA* and *sboA* promoters contain the TTCA repeats. The next question was whether both TTCA sequences are important for ResD-dependent transcription of *resA* and *sboA* or whether the promoter-distal TTCA sequence is sufficient for transcription. The result obtained from mutational analysis of the *resA* promoter showed that the promoter-distal TTCA site is critical for transcription as shown by the substitution of T at –60 or C at –59, which greatly reduced promoter activity (Fig. 2.2C). In the downstream TTCA sequence, the second T (–49T), which corresponds to –48T in the *fnr* promoter, is the most critical residue, as is the case with *fnr*. The –48C to T change led to 60% reduction of transcription, which is consistent with the result that the –47T to C change in *fnr* moderately increased transcription. In contrast, the base substitution of T at –50 or A at –47 did not show any significant effect on *resA* transcription. These results support the conclusion obtained from the mutational analysis of the *fnr* promoter that TTCA (–60 to –57 of *fnr*) and –48T are essential for ResD-dependent transcription activation.

### 2.2.3 DNase I footprinting analysis of the *fnr* promoter

In order to determine whether ResD~P binds to the TTCA sequence in *fnr*, we carried out DNase I footprinting analysis of the wild-type and mutant *fnr* promoters. We

used two mutant promoters with T–48G and T–47C, the nucleotide substitutions of which resulted in defective and slightly increased transcription, respectively (Fig. 2.2B). It was noticed that cleavage patterns of free DNA between positions –46 and –50 reproducibly changed when T at –48 was substituted with G (see Fig. 2.3 to 2.5). In the wild-type promoter, sensitivity to DNase I was similar among nucleotides at –47, –48, and –50 of the coding strand, whereas in the mutant promoter the position at –50 was hypersensitive to DNase I attack. On the noncoding strand of the wild-type promoter, cleavage sites were located at –46, –48, and –50. When T at –48 was substituted with G, residues –48 and –50 became resistant to DNase I, and a new hypersensitive site appeared at position –49. In the mutant T–47C promoter, a cleavage pattern around this region was similar to that of the wild-type DNA, except –46 of the coding strand became slightly more sensitive to cleavage.

Figure 2.3 indicated that ResD~P alone did not bind to either the wild-type or mutant promoters (lanes 9, 14, and 19). Increasing the concentration of ResD~P up to 12 μM did not protect the *fnr* promoter, although 4 μM ResD~P fully protected *nasD* (data not shown). RNAP alone induced reproducible changes in cleavage patterns of the promoter DNA (lanes 6, 11, and 16), suggesting a distortion of DNA. In the presence of both ResD~P and RNAP, the region between positions –63 and –40 was strongly protected in the wild-type and T–47C promoters, and a region downstream from –32 was also weakly protected (lanes 7, 8, 17, and 18). In contrast, the protection was not detected in the T–48G promoter (lanes 12 and 13), which is consistent with the in vivo result showing that the T–48G mutation impaired transcription.

The region protected in the presence of ResD~P and RNAP included the critical T at –48 (shown by an arrow in Fig. 2.3) and the TTCA sequence (–60 to –57; marked with a box), which we proposed as the ResD-binding site. This result indicated that ResD~P in the presence of RNAP binds to both the TTCA sequence and the sequence around –48 or that ResD~P binds to TTCA, while a subunit of RNAP makes contact with T at –48. If the latter is the case, the C-terminal domain of the α subunit is a likely candidate, given the position of –48 with respect to the promoter DNA. Consistent with this notion, a region around –48 was strongly protected by purified α (Fig. 2.4A and B). Some weaker protection by α was observed in upstream and downstream regions of –48, which may be

caused by nonspecific binding. When αCTD (amino acid residues 239 to 314) was used instead of α, the extended protection disappeared and only the region between –38 and –50 was protected (Fig. 2.4C and D). This result suggested that αCTD interacts with the region around –48; however, the substitution of T at –48 to G did not decrease the binding of either α or αCTD itself, suggesting that T at –48 is not involved in a direct contact with αCTD. A possible effect of the T–48G mutation on *fnr* transcription will be further discussed in the Discussion.

When ResD~P was present, the protection by α was further extended to a region including the TTCA sequence in the wild-type promoter and the T–47C promoter (Fig. 2.5). Consistent with the footprinting results with ResD~P and RNAP (Fig. 2.3), no cofootprinting with ResD~P and α in the T–48G promoter was observed. These results indicated that interaction between α and ResD~P stabilizes ResD~P binding to the *fnr* promoter and strongly suggested that ResD~P makes contact with TTCA and the change of T at –48 to G adversely affects formation of the RNAP-ResD~P-DNA ternary complex.

### 2.2.4 Effects of amino acid substitutions in αCTD on *fnr* expression

The results of DNase I footprinting analysis described above strongly suggested that interaction of ResD~P with α, very likely αCTD, is important in transcriptional activation of *fnr*. To examine this possibility, we took advantage of a recently constructed αCTD mutant library (Zhang *et al.*, 2006). This mutant library was constructed by replacing the wild-type *rpoA* with a mutant allele bearing an alanine codon substitution. *B. subtilis* strains carrying substitutions E255A, R261A, R268A, R289A, and G292A could not be isolated probably because these residues are essential for transcription of housekeeping genes. Residues 269, 278, and 301 are alanine in the wild-type α, and we substituted only residue 269 with isoleucine. Within the residues 251 to 314, substitutions Y263A, K267A, A269I, and N290A strongly reduced *fnr* expression (Fig. 2.6). I253A, E254A, V260A, L266A, and G309A had a moderate effect on transcription (30 to 50% of the wild-type expression). A previous study showed that substitutions of these residues had no significant effect on expression of *rpsD* encoding ribosomal S6, except that Y263C and Y263A led to 50% and 40% reduction in *rpsD* expression, respectively

(Zhang *et al.*, 2006), indicating that the effect of these substitutions in transcriptional activation is specific to *fnr*.

### 2.2.5 K267 of αCTD is essential for interaction with ResD~P

One possible explanation of the reduced *fnr* expression in cells producing certain αCTD mutants is that the mutated residues are essential for interaction with ResD~P. To examine this possibility, DNase I footprinting analysis of the wild-type *fnr* promoter was carried out using the αCTD (K267A) protein. As shown in Fig. 2.7, the mutant αCTD binds to the region between –38 and –50 with an affinity similar to that of the wild-type αCTD (lanes 6 to 8 and 12 to 14). In the presence of ResD~P, the wild-type αCTD was able to bind to DNA at a concentration lower than the αCTD alone, and the protected region extended to the upstream region including the TTCA sequence (lanes 9 to 11), indicating that interaction between αCTD and ResD~P stabilizes binding of both proteins to DNA. In contrast, ResD~P did not significantly increase binding of the mutant αCTD to DNA, and the mutant αCTD did not stimulate binding of ResD~P to the TTCA sequence (lanes 15 to 17). We concluded from these results that K267 of αCTD is essential for interaction with ResD~P at the *fnr* promoter and that the interaction accelerates transcription initiation of *fnr*.

## 2.3 DISCUSSION

On the basis of the results described here, we propose that ResD~P binds to the TTCA sequence in the *fnr* and *resA* promoters and likely the sequence in the *sboA* promoter, which supports the hypothesis that TGAA in the proposed consensus sequence a (Fig. 2.1A) is important for binding of ResD~P to activate transcription of *ctaA*, *ctaB*, and *yclJ*. Given that either TTCA or TGAA is likely used as a ResD-binding site in divergently transcribed *ctaA* and *ctaB* as well as *yclI* and *yclJ*, we assume that ResD~P could bind to DNA in both orientations. ResD~P dissociation constants for the *ctaA* genes calculated by Zhang and Hulett were as follows: 8.9 nM for *ctaA1*, 200 nM for *ctaA2*, and

47 nM for *ctaA3* (Zhang and Hulett, 2000). This may suggest that ResD~P binds to a direct repeat (TTCA $N_7$ TTCA or TGAA $N_7$ TGAA present in *ctaA1* and *ctaA3*) with an affinity higher than that to a single TGAA in *ctaA2*. TTCA $N_7$ TTCA is similar to a half-site of the PhoP-binding sequence [TT(A/T/C)ACA $N_{4-6}$ TT(A/T/C)ACA] that accommodates a PhoP dimer (Hulett, 2001; Liu and Hulett, 1998). Previous work showed that *resA* expression is positively regulated by the ResDE and PhoPR signal transduction systems and that PhoP~P binds to the *resA* promoter region that includes the direct repeat sequence shown in Fig. 2.2 (Birkey *et al.*, 1998), indicating that the same sequence (or overlapping sequence) of *resA* is used for binding of the two response regulators.

We have shown by hydroxyl radical footprinting that ResD~P binds to five distinct regions of *nasD* (positions –90 to –45) and *hmp* (–80 to –40), and we have shown by deletion analysis that *cis* regions required for activation by ResD~P reside downstream of –87 (*nasD*) or –67 (*hmp*). Within these regions, no TGAA sequence is identified, but a single TTCA sequence (–87 to –84 in *nasD* and –48 to –45 in *hmp*) is detected, suggesting that ResD~P binds to and activates these promoters in a way different from *cta* and *ycl* that contain the direct repeat. In light of consensus sequence a and b, we propose TTGTGAA$N_3$TTT$N_4$A (Fig. 2.1C) as a consensus ResD box (consensus sequence c) for *fnr*, *nasD*, and *hmp*. Consensus sequence c is composed of two half-sites of 9 bases—site a (TTGTGAANN), which is a part of consensus sequence a, and site b (NTTT$N_4$A), which is similar to the 3' end of consensus sequence b. Figure 2.1C shows a possible alignment of these sites in the *nasD*, *hmp*, and *fnr* promoters. Each regulatory sequence listed in Fig. 2.1C was shown to be sufficient for full activation by ResD~P (Nakano *et al.*, 2000b). Two consensus sequences (8 of 11 matches) are present in *nasD*, which likely accommodate two ResD~P dimers. Upstream of the two putative ResD~P dimer-binding sites, half-site a is present in the opposite orientation. The *hmp* and *fnr* promoters each contain a single full-site and one half-site a that is oriented oppositely to the full site. The half-site resides upstream (in *fnr*) or downstream (in *hmp*) of the full site. Site a, but not site b, is able to serve as a stand-alone half-site, suggesting that ResD~P binds to site b only after site a is occupied by ResD~P. We reexamined the result of base substitutions in the *fnr* promoter in the context of the newly proposed consensus ResD-binding sequence. Among the bases in *fnr*-2a (TTGTTAG), the first G (–49), and the third

T (–48) in particular, are important for ResD-dependent transcription because the change from TTGTTAG to either TTTTTAG or TTGGTAG led to moderate and severe reduction of *fnr* transcription (Fig. 2.2A), respectively. This result is in good agreement with the proposed consensus sequence half-site a ($TTGTGAAN_2$). The change of A at –46 to G (resulting in TTGTTGG) and G at –45 to A (TTGTTAA) did not significantly decrease or increase transcription, indicating that the two A residues in the proposed half-site a are not critical. We showed that the adverse effect of the G–49T mutation was suppressed by the T–47C and G–45A mutations (Fig. 2.2B). We interpreted this result as meaning that the TTCA direct repeat does not increase *fnr* transcription; however, we now suggest another interpretation. The G–49T substitution (TTTT) impairs the function of the critical TTGT sequence in consensus sequence c, thereby adversely affecting *fnr* transcription. Introduction of the T–47C mutation in the G–49T promoter generates TTCA, and the resultant TTCA $N_7$ TTCA sequence now functions as a ResD-binding site like those found in *cta* and *ycl*. Further studies of base substitutions, including the first two T residues in the $TTGTGAAN_2$ sequence, are required to properly evaluate the putative consensus sequence c and to understand how differences in the half-site arrangement and orientation in various promoters affect binding and activation by ResD~P.

This study also uncovered the critical role of αCTD for ResD-dependent *fnr* transcription. Transcriptional activators bind to specific DNA sequences and often interact with αCTD. This protein-protein interaction increases the affinity of RNAP to the promoter site to initiate transcription (reviewed in reference (Busby and Ebright, 1994). Such an activator-dependent transcription is usually observed in promoters lacking the consensus –35 sequence as is the case with ResD-controlled promoters. The results of our footprinting analysis showed that ResD~P alone does not bind to the *fnr* promoter, unlike the *nasD* or *hmp* promoter (Geng *et al.*, 2004) and the *cta* (Zhang and Hulett, 2000) or *ycl* (Härting *et al.*, 2004) promoter. Hence, a simple recruiting model in which ResD~P binds to the ResD box and recruits RNAP to the promoter seems inapplicable to explain how ResD~P activates *fnr* transcription. A ternary complex by ResD~P, RNAP, and *fnr* is formed presumably through direct interaction between ResD~P and αCTD. Screening of the αCTD alanine substitution library identified residues that are important for *fnr*

activation by ResD~P. Among these residues, E254, V260, Y263, K267, and A269 form a surface-exposed patch (Fig. 2.8). Residues D258, K271, A272, E273, and L289 in *E. coli* αCTD, which correspond to E254, K267, R268, A269, and M285, respectively, in *B. subtilis* αCTD, are proposed to interact with the Fis transcriptional activator (Aiyar *et al.*, 2002; McLeod *et al.*, 2002). Therefore, ResD~P likely interacts with a surface of αCTD similar to the "273 determinant" that interacts with Fis. N290, which is important for ResD-dependent activation of *fnr*, is not a part of this surface patch proposed to interact with ResD~P, and might be involved in interaction with DNA. In fact, the corresponding residue (N294) in *E. coli* αCTD was shown to interact with DNA (Gaal *et al.*, 1996). Our DNase I footprinting analysis demonstrated that αCTD carrying the K267A mutation binds to *fnr* DNA nearly as well as the wild-type αCTD does; however, the mutant αCTD, unlike the wild-type protein, does not facilitate binding of ResD~P to the TTCA sequence, confirming our hypothesis that the surface including K267 interacts with ResD~P. The residue K267, as well as Y263, C265, and L266, was shown to be required for optimal ComA-dependent activation of the *srf* operon encoding proteins that function in the control of competence development and in nonribosomal peptide synthesis (Zhang *et al.*, 2006). The C265A mutation that most severely affected *srf* expression had no effect on *fnr* expression. Conversely, A269I conferred severely reduced *fnr* expression but only moderately affected *srf* transcription. Therefore, it is likely that ResD~P and ComA~P interact with overlapping but distinct surfaces of αCTD.

The ResD-αCTD-DNA ternary complex was not formed with *fnr* carrying the T–48G mutation, suggesting that the in vivo defect in transcription caused by the mutation could be due to the lack of cooperative binding between ResD~P and αCTD. Purified αCTD protects a region around –48, raising the possibility that αCTD might make direct contact with T at –48. However, the change of –48T to G did not weaken the binding of αCTD (Fig. 2.4), instead we observed DNase I-hypersensitive sites unique to T–48G DNA (Fig. 2.3 to 2.5). One could imagine that the substitution of T at –48 with G introduces a kink around –50, which results in interference between ResD~P and RNAP, thus disrupting the ResD~P-RNAP-DNA ternary complex.

Although αCTD alone binds to a region between –50 and –38, it remains unclear whether the protection of the region in the presence of αCTD and ResD~P is caused by

binding of αCTD, ResD~P, or both because the sequence between –51 and –34 contains two half-sites that show similarity to consensus sequence c (Fig. 2.1C). One possibility is that ResD~P and αCTD interact by binding to the same region but to different faces of the DNA helix as seen with BvgA and αCTD in *E. coli* (Boucher *et al.*, 2003). DNase I footprinting experiments in Fig. 2.3 showed that hypersensitive sites that were observed in the presence of RNAP disappeared when both RNAP and ResD~P were present, indicating that the interaction with ResD~P and αCTD remodels RNAP-promoter interaction to initiate transcription.

## 2.4 MATERIALS AND METHODS

### 2.4.1 Strains and plasmids

All *B. subtilis* strains used in this study are derivatives of *B. subtilis* JH642 and listed in Table 2.1. Construction of *rpoA* with alanine substitution at each residue in αCTD was previously described (Zhang *et al.*, 2006). Plasmids are also listed in Table 2.1.

### 2.4.2 Measurement of β-galactosidase activity

*B. subtilis* cells were grown anaerobically in 2x yeast extract-tryptone (2xYT) medium (Nakano *et al.*, 1988) supplemented with 1% glucose and 0.2% $KNO_3$ (the starting optical density at 600 nm was 0.02) with 5 μg/ml of chloramphenicol. Samples were taken at 1-h intervals, and β-galactosidase activity was measured as described previously (Miller, 1972). The activity reaches its maximum level around T1 (1 h after the end of exponential growth), which was shown in Fig. 2.2.

### 2.4.3 Mutational analysis of the *fnr* and *resA* promoters

Base substitutions in the *fnr* promoter were generated by PCR amplification using an upstream mutagenic oligonucleotide (oligonucleotides listed in Table 2.2) and a downstream oligonucleotide, oMN97-3 with chromosomal DNA isolated from *B. subtilis*

JH642 as the template. Base substitutions in the *resA* promoter were generated by two-step PCR amplification. Briefly, two PCR products were generated from pMMN649 using one of the complementary mutagenic oligonucleotides (Table 2.2) together with either oMN06-305 or oMN06-306. The two PCR products were annealed and extended, and the resulting product was used as the template in a second PCR with oMN06-305 and oMN06-306. The resultant PCR products carrying the *fnr* (positions –62 to +265 relative to the transcription start site) and *resA* (–134 to +65) promoters were digested with EcoRI and BamHI and inserted into pTK*lac* (Kenny and Moran Jr., 1991) which had been digested with the same enzymes, to generate transcriptional *lacZ* fusions. Each mutation was verified by sequencing. The *fnr-lacZ* and *resA-lacZ* constructs were integrated into the SPβ locus of JH642 chromosome as described previously (Zuber and Losick, 1987).

### 2.4.4 Protein purification

Purification of ResD and ResE proteins were described elsewhere (Geng *et al.*, 2004). RNA polymerase (RNAP) was purified from *B. subtilis* MH5646 that produces the RNAP β' subunit fused to a 10x His tag by $Ni^{2+}$-nitrilotriacetic acid chromatography as described previously (Qi and Hulett, 1998), followed by HiQ column chromatography (Nakano *et al.*, 2005). $\sigma^A$, α, and αCTD (between residues 239 and 314) proteins were produced in *Escherichia coli* carrying pSN64, pSN28, and pSN106, respectively, and purified as described in previous papers (Nakano *et al.*, 2006; Nakano *et al.*, 2003b; Nakano *et al.*, 2005). αCTD protein with the K267A mutation was produced using *E. coli* carrying pZY18 and purified by the procedure used for the wild-type αCTD purification (Nakano *et al.*, 2005). pZY18 was constructed in the same manner as pSN106 was (Nakano *et al.*, 2005), except chromosomal DNA isolated from *B. subtilis* ORB5262 [*rpoA* encoding αCTD with the K267A mutation (Zhang *et al.*, 2006)] was used as the template for PCR.

### 2.4.5 DNase I footprinting

A fragment carrying the wild-type *fnr* promoter (positions –136 to +20) was amplified by PCR using oligonucleotides oHG-2 and oHG-9 with pMMN408 as the

template. Mutant promoters (–47T to C and –48T to G) were amplified using the same oligonucleotides with pHG45 and pHG71, respectively, as the template. Two PCR products were generated from pMMN408 using oligonucleotide pairs, oMMN99-24/oHG66 and oMMN99-25/oHG65 where oHG65 and oHG66 are complementary mutagenic oligonucleotides. The second PCR was carried out using the first PCR products with oMN99-24 and oMMN99-25. The second PCR product digested with EcoRI and BamHI was cloned into pUC18 digested with the same enzymes to generate pHG45. pHG71 was generated similarly, except oHG101 and oHG102 were used for mutagenic oligonucleotides. To label the coding or noncoding strand, one of the primers was phosphorylated with T4 polynucleotide kinase and [$\gamma$-$^{32}$P] ATP. The labeled PCR products were separated on a nondenaturing polyacrylamide gel and purified as previously described (Nakano *et al.*, 2000b), and DNase I footprinting was carried out as previously described (Härting *et al.*, 2004). The dideoxynucleotide sequence ladder was obtained by using the Thermo Sequenase cycle sequencing kit (United States Biochemical) using the labeled primer and pMMN408 as the template.

**TABLE 2.1 *Bacillus subtilis* strains and plasmids**

| Strain or plasmid | Relevant feature | Reference |
|---|---|---|
| Strains | | |
| JH642 | Parental strain (*trpC2 pheA1*) | J. A. Hoch |
| LAB2714 | SPβc2Δ2::Tn*917*::pMMN350 | This study |
| LAB2761 | SPβc2Δ2::Tn*917*::pMMN380 | This study |
| LAB2764 | SPβc2Δ2::Tn*917*::pMMN351 | This study |
| ORB3502 | SPβc2Δ2::Tn*917*::pYZ32 | This study |
| ORB3503 | SPβc2Δ2::Tn*917*::pYZ33 | This study |
| ORB3504 | SPβc2Δ2::Tn*917*::pYZ34 | This study |
| ORB3505 | SPβc2Δ2::Tn*917*::pYZ35 | This study |
| ORB5001 | SPβc2Δ2::Tn*917*::pMMN580 | This study |
| ORB5019 | SPβc2Δ2::Tn*917*::pKM3 | This study |
| ORB5090 | SPβc2Δ2::Tn*917*::pKM4 | This study |
| ORB5262 | *rpoA* (K267A) | (Zhang *et al.*, 2006) |
| ORB6462 | SPβc2Δ2::Tn*917*::pMMN650 | This study |
| ORB6463 | SPβc2Δ2::Tn*917*::pMMN652 | This study |
| ORB6466 | SPβc2Δ2::Tn*917*::pMMN651 | This study |
| ORB6467 | SPβc2Δ2::Tn*917*::pMMN653 | This study |
| ORB6513 | SPβc2Δ2::Tn*917*::pMMN649 | This study |
| ORB6589 | SPβc2Δ2::Tn*917*::pMMN658 | This study |
| ORB6590 | SPβc2Δ2::Tn*917*::pMMN659 | This study |
| ORB6613 | SPβc2Δ2::Tn*917*::pCSZ4 | This study |
| ORB6614 | SPβc2Δ2::Tn*917*::pCSZ5 | This study |
| ORB6615 | SPβc2Δ2::Tn*917*::pCSZ6 | This study |
| ORB6637 | SPβc2Δ2::Tn*917*::pMMN670 | This study |

| Plasmids | | |
|---|---|---|
| pTK*lac* | Promoter-probe vector; $Amp^r$ $Cm^r$ | (Kenny and Moran Jr., 1991) |
| pCSZ4 | pTK*lac* with *resA* promoter (–134 to +65, –48C to T) | This study |
| pCSZ5 | pTK*lac* with *resA* promoter (–134 to +65, –60T to G) | This study |
| pCSZ6 | pTK*lac* with *resA* promoter (–134 to +65, –59C to T) | This study |
| pHG45 | pUC18 with *fnr* promoter (–169 to +96, –47T to C) | This study |
| pHG71 | pUC18 with *fnr* promoter (–169 to +96, –48T to G) | This study |
| pKM3 | pTK*lac* with *fnr* promoter (–62 to +265, –47T to C) | This study |
| pKM4 | pTK*lac* with *fnr* promoter (–62 to +265, –45G to A) | This study |
| pMMN350 | pTK*lac* with *fnr* promoter (–62 to +265, –59T to G) | This study |
| pMMN351 | pTK*lac* with *fnr* promoter (–62 to +265, –57A to C) | This study |
| pMMN380 | pTK*lac* with *fnr* promoter (–62 to +265, –48T to G) | This study |
| pMMN408 | pUC18 with *fnr* promoter (–169 to +96) | (Nakano *et al.*, 2000b) |
| pMMN580 | pTK*lac* with *fnr* promoter (–62 to +265, –49G to T) | This study |
| pMMN649 | pTK*lac* with *resA* promoter (–134 to +65) | This study |
| pMMN650 | pTK*lac* with *fnr* promoter (–62 to +265, –55A to G) | This study |
| pMMN651 | pTK*lac* with *fnr* promoter (–62 to +265, –46A to G) | This study |
| pMMN652 | pTK*lac* with *fnr* promoter (–62 to +265, –49G to T, –47T to C, –45G to A) | This study |
| pMMN653 | pTK*lac* with *fnr* promoter (–62 to +265) | This study |
| pMMN658 | pTK*lac* with *resA* promoter (–134 to +65, –50T to G) | This study |
| pMMN659 | pTK*lac* with *resA* promoter (–134 to +65, –47A to G) | This study |
| pMMN670 | pTKlac with *resA* promoter (–134 to +65, –49T to G) | This study |
| pSN28 | pTYB2 with *rpoA* | (Nakano *et al.*, 2003a) |
| pSN64 | pTYB4 with *sigA* | (Nakano *et al.*, 2006) |
| pSN106 | pTYB2 with *rpoA* encoding αCTD | (Nakano *et al.*, 2005) |
| pYZ32 | pTK*lac* with *fnr* promoter (–62 to +265, –60T to C) | This study |

| | | |
|---|---|---|
| pYZ33 | pTK*lac* with *fnr* promoter (–62 to +265, –58C to T) | This study |
| pYZ34 | pTK*lac* with *fnr* promoter (–62 to +265, –56C to T) | This study |
| pYZ35 | pTK*lac* with *fnr* promoter (–62 to +265, –54A to G) | This study |
| pZY18 | pTYB2 with *rpoA* encoding αCTD (K267A) | This study |

**TABLE 2.2 Oligonucleotides**

| Oligonucleotide | Sequence (5' to 3')[a] | Use |
|---|---|---|
| oHG-2 | CTCGAAGAAAGTCACGTTGT | DNase I footprinting |
| oHG-9 | GGATGTATTGGCAGGAAAG | DNase I footprinting |
| oHG-65 | CACAAGATTGTCAGTTTTTTCTC | pHG45 |
| oHG-66 | GAGAAAAAACTGACAATCTTGTG | pHG45 |
| oHG-101 | CACAAGATTGGTAGTTTTTTCTC | pHG71 |
| oHG-102 | GAGAAAAAACTACCAATCTTGTG | pHG71 |
| oMN97-3 | CGGGATCCGACGATATCATGCT | *fnr* downstream |
| oMN97-4 | GGAATTCATGCACAAGATTGTTA | pMMN350 |
| oMN97-5 | GGAATTCATTCCCAAGATTGTTA | pMMN351 |
| oMN97-6 | GGAATTCATTCACAAGATTGGTAGTT | pMMN380 |
| oMN98-24 | GGAATTCAGAGGTGGCGTTA | pMMN408 |
| oMN98-25 | CGGGATCCAGCAATTCATAC | pMMN408 |
| oMN99-83 | GGAATTCACTCACAAGATTGTT | pYZ32 |
| oMN99-84 | GGAATTCATTTACAAGATTGTT | pYZ33 |
| oMN99-85 | GGAATTCATTCATAAGATTGTT | pYZ34 |
| oMN99-86 | GGAATTCATTCACAGGATTGTT | pYZ35 |
| oMN04-256 | GGAATTCATTCACAAGATTTTTAGTTTTTTCTC | pMMN580 |
| oMN04-257 | GGAATTCATTCACAAGATTGTCAGTTTTTTCTC | pKM3 |
| oMN04-263 | GGAATTCATTCACAAGATTGTTAATTTTTTCTC | pKM4 |
| oMN06-305 | GAGAATTCGATGCCAGAGAGTTA | pMMN649 |
| oMN06-306 | TTAGGATCCGGTCCGAATGAATAA | pMMN649 |
| oMN06-307 | GGAATTCATTCACAAGATTGTT | pMMN653 |
| oMN06-308 | GGAATTCATTCACGAGATTGTT | pMMN650 |
| oMN06-309 | GGAATTCATTCACAAGATTGTTGGTTTTTTCT | pMMN651 |

| | | |
|---|---|---|
| oMN06-310 | GGAATTCATTCACAAGATTTTCAATTTTTTCT | pMMN652 |
| oMN06-312 | ATTTCACATAACCGTCAAAAAGTAAGA | pMMN658 |
| oMN06-313 | TTCACATAACCTTCGAAAAGTAAGAAA | pMMN659 |
| oMN06-314 | TCTTACTTTTTGACGGTTATGTGAAAT | pMMN658 |
| oMN06-315 | TTTCTTACTTTTCGAAGGTTATGTGAA | pMMN659 |
| oMN06-324 | TTTCACATAACCTTTAAAAAGTAAGAAA | pCSZ4 |
| oMN06-325 | TTTCTTACTTTTTAAAGGTTATGTGAAA | pCSZ4 |
| oMN06-326 | GCTTTCTAAATTGCACATAACCTTC | pCSZ5 |
| oMN06-327 | GAAGGTTATGTGCAATTTAGAAAGC | pCSZ5 |
| oMN06-328 | CGCTTTCTAAATTTTACATAACCTTCA | pCSZ6 |
| oMN06-329 | TGAAGGTTATGTAAAATTTAGAAAGCG | pCSZ6 |
| oMN06-332 | TTTCACATAACCTGCAAAAAGTAAGAAA | pMMN670 |
| oMN06-333 | TTTCTTACTTTTTGCAGGTTATGTGAAA | pMMN670 |
| oSN03-88 | GGAATTCCATATGGAAAAAGAAGAAGATCAAAAAG | pZY18 |
| oSN03-89 | ATCGTCTTTGCGAAGTCCGAGTC | pZY18 |

[a] Restriction enzyme sites and base substitutions are underlined.

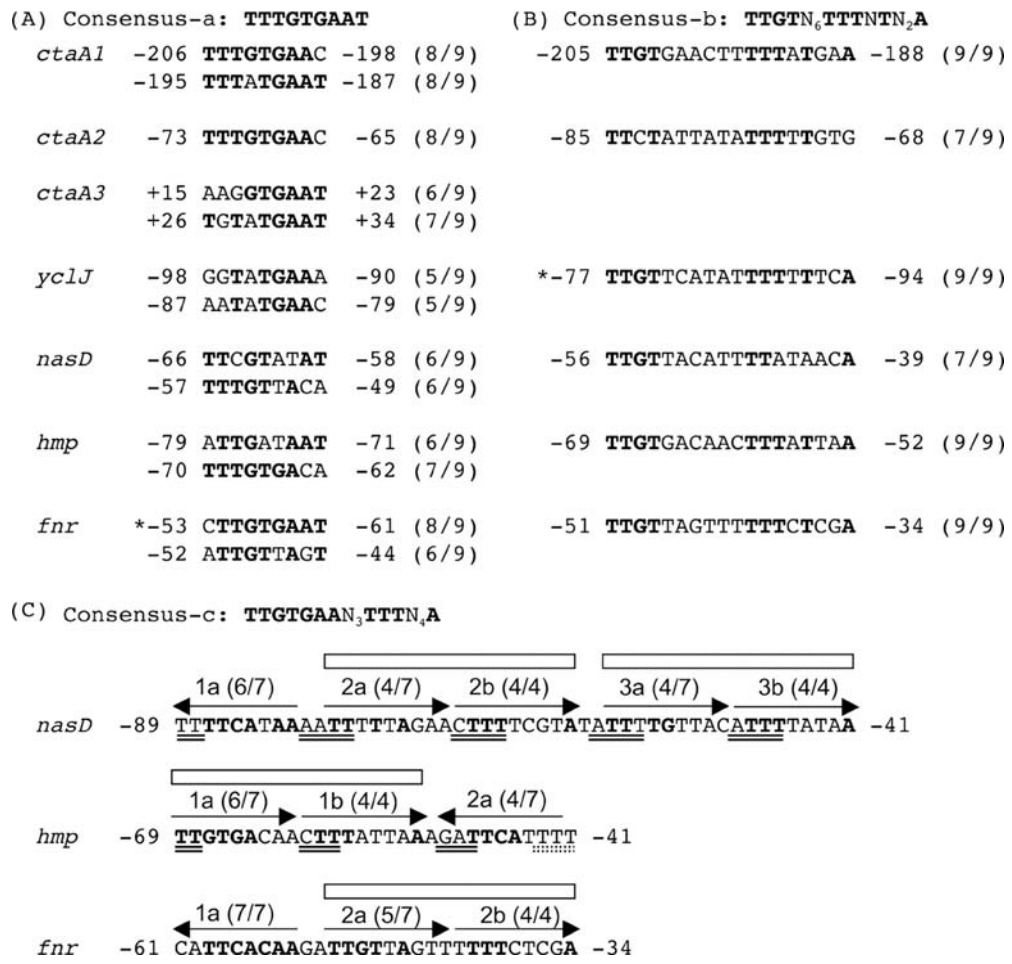


FIG. 2.1. Comparison of proposed consensus ResD-binding sequences. (A) Consensus sequence a was proposed by a sequence alignment of experimentally detected ResD-binding regions (Nakano *et al.*, 2000; Zhang and Hulett, 2000). Numbering is relative to the transcription start site. *ctaA* has three distinct ResD-binding regions (Zhang and Hulett, 2000). Bases identical to those in the consensus sequence are indicated by bold type, and the numbers in parentheses show the number of bases of the total number of bases that match those in the consensus sequence. An asterisk indicates the sequence on the complementary strand. (B) Consensus sequence b was defined by bioinformatics approach (Härting *et al.*, 2004). Sequence similarity with consensus sequence b is not detected in *ctaA3*. (C) A newly proposed consensus ResD-binding sequence (consensus sequence c) for *nasD*, *hmp*, and *fnr*. The full consensus sequence is shown by a box above the sequence. Half-site a is TTGTGAAN2, and half-site b is NTTTN4A. The arrows show the orientation of each half-site. Nucleotides doubly underlined are the nucleotides protected by ResD~P from attack by hydroxyl radicals, and a dotted double line shows protected nucleotides located on the opposite face of DNA helix (Geng *et al.*, 2004).

(A)

| | | |
|---|---|---|
| *PsboA* | −63 | TTTTCACATTTTTTTCAAAATATA −40 |
| *PresA* | −63 | ATTTCACATAACCTTCAAAAAGTA −40 |
| *Pfnr* | −62 | CATTCACAAGATTGTTAGTTTTTT −39 |

(B)

| Plasmid | Base substitution | *fnr-lacZ* (Miller units) |
|---|---|---|
| pMMN653 | Wild-type | 547 ± 4.9 |
| pYZ32 | −60T to C | 48 ± 0 |
| pMMN350 | −59T to G | 43 ± 4.2 |
| pYZ33 | −58C to T | 47 ± 7.1 |
| pMMN351 | −57A to C | 53 ± 18 |
| pYZ34 | −56C to T | 169 ± 10 |
| pMMN650 | −55A to G | 356 ± 52 |
| pYZ35 | −54A to G | 413 ± 63 |
| pMMN580 | −49G to T | 128 ± 1.0 |
| pMMN380 | −48T to G | 15 ± 2.8 |
| pKM3 | −47T to C | 693 ± 71 |
| pMMN651 | −46A to G | 653 ± 63 |
| pKM4 | −45G to A | 557 ± 54 |
| pMMN652 | −49G to T, −47T to C, and −45G to A | 434 ± 21 |

(C)

| Plasmid | Base substitution | *resA-lacZ* (Miller units) |
|---|---|---|
| pMMN649 | Wild-type | 744 ± 68 |
| pCSZ5 | −60T to G | 50 ± 7.2 |
| pCSZ6 | −59C to T | 62 ± 16 |
| pMMN658 | −50T to G | 651 ± 55 |
| pMMN670 | −49T to G | 88 ± 3.5 |
| pCSZ4 | −48C to T | 307 ± 56 |
| pMMN659 | −47A to G | 644 ± 41 |

FIG. 2.2. Mutational analysis of the *fnr* and *resA* promoters. (A) Comparison of the promoter regions of ResDE-dependent *sboA*, *resA*, and *fnr*. The positions of nucleotides are relative to the transcription start site. The conserved TTCA sequence is underlined. (B) Effects of base substitutions in the *fnr* promoter region on transcription. (C) Effects of base substitutions in the *resA* promoter region on transcription. Cells were grown anaerobically in 2xYT supplemented with 1% glucose and 0.2% $KNO_3$, and β-galactosidase activities were measured as described in Materials and Methods. Experiments were repeated two to six times, and the averages of maximal activities around T1 (1 h after the end of exponential growth) are shown with standard deviations.

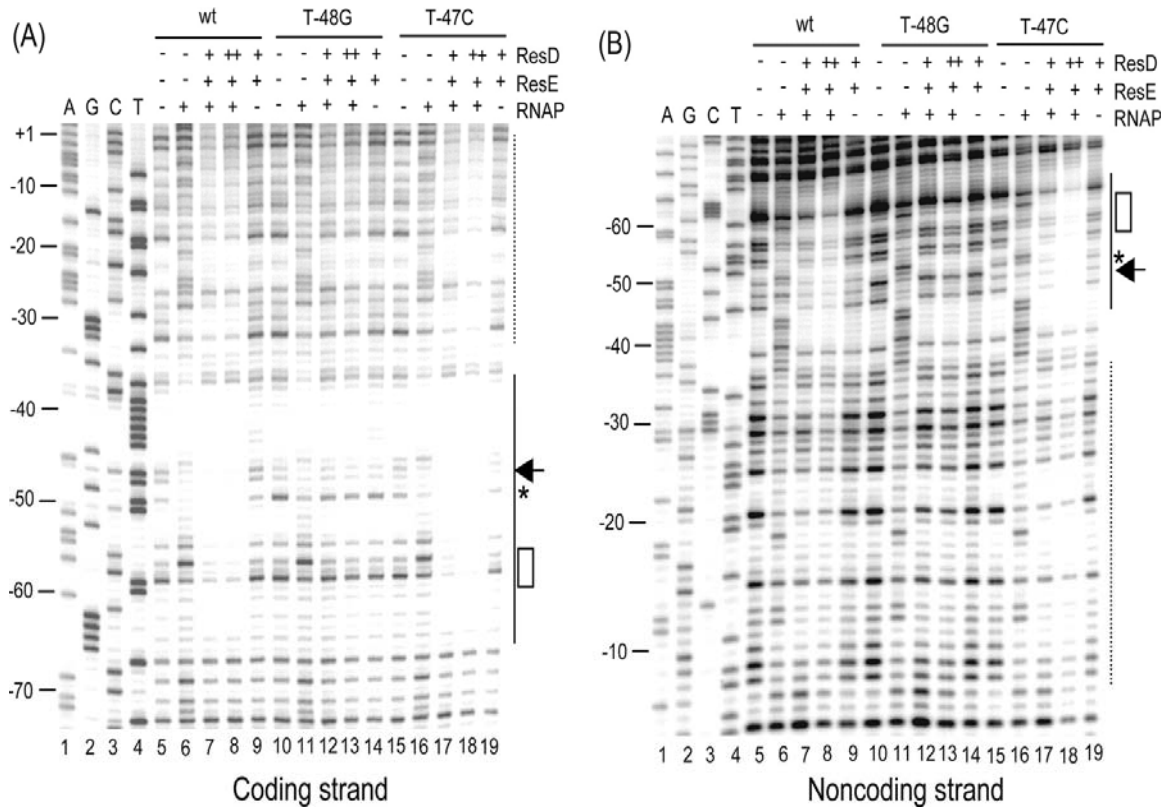


FIG. 2.3. DNase I footprinting analysis of the wild-type (wt) and mutant (T–47C and T–48G) promoters in the presence of ResD~P and RNAP. The coding (A) and noncoding (B) strands of each promoter fragment were labeled as described in Materials and Methods. ResD was phosphorylated with 2 μM ResE. Two concentrations of ResD, 3 μM (+) or 6 μM (++), were used (–, none). *B. subtilis* RNAP (50 nM) was used with 50 nM purified $\sigma^A$. Regions of strong and weak protection from DNase I digestion are marked by solid and dotted lines, respectively. A box and an arrow show TTCA (positions –60 to –57) and T at –48, respectively, which are critical for *fnr* transcription. A hypersensitive site detected in the T–48G promoter is shown with an asterisk. Dideoxynucleotide sequencing reactions are also shown, and nucleotide positions are marked relative to the transcription start site.

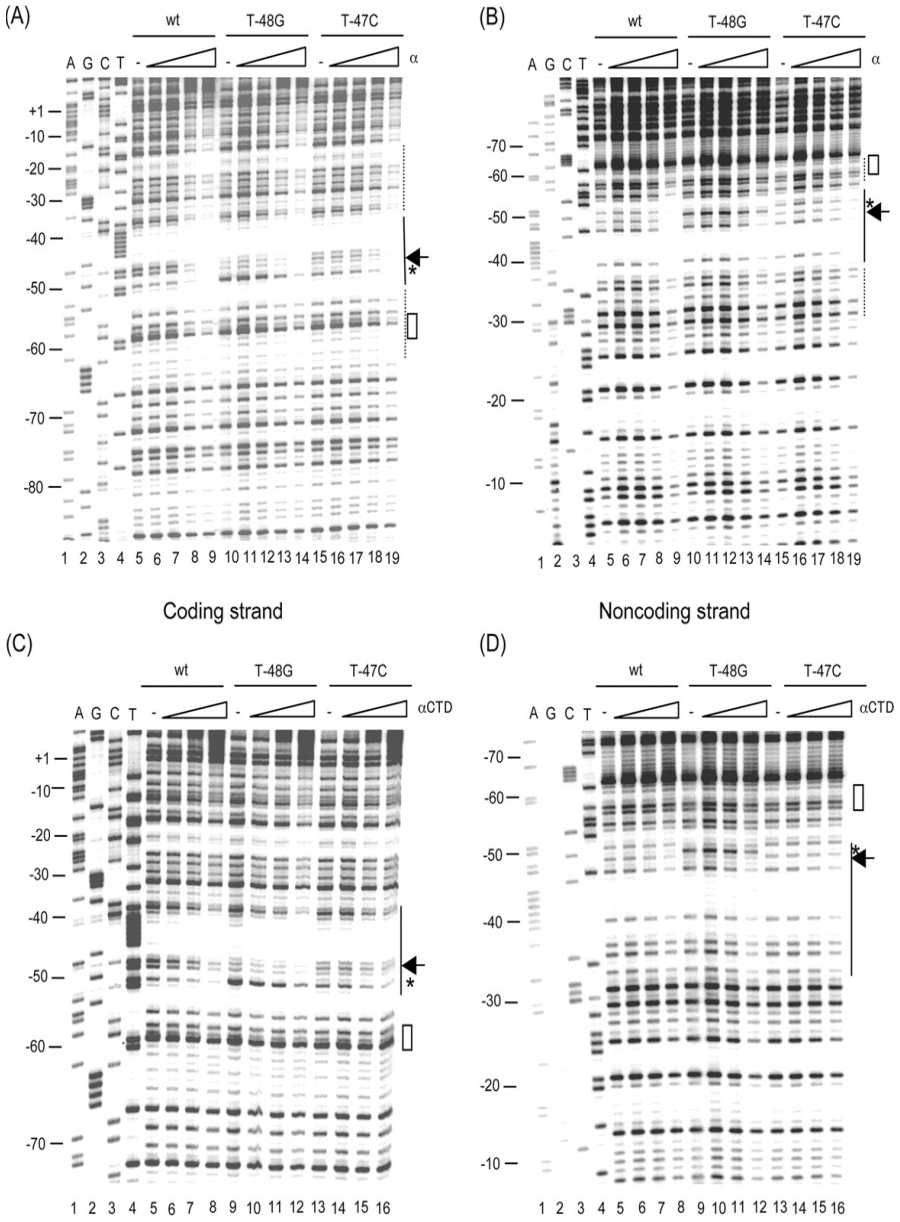


FIG. 2.4. DNase I footprinting analysis of the wild-type (wt) and mutant (T–47C and T–48G) promoters in the presence of α or αCTD. The coding (A and C) and noncoding (B and D) strands of each promoter fragment were labeled as described in Materials and Methods. (A and B) Increased concentrations of α (0.75, 1.5, 3, and 6 μM) were used. –, none. (C and D) Increased concentrations of αCTD (12.5, 25, and 50 μM) were used. Regions of strong and weak protection from DNase I digestion are marked by solid and dotted lines, respectively. A box and an arrow show TTCA (positions –60 to –57) and T at –48, respectively. A hypersensitive site detected in the T–48G promoter is shown with an asterisk. Dideoxynucleotide sequencing reactions are also shown, and nucleotide positions are marked relative to the transcription start site.

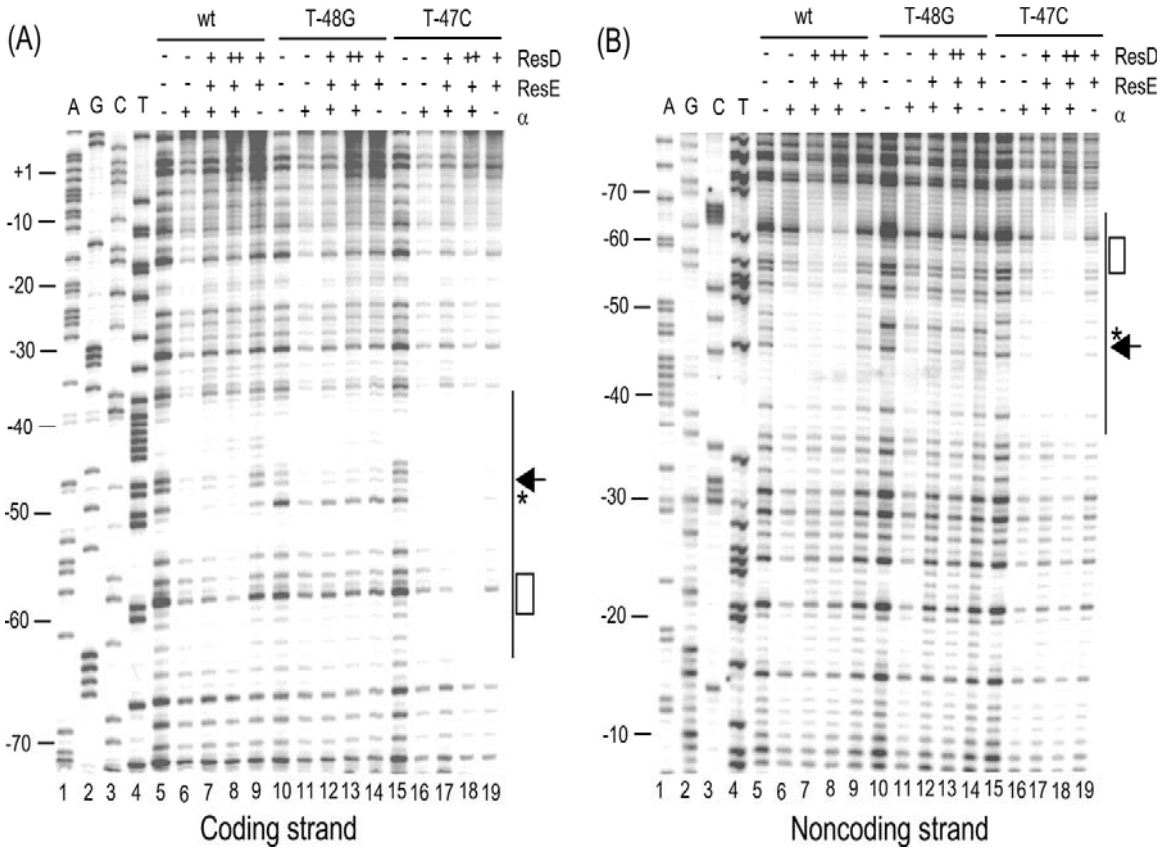


FIG. 2.5. DNase I footprinting analysis of the wild-type (wt) and mutant (T–47C and T–48G) promoters in the presence of ResD~P and α. DNase I footprinting analysis was carried out in a manner similar to that described in the legend to Fig. 2.3, except that 3 μM α was used instead of RNAP. A box and an arrow show TTCA (–60 to –57) and T at –48, respectively. A hypersensitive site detected in the T–48G promoter is shown with an asterisk. Dideoxynucleotide sequencing reactions are also shown, and nucleotide positions are marked relative to the transcription start site.

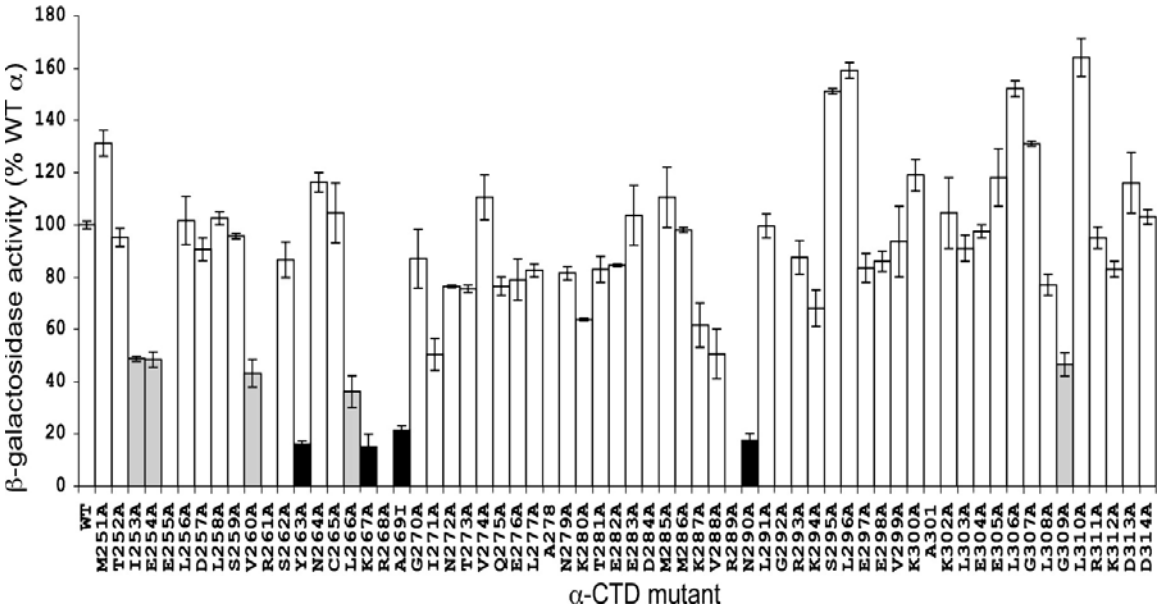


FIG. 2.6. Effects of single alanine substitutions in αCTD on *fnr-lacZ* expression. The wild-type (WT) strain and each αCTD mutant were grown anaerobically in 2xYT supplemented with 1% glucose and 0.2% $KNO_3$, and ß-galactosidase activities were measured as described in Materials and Methods. Experiments were repeated two or three times, and the average of maximal activities around T1 (1 h after the end of exponential growth) is expressed as percentage of the activity in cells carrying the wild-type *rpoA* gene. Black bars indicate substitutions that reduced the expression to less than 20% of the wild-type level, and gray bars indicate substitutions that reduced expression to 30 to 50%. Substitutions of residues with no data indicate those substitutions not obtained in *B. subtilis* (Zhang *et al.*, 2006).

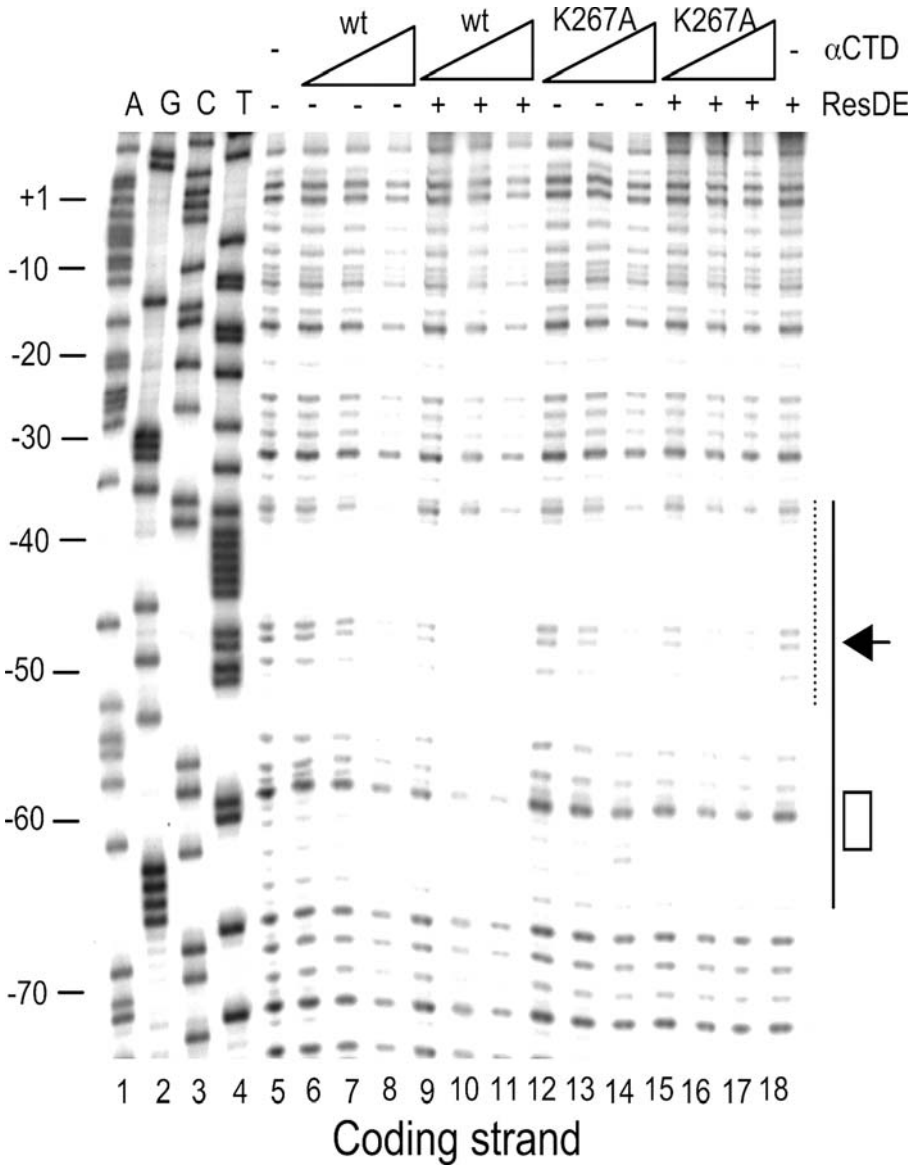


FIG. 2.7. DNase I footprinting analysis of the wild-type *fnr* promoter in the presence of ResD~P and the wild-type or K267A αCTD. The coding strand of the *fnr* promoter was labeled as described in Materials and Methods. ResD (6 μM) was phosphorylated with 2 μM ResE. An increased concentration (25, 50, and 100 μM) of the wild-type (wt) or K267A αCTD was included when indicated. The dotted line indicates the region protected by αCTD, and the solid line indicates the region protected in the presence of the wild-type αCTD and ResD~P. The box and arrow show TTCA (–60 to –57) and T at –48, respectively. Dideoxynucleotide sequencing reactions are also shown, and nucleotide positions are marked relative to the transcription start site. –, none.

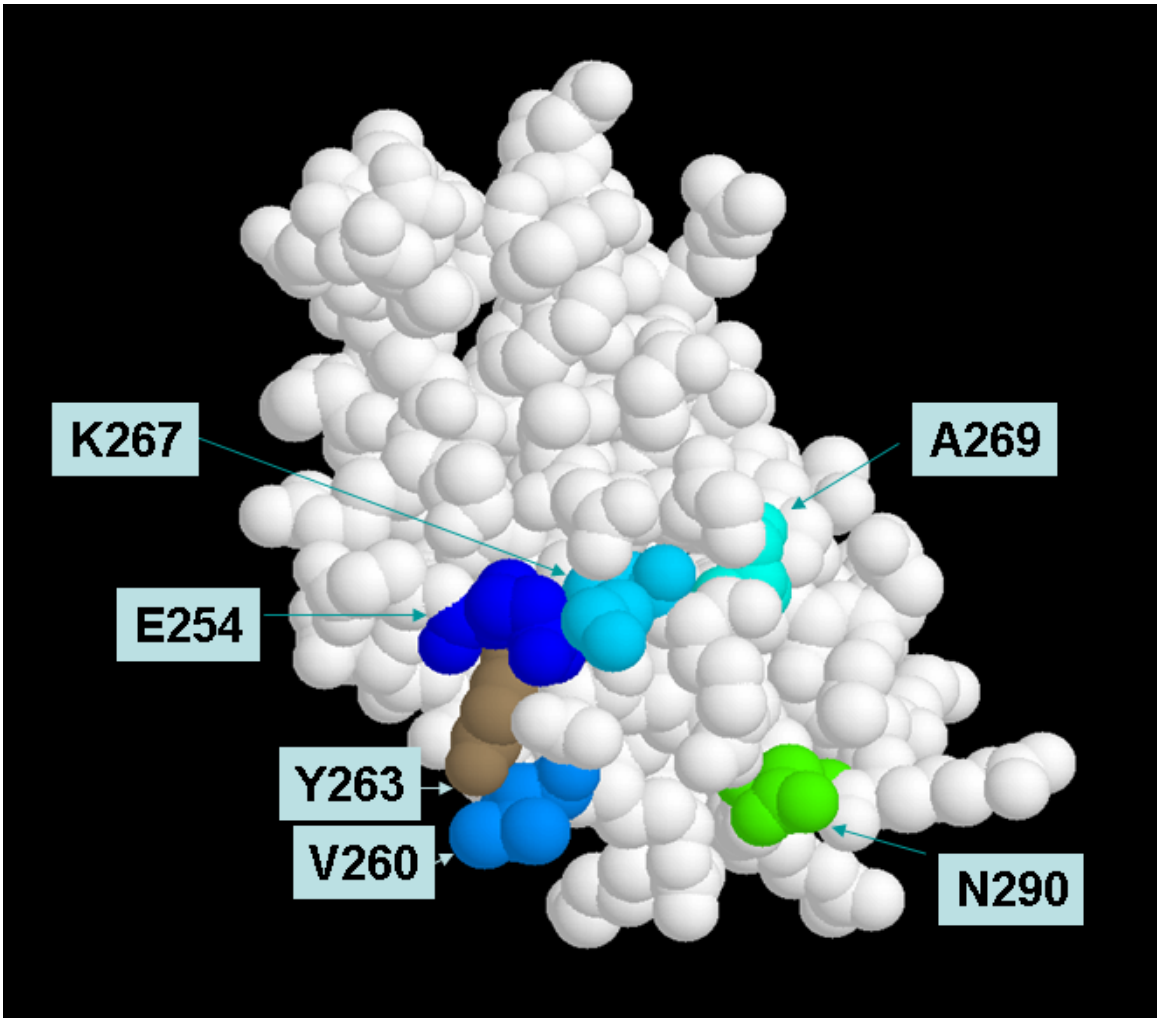


FIG. 2.8. Structure of *B. subtilis* αCTD (Newberry *et al.*, 2005, PDB ID: 1Z3E) indicating the residues identified as important for ResD-dependent activation of *fnr*. Amino acid residues with more than twofold effect are labeled.

# CHAPTER 3
# TRANSCRIPTIONAL ACTIVATION BY BACILLUS SUBTILIS RESD: TANDEM BINDING TO TARGET ELEMENTS AND PHOSPHORYLATION-DEPENDENT AND -INDEPENDENT TRANSCRIPTIONAL ACTIVATION[2]

## 3.1 INTRODUCTION

*Bacillus subtilis* senses extracellular oxygen limitation and adapts to a new environment by switching to anaerobic metabolism [for reviews see references (Nakano and Zuber, 1998) and (Nakano and Zuber, 2002)]. When nitrate is available under anaerobic conditions, *B. subtilis* undergoes nitrate respiration. To successfully switch from aerobic growth to nitrate respiration, the ResD-ResE two-component signal transduction system must be activated, which allows induction of genes that function in nitrate respiration, including *fnr* (encoding the anaerobic gene regulator Fnr), *nasDEF* (encoding the nitrite reductase operon), and *hmp* (encoding the flavohemoglobin) (Harrison-McMonagle *et al.*, 1999; Nakano *et al.*, 1996; Nakano *et al.*, 1998; Sun *et al.*, 1996b). Activation of the signal transduction system commences by sensing by ResE of unidentified signals, followed by autophosphorylation at the conserved histidine residue. The phosphoryl residue is then transferred to the N-terminal aspartate of the cognate response regulator ResD, which leads to activation of the target genes.

Most response regulators consist of two domains, a conserved N-terminal receiver (regulatory) domain and a variable C-terminal effector domain [for a review see reference (Stock *et al.*, 2000)]. The receiver domains of response regulators are doubly wound α/β

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Geng, H., Nakano, S., and Nakano, M.M. (2004) Transcriptional activation by *Bacillus subtilis* ResD: Tandem binding to target elements and phosphorylation-dependent and -independent transcriptional activation. *J. Bacteriol. 186*: 2028-2037.

proteins with a central five-strand parallel β sheet surrounded by five α helices, suggesting that there is a common mechanism for phosphorylation and phosphorylation-mediated signal transmission. ResD belongs to an OmpR subfamily whose C-terminal region has a winged helix-turn-helix motif [for reviews see references (Kenney, 2002) and (Martinez-Hackert and Stock, 1997a)]. Structural analyses of response regulators of this class have been described for each domain (Blanco *et al.*, 2002; Kondo *et al.*, 1997; Martinez-Hackert and Stock, 1997b; Okamura *et al.*, 2000; Sola *et al.*, 1999) and for a full-length protein (Buckler *et al.*, 2002; Robinson *et al.*, 2003). These structural studies revealed an overall similarity, as well as characteristic differences, among the members of the same family of response regulators. The most apparent differences are seen in the recognition helix, α3, and in the activation loop between α2 and α3 in the effector domain (Buckler *et al.*, 2002; Okamura *et al.*, 2000). Despite the assignment of OmpR and PhoB to the same subfamily, with the activation loop implicated as the region that directly interacts with RNA polymerase (RNAP) in each case, the former interacts with the C-terminal domain (CTD) of the RNAP α subunit (αCTD) (Aiba *et al.*, 1994; Pratt and Silhavy, 1994; Slauch *et al.*, 1991) and the latter interacts with $\sigma^{70}$ (Makino *et al.*, 1993).

OmpR and PhoB bind to the target sites in a tandem array; the recognition helix of each protein penetrates the major groove, and a β hairpin wing interacts with the minor groove (Blanco *et al.*, 2002; Harlocker *et al.*, 1995; Harrison-McMonagle *et al.*, 1999; Head *et al.*, 1998; Huang and Igo, 1996; Makino *et al.*, 1996). PhoB has been shown to dimerize upon phosphorylation (Fiedler and Weiss, 1995), which increases the DNA binding affinity to the target element. In addition, the receiver domain of PhoB interferes with the DNA binding activity in the C terminus. The isolated CTD of PhoB efficiently binds the target DNA in vitro and activates the *pho* regulon in vivo, indicating that the receiver domain silences the DNA binding activity, which is released by phosphorylation (Ellison and McCleary, 2000; Makino *et al.*, 1996). In the case of OmpR, the effect of phosphorylation on the activity of the effector domain is more complex. Unlike PhoB, neither unphosphorylated nor phosphorylated OmpR forms a dimer in solution (Aiba *et al.*, 1989b), yet phosphorylation increases the DNA binding affinity of OmpR (Aiba *et al.*, 1989b; Head *et al.*, 1998). The isolated CTD of OmpR is unable to activate transcription (Tsuzuki *et al.*, 1994; Tsuzuki *et al.*, 1995; Walthers *et al.*, 2003) unless a stable dimer is

artificially generated by introduction of a Cys residue into the C terminus (Tsuzuki *et al.*, 1994). These results suggest that the contribution of phosphorylation to activation of the effector domain in OmpR is not to relieve inhibition by the receiver domain. One possible reason for this difference in intramolecular communication between the two response regulators is the difference in the linker region connecting the two domains. Both the length of the linker (Mattison *et al.*, 2002) and the extent of the domain interface (Robinson *et al.*, 2003) are believed to contribute differently to the activation mechanisms of response regulators through intramolecular communication.

A novel mode of a dimerization interface between receiver domains was recently reported for *B. subtilis* PhoP, a response regulator belonging to the OmpR/PhoB subfamily that is involved in *pho* regulation [for a review see reference (Hulett, 2001)]. The protein-protein interface includes surface A (α4, β5 loop β5-α5, and α5) of one monomer and surface B (α3, loop β4-α4, and α4) of another monomer. This asymmetric interface formation leaves free surfaces A and B from each monomer, which function in multimer formation (Birck *et al.*, 2003; Chen *et al.*, 2003b). This unique interface interaction satisfactorily explains the previous observation that unphosphorylated PhoP forms a dimer and binds the target DNA, although phosphorylation moderately stimulates the binding affinity (Liu and Hulett, 1997). Phosphorylation of PhoP may enhance cooperative DNA binding or interactions with other proteins involved in transcriptional activation (Birck *et al.*, 2003; Liu and Hulett, 1997).

As shown by the studies described above, each response regulator in the OmpR/PhoB subfamily exhibits unique properties for dimerization, the effect of phosphorylation on the DNA binding ability, and transcriptional activation. In order to obtain a full understanding of how ResD activates transcription of the target genes, detailed analysis of ResD both in vivo and in vitro is required. ResD was shown to be a monomer regardless of phosphorylation (Zhang and Hulett, 2000). Previous studies showed that ResD activates transcription by directly interacting with upstream regulatory regions of the target genes (Nakano *et al.*, 2000b; Zhang and Hulett, 2000). Unphosphorylated ResD is able to bind the target DNA, and the binding affinity was only moderately stimulated by phosphorylation. In this study we further examined how ResD interacts with DNA to activate transcription and whether phosphorylation is essential for

the activation. Our results indicate that ResD activates transcription of the target genes in a phosphorylation-dependent and -independent manner when *B. subtilis* encounters oxygen limitation.

## 3.2 RESULTS

### 3.2.1 ResD binds tandemly to upstream regions of the *hmp* and *nasD* promoters

A previous DNase I footprinting analysis revealed that ResD interacts directly with upstream regulatory regions of the anaerobically induced genes *hmp*, *nasD*, and *fnr* (Nakano *et al.*, 2000b). ResD binds to the region at approximately positions -40 to -75 relative to the transcription start sites of the *hmp* and *nasD* promoters and to the region at approximately positions -48 to -62 of the *fnr* promoter (Nakano *et al.*, 2000b). Because some regions showed intrinsic resistance to DNase I, it was difficult to delimit the binding sites. In order to localize the binding sites more precisely and also to examine the contacts between ResD and the DNA backbone, a hydroxyl radical footprinting analysis was carried out (Fig. 3.1). ResD protected five regions between positions -40 and -85 of the coding and noncoding strands of *hmp*. The protected regions were separated by approximately 10 bp except for the two binding sites proximal to the promoter, which were separated by 5 bp. There are five protected regions separated by 10 bp between positions -45 and -95 of the coding and noncoding strands of *nasD*. The protected regions obtained by using ResD in the presence of ResE were similar to those obtained with ResD alone; however, the phosphorylation of ResD stimulated the binding of ResD to the target DNA, especially in the *hmp* regulatory region. The protection of the *hmp* coding strand by ResD was not enhanced in the presence of ResE if ATP was omitted from the reaction mixture, indicating that the stimulatory effect of ResE is dependent on ATP. We did not obtain reproducible results for hydroxyl radical footprinting for *fnr*, probably due to the low binding affinity of ResD to the *fnr* regulatory region, as shown previously (Nakano *et al.*, 2000b). In the hydroxyl radical experiment, we did not use ResD at the high concentration that ever used in the previous DNase I footprinting experiment (Nakano *et al.*, 2000b), because the high concentration proteins may affect Fenton reaction that generates hydroxyl radical, thus decreasing digestion of DNA.

### 3.2.2 ResD activates in vitro transcription of *hmp*, *nasD*, and *fnr*, and phosphorylation of ResD markedly stimulates transcription

The hydroxyl radical footprinting experiment and the DNase I footprinting analysis indicated that ResD activates transcription of *hmp*, *nasD*, and probably *fnr* by directly interacting with the promoters. Previous work demonstrated that *ctaA* transcription in vitro was stimulated by phosphorylated ResD (ResD~P) (Paul *et al.*, 2001). It has also been shown that *hmp* transcription in vitro is activated by ResD~P, but the question of how phosphorylation affects ResD-dependent transcription was not addressed (Nakano *et al.*, 2003b). We wished to determine whether ResD is also sufficient to activate the transcription of anaerobically induced genes for two reasons. First, ResD is needed for both aerobic and anaerobic respiration. Hence, it is possible that to achieve higher gene expression under anaerobic conditions, ResD must be assisted by another coactivator. Alternatively, the stronger induction might be caused by a higher ResD phosphorylation level. Second, a previous gel retardation analysis showed that larger amounts of ResD are required for binding to *fnr* than for binding to *hmp* and *nasD*, and phosphorylation of ResD had no significant stimulatory effect on the binding affinity to *fnr*, unlike the effect on the binding affinity to *hmp* and *nasD* (Nakano *et al.*, 2000b). This result could be explained by involvement of another protein that stabilized a complex between ResD and the *fnr* promoter, leading to activation of transcription. Therefore, we tested these possibilities by examining in vitro runoff transcription of the anaerobically induced promoters using *B. subtilis* RNAP, ResD, and ResE.

When ResD and ResE were purified by one-step affinity column chromatography as described previously (Nakano *et al.*, 2000b), we found that in vitro transcription was activated by ResD but the phosphorylation state of ResD had only a minor effect on stimulation, probably due to DNA present in purified ResD that was caused by nonspecific binding in *E. coli*. Therefore we made two modifications to the purification method. First, nucleic acids were removed from the ResD preparation by precipitation with streptomycin sulfate (the absence of contaminated nucleic acids in the purified ResD preparation was confirmed by agarose gel electrophoresis followed by staining with ethidium bromide). Second, the ResD protein that eluted from the chitin affinity column was further purified by DEAE-Sepharose chromatography (see Materials and Methods).

Similarly, ResE was purified by affinity chromatography, followed by purification by Hi-Q column chromatography.

In vitro transcription by using the ResD and ResE proteins prepared by the new purification method showed that phosphorylation markedly increased the activity of ResD; therefore, all experiments described in this paper were carried out with these proteins. In the absence of ResD and ResE, the levels of the transcripts of *hmp* (Fig. 3.3A), *nasD* (Fig. 3.3B), and *fnr* (Fig. 3.3C) were very low or the transcripts were hardly detected (lanes 1). Increased amounts of unphosphorylated ResD slightly stimulated transcription (Fig. 3.3, lanes 2 to 4). In addition to the transcripts of the expected sizes (86 nucleotides for *hmp*, 96 nucleotides for *nasD*, and 101 nucleotides for *fnr*; note that extra nucleotides in the *hmp* and *fnr* transcripts originated from the PCR primers used for amplification of the templates), additional longer transcripts were detected for *hmp* (126 and 97 nucleotides) and for *nasD* (112 nucleotides) (the sizes of the transcripts were estimated by sequence ladders, which are not shown in Fig. 3.3, together with RNA markers). Transcription was greatly stimulated in the presence of ResD and ResE, indicating that phosphorylation of ResD enhances transcription initiation (lanes 5 to 7).

Amino acid sequence alignment of ResD with other response regulators suggested that Asp57 is the phosphorylation site. To further examine the effect of ResD phosphorylation on transcription, the aspartate residue was replaced by alanine, and the mutant ResD was overproduced and purified from *E. coli* as described in Materials and Methods. Transcription was stimulated by the mutant ResD (D57A) protein to a level similar to that stimulated by unphosphorylated wild-type ResD (Fig. 3.3, compare lanes 2 to 4 and lanes 8 to 10), and the amounts of the transcripts were not further increased in the presence of ResE (lanes 11 to 13). These results demonstrated that ResD alone is sufficient to activate transcription of *hmp*, *nasD*, and *fnr* and that phosphorylation at Asp57 strongly enhances the transcriptional activity of ResD. However, unphosphorylated ResD, both wild type and mutant, can activate transcription, albeit to a lesser extent.

Although *hmp* transcription and *nasD* transcription were shown to start in vivo at a single site (Harrison-McMonagle *et al.*, 1999) and two adjacent sites (Nakano *et al.*, 1998), respectively, in vitro transcription also produced longer transcripts, indicating that

additional transcription start sites were utilized in vitro. Since the amounts of the longer transcripts were also increased by ResD and by ResD~P in particular, we examined how these transcripts were generated by using new templates for *hmp* (positions -185 to 61) and *nasD* (positions -138 to 65) for in vitro transcription. Each new template has the same upstream end as the other template and a shortened downstream end. If the additional transcription started upstream of the transcription start site utilized in vivo and transcription proceeded in the same direction, *hmp* transcripts should have been shorter with the new templates (68, 79, and 108 nucleotides instead of 86, 97, and 126 nucleotides). Similarly, the sizes of the *nasD* transcripts should have been reduced to 71 and 87 nucleotides (again, extra nucleotides were generated from oligonucleotides used to amplify the templates by PCR). If the additional transcription proceeded in the opposite direction by using the complementary strand as a template, the size of the shortest transcript, but not the size of the larger transcript(s), should have been changed. The results shown in Fig. 3.3D indicate that the sizes of the transcripts corresponded well to the sizes predicted by the first possibility. A sequence similar to the -10 sequence (TATAAT) recognized by $\sigma^A$-RNAP was present upstream of each transcription start site (Fig. 3.2). These results showed that ResD activated *hmp* and *nasD* transcription in vitro at an additional transcription start site(s) in addition to the native transcription start site (see Discussion).

### 3.2.3 Aspartate 57 of ResD is required for phosphorylation by ResE

The ResD (D57A) mutant was shown to activate in vitro transcription of *hmp*, *nasD*, and *fnr*. Although the stimulatory effect was much lower than that of phosphorylated ResD, the mutant ResD possessed an activity similar to that of unphosphorylated wild-type ResD to activate the transcription in vitro. One possible reason for this is that ResD has an alternative phosphorylation site. The phosphorylation site mutant (D57N) of the chemotaxis response regulator CheY can be phosphorylated at Ser56 by the CheA sensor kinase (Appleby and Bourret, 1999). However, the amino acid corresponding to Ser56 of CheY is Leu in ResD, and the CheY (D57A) mutant was not phosphorylated at Ser56, suggesting that the alternative phosphorylation of ResD is unlikely. To confirm this, we carried out in vitro phosphorylation of ResD by ResE.

Figure 3.4 shows that wild-type ResD was phosphorylated in the presence of ResE and [$\gamma$-$^{32}$P]ATP, but no phosphorylation was detected in the mutant ResD. This result clearly demonstrated that Asp57 is the phosphorylation residue and that the D57A mutant cannot be phosphorylated by ResE.

### 3.2.4 Unphosphorylated ResD is able to activate the ResDE regulon in vivo by responding to oxygen limitation

At least in vivo, mutations in *resE* impair ResD-dependent gene regulation. Hence, it is reasonable to conclude that only phosphorylated ResD can activate transcription of the target genes. However, this conclusion should be carefully drawn because the transcription of *resDE* from the major *resA* operon promoter requires ResD and ResE (Sun *et al.*, 1996b). In fact, in vitro experiments described above indicated that unphosphorylated ResD (D57A) is able to activate the transcription of ResDE-dependent genes in vitro to a lesser extent. Therefore, we decided to examine whether the D57A mutant is able to activate transcription in vivo and, if so, whether the activation is still dependent on oxygen limitation. In order to express the *resD* gene independently of ResDE, the wild-type and mutant *resD* genes, placed under control of the IPTG-inducible P*spank-hy* promoter (Britton *et al.*, 2002), were introduced into the *amyE* locus of the *resDE* mutant strain. Therefore, in the resulting strains, ResE was absent and ResD was produced solely from the IPTG-inducible *resD* construct. The expression of three *lacZ* fusions to the *fnr*, *nasD*, and *hmp* promoters was examined in cells grown under either aerobic or anaerobic conditions.

The expression of *nasD* and *hmp* in strains carrying the wild-type *resD* gene was greatly activated by oxygen limitation, and the expression was dependent on IPTG (Fig. 3.5B and C). In contrast, *fnr-lacZ* expression was similar in cells grown under aerobic and anaerobic conditions, indicating that the oxygen-dependent regulation of *fnr* was largely eliminated by circumventing the autoregulatory pathway of *resD* expression (Fig. 3.5A). This may have been due to the lower *fnr* induction by oxygen limitation relative to *nasD* and *hmp* induction, as well as the stimulatory effect under aerobic conditions of increasing amounts of ResD brought about by an oxygen-independent promoter. The expression of *nasD-lacZ* and *hmp-lacZ* in cells carrying P*spank-hy resD* (D57A) was

activated only in the presence of IPTG under anaerobic conditions (Fig. 5E and F), although the expression was two- to fourfold lower than that observed in cells bearing the wild-type *resD* gene. The latter result suggests that the wild-type ResD may be phosphorylated by a noncognate kinase(s) or by small compounds with high-energy phosphate in the absence of ResE, thus leading to higher gene expression. Apparently, this ResE-independent phosphorylation also responds to oxygen limitation. A more important observation in this experiment is that ResD (D57A) was able to activate the ResDE regulon in response to oxygen limitation. This result, together with the in vitro transcription results described here, clearly demonstrated that ResD, without phosphorylation, is able to respond to the redox state of cells and to activate transcription.

### 3.3 DISCUSSION

In this study we examined both in vivo and in vitro how the response regulator ResD activates the transcription of genes that function in nitrate respiration under oxygen limitation conditions. Previous DNase I footprinting studies demonstrated that ResD binds to the promoter regions of *hmp*, *nasD*, and *fnr*, as well as *ctaA*, which suggests that ResD directly activates transcription of these genes (Nakano *et al.*, 2000b; Zhang and Hulett, 2000). Hydroxyl radical footprinting experiments described here revealed that ResD binds tandemly to the same face of the DNA helix at the *nasD* promoter. OmpR is also known to bind to the same face of the DNA helix as a head-to-tail dimer, with each monomer occupying 10 bp of DNA (Harlocker *et al.*, 1995; Harrison-McMonagle *et al.*, 1999; Huang and Igo, 1996). OmpR is unable to form a stable complex with DNA as a monomer (Harlocker *et al.*, 1995; Huang and Igo, 1996). Similarly, several monomers of PhoB bind head to tail to successive 11-base direct repeats in the target DNA (Blanco *et al.*, 2002; Makino *et al.*, 1996; Okamura *et al.*, 2000). The *ugpB* promoter has 3.5 *pho* boxes, and in vitro analysis showed that PhoB binds to the *pho* half site, although deletion of the half site did not affect the expression of *ugp* in vivo (Kasahara *et al.*, 1991). Therefore, unlike OmpR, PhoB is able to bind DNA as a monomer. Our results also showed that ResD is likely to bind as a monomer to 10-bp regions of the target DNA. The most proximal ResD binding site of *hmp* is situated on a different helix face of the DNA

than the rest of the binding sites. At present, it is not known how this unusual pattern of binding sites in *hmp* affects transcriptional activation of *hmp* by ResD. The hydroxyl radical footprinting analysis, together with sequence comparisons, did not provide any solid consensus sequence data for ResD except that the A(A/T)TT sequence is commonly protected.

Our in vitro transcription experiment indicated that ResD is required and sufficient to activate transcription of the anaerobically induced genes. The extent of the stimulation by phosphorylation of ResD varies depending on the target gene. Phosphorylation had the most drastic stimulatory effect on *hmp* transcription, had a slightly smaller effect on *nasD* transcription, and had the least effect on *fnr* transcription, which corresponds well to the level of in vivo activation of each gene observed upon oxygen limitation. Additional transcription start sites detected in *hmp* and *nasD* are likely artifacts only seen in vitro. One possible reason for this is that some transcription initiates when less than five ResD monomers bind to the regulatory region and the most upstream or most downstream ResD interacts with RNAP. Alternatively but not mutually exclusively, if the αCTD of RNAP interacts with ResD, the linker of the α subunit, which connects the N-terminal and C-terminal domains, allows flexible contact between RNAP and ResD. A previous study showed that the α subunit is flexible enough to allow the CTD to move freely at least 30 bp along the promoter (Murakami *et al.*, 1997). This suggests that ResD interacts with αCTD to activate transcription. Several lines of circumstantial evidence support this possibility. First, OmpR, which is known to interact with the αCTD, binds to a region at positions -107 to -39 of *ompF*, and PhoB binds to a region at positions -63 to -20 of *pstS*, closer to the promoter. Our footprinting data indicated that ResD binds to the region between positions -41 and -83 of *hmp* and to the region between positions -46 and -92 of *nasD*. Second, the *ctaA* gene was transcribed from two promoters utilized by $\sigma^A$ and $\sigma^E$ holo-RNAP, and transcription from both was shown to be stimulated by ResD~P in vitro (Paul *et al.*, 2001). These results support the hypothesis that there is probably an interaction between ResD and α but not between ResD and σ. Third, ResDE-dependent transcription of *hmp* was shown to be inhibited by Spx, a newly discovered anti-α factor (Nakano *et al.*, 2003b). Last, we have isolated certain amino acid substitutions of the αCTD which affect ResDE-dependent gene

regulation (Geng *et al.*, 2007)(Chapter 4). It remains to be determined whether ResD interacts with αCTD and which step of transcription initiation is controlled by ResD.

Phosphorylation of the N-terminal aspartate has been shown to be the key step in two-component regulatory pathways. Structural analyses of the receiver domains of response regulators have revealed a conformational change involving displacement of β strands 4 and 5, as well as α helices 3 and 4, away from the active site (Birck *et al.*, 1999; Cho *et al.*, 2000; Kern *et al.*, 1999; Lee *et al.*, 2001; Lewis *et al.*, 1999; Lewis *et al.*, 2000). These structural rearrangements in the receiver domain result in changes that affect the activity of output domains, including dimer-multimer formation (Asayama *et al.*, 1998; Fiedler and Weiss, 1995; Jeon *et al.*, 2001; Lewis *et al.*, 2002; Porter *et al.*, 1993), and/or that relieve the inhibitory effect by the receiver domain (Allen *et al.*, 2001; Baikalov *et al.*, 1998; Da Re *et al.*, 1999; Ellison and McCleary, 2000; Ireton *et al.*, 1993; Makino *et al.*, 1996; Webber and Kadner, 1997; Zhang and Hulett, 2000). Regardless of the mechanism by which phosphorylation affects the output activity, most of response regulators are active in vivo in phosphorylated forms, as shown by the finding that mutations in the cognate kinase gene impair or greatly reduce target gene expression. Some, although not many, exceptions have been reported. The *B. subtilis* DegU regulator required for production of degradative enzymes and competence development has been shown to function as either a phosphorylated form or an unphosphorylated form depending on the target gene [for a review see reference (Msadek *et al.*, 1995)]. The conserved aspartate residue is not essential for the activity of BldM, a response regulator involved in the developmental cycle of *Streptomyces coelicolor* (Molle and Buttner, 2000). The AlgB response regulator, together with the KinB cognate kinase, constitutes a two-component signal transduction system of *Pseudomonas aeruginose* (Ma *et al.*, 1998). AlgB, like DegU, may have dual function i.e. unphosphorylated AlgB is required for alginate overproduction, while the phophorylated form has different target genes (Ma *et al.*, 1998). The response regulators Hp1043 and Hp1021 of *Helicobacter pylori* also have phosphorylation-independent activity (Schar *et al.*, 2005). Hp1043 is essential for the growth under standard culture conditions (Schar *et al.*, 2005). Hp1021 activates the transcription of the housing keeping genes such as *nifS* and *nifU*, and genes involved in

actone metabolism (Pflock *et al.*, 2007). However unlike typical response regulators, no cognate kinase has been identified for Hp1043 and Hp1021 (Schar *et al.*, 2005).

Our finding that the ResD (D57A) mutant retains the ability to respond to oxygen limitation is unexpected but interesting because, unlike phosphorylated and unphosphorylated DegU, phosphorylated ResD and unphosphorylated ResD activate the same target genes. *E. coli* UhpA, a response regulator involved in sugar phosphate transport, also has phosphorylation-independent activity. However, in this case UhpA carrying a substitution of phosphorylated Asp54 with Asn has constitutive activity only when it is overproduced (Webber and Kadner, 1997). To our knowledge, this is the first example of a response regulator that is activated by a phosphorylation-independent mechanism and yet responds to the same conditions that are sensed by the cognate sensor kinase. Many unphosphorylated response regulators have been shown to activate transcription in vitro; however, this phenomenon was brought into question by the recent finding that purified Spo0A from *E. coli* is already phosphorylated (Ladds *et al.*, 2003). This may explain why the unphosphorylated response regulator PrrA has more activity in vitro than the protein with the phosphorylation site mutation (Comolli *et al.*, 2002). In contrast, our results showed that unphosphorylated ResD and the mutant ResD have similar activities for in vitro transcription. Furthermore, compelling evidence that unphosphorylated ResD activates transcription of the target genes was obtained by the in vivo study performed with strains carrying the mutant ResD. At this time we cannot completely eliminate the possibility that a coactivator, which binds tightly to ResD, is responsible for redox sensing. In future studies we will focus on determining whether ResD itself responds to oxygen limitation and, if so, how the redox-related signal is sensed by ResD to activate transcription.

## 3.4 MATERIALS AND METHODS

### 3.4.1 Bacterial strains, plasmids, and media

All *B. subtilis* strains used in this study are derivatives of JH642 (*trpC2* pheA1) (Table 3.1). *Escherichia coli* DH5α was used to propagate plasmids, and *E. coli* transformants were selected on Luria broth (LB) agar supplemented with 25 μg of

ampicillin per ml. ResD and ResE proteins were overproduced in *E. coli* ER2566 and were purified by using the IMPACT system (New England Biolabs), in which the inducible self-cleaving intein tag is used.

To express wild-type *resD* and a mutant (D57A) *resD* from the isopropyl-β-thiogalactopyranoside (IPTG)-inducible P*hyperspank* (*Pspank-hy*) promoter, plasmids pMMN546 and pMMN547 were constructed. The wild-type *resD* gene was amplified by PCR by using oligonucleotides oMN02-205 and oMN02-206 (the oligonucleotides used in this study are listed in Table 3.2). The PCR product, after digestion with SalI and SphI, was cloned into pDR111 (Britton *et al.*, 2002) that was digested with the same enzymes to generate pMMN546. The mutant *resD* (D57A) gene was amplified by a two-step PCR. Two overlapping PCR products were generated with the oMN02-205-oMN03-221 and oMN02-206-oMN03-220 primer pairs. Primers oMN03-220 and oMN03-221 are mutagenic primers. The resultant PCR products were used as templates for a second PCR in which primers oMN02-205 and oMN02-206 were used. The product of the second PCR was cloned into pDR111 by using a method similar to that used for construction of pMMN546 in order to generate pMMN547. The *resD* primary structure in pMMN546 and pMMN547 was verified by DNA sequencing. pMMN546 and pMMN547 were used to transform JH642 with selection for spectinomycin resistance ($Spc^r$) (75 μg/ml), and transformants (ORB4599 and ORB4600, respectively) were screened for the amylase-negative phenotype that is indicative of a double recombination event at the *amyE* locus. LAB2135 (Δ*resDE*) was transformed with chromosomal DNA prepared from ORB4599 and ORB4600 to generate ORB4605 and ORB4606, respectively. SPβ phage lysate carrying *fnr-lacZ* (Nakano *et al.*, 1996), *nasD-lacZ* (Nakano *et al.*, 1995), or *hmp-lacZ* (Nakano *et al.*, 2000b) was used to transduce ORB4599 and ORB4600 with selection for chloramphenicol resistance (5 μg/ml) (the resulting strains ORB4612 to ORB4614 and ORB4616 to ORB4618 are listed in Table 3.1).

ResD (D57A) was overproduced in ER2566 carrying pMMN539. The mutant *resD* gene was amplified by PCR by using oligonucleotide primers oMN00-102 and oMN00-103 and pMMN547 as the template. The PCR product was digested with NcoI and SmaI and ligated with pTYB4 (New England Biolabs), which was digested with the same enzymes, to generate pMMN539.

### 3.4.2 Purification of proteins

ResD and ResE were overproduced in *E. coli* ER2566 carrying pXH22 (Zhang and Hulett, 2000) and pMMN424 (Nakano *et al.*, 2000b), respectively. For ResE purification, *E. coli* was grown in LB with ampicillin at 30°C until the optical density at 600 nm reached 0.5 to 0.6, at which point IPTG was added to a final concentration of 0.5 mM. The cells were then incubated at 30°C for an additional 4 h before they were harvested. The cell pellets were suspended in buffer A (25 mM Tris-HCl [pH 8.0], 500 mM NaCl, 5% glycerol) and were broken by passage through a French press. The cell lysates were centrifuged at 15,000 x *g* to remove cell debris, and the supernatant was applied to a chitin column. After the column was washed with buffer B (24 mM Tris-HCl [pH 8.0], 100 mM NaCl, 5% glycerol), it was flushed with buffer B containing 50 mM dithiothreitol (DTT) and kept at 4°C overnight to cleave the intein tag. After elution with buffer B, pooled fractions containing ResE proteins were applied to a High-Q column, and the proteins were eluted with a linear salt gradient (100 to 500 mM NaCl) in buffer B. The fractions containing ResE were combined, dialyzed against 25 mM Tris-HCl (pH 8.0)-100 mM NaCl-5% glycerol, and stored at -70°C.

For ResD purification *E. coli* ER2566 strains carrying pXH22 (wild-type ResD) and pMMN539 (D57A mutant) were grown in LB at 30°C, and the expression of *resD* was induced as described above for *resE* heterologous overexpression. The cell lysate was prepared by using a method similar to the method used for ResE, except that streptomycin sulfate (1.5%, wt/vol) was added to precipitate nucleic acids with centrifugation at 40,000 x *g* at 4°C for 30 min. The supernatant was loaded onto a chitin column and washed with buffer C (25 mM Tris-HCl [pH 8.0], 500 mM NaCl, 5% glycerol, 5 mM $MgCl_2$) and then with buffer D (25 mM Tris-HCl [pH 8.0], 25 mM NaCl, 5% glycerol, 5 mM $MgCl_2$). After cleavage of the intein tag with 30 mM DTT, ResD was eluted with buffer D. Pooled ResD-containing fractions were loaded onto a DEAE-Sepharose CL-6B column equilibrated with buffer D. ResD was eluted with buffer E (25 mM Tris-HCl [pH 8.0], 100 mM NaCl, 5% glycerol, 5 mM $MgCl_2$). The protein was dialyzed against buffer F (25 mM Tris-HCl [pH 8.0], 50 mM NaCl, 5 mM $MgCl_2$) and stored at -70°C.

RNAP was purified from *B. subtilis* MH5636 (wild type) (Qi and Hulett, 1998), which produces the RNAP β' subunit fused to a 10-His tag. Cells were grown in 2xYT (Nakano *et al.*, 1988) and harvested around 2 h after the end of exponential growth. Purification of RNAP by using Ni-nitrilotriacetic acid has been described elsewhere (Liu and Zuber, 2000; Qi and Hulett, 1998).

### 3.4.3 Hydroxyl radical footprinting

The DNA probes *hmp* (positions -133 to 27 with respect to the transcription start site) and *nasD* (positions -185 to 66), which were used for footprinting, were amplified by PCR by using primers oHG-5 and oHG-6 and primers oMN98-19 and oMN98-20, respectively. To end label coding or noncoding strands, one member of each primer pair was treated with T4 polynucleotide kinase and [$\gamma$-$^{32}$P] ATP. DNA probes generated by PCR by using one labeled primer and one unlabeled primer were separated on a 6% nondenaturing polyacrylamide gel and purified with an Elutip-d column (Schleicher and Shuell) as described previously (Nakano *et al.*, 2000b).

Hydroxyl radical footprinting was performed as described previously (Zaychikov *et al.*, 2001), with minor modifications. ResD or ResE or both were incubated in 20 μl of binding buffer (25 mM Tris-HCl [pH 7.5], 100 mM KCl, 1 mM EDTA, 4 mM DTT, 4 mM $MgCl_2$, 0.25 mM ATP) for 10 min at room temperature. Labeled DNA probe (50,000 cpm) was added, and the reaction mixture was then incubated for 30 min at room temperature. The cleavage reaction was initiated by simultaneously mixing 2 μl of 1 mM Fe EDTA-0.1 M DTT-1% $H_2O_2$ with the protein-DNA complex. After incubation for 2 min at room temperature, the cleavage reaction was stopped by adding 25 μl of stop solution (4% glycerol, 0.6 M sodium acetate [pH 5.0], 0.1 mg of yeast RNA per ml). The reaction mixture was extracted with 50 μl of phenol-chloroform-isoamyl alcohol (pH 6.8) and was precipitated with ethanol. The pellet was resuspended with 3.5 μl of loading buffer (90% formamide, 0.04% bromophenol blue, 0.04% xylene cyanol in Tris-borate buffer) and heated for 1.5 min at 90°C before it was loaded onto an 8% polyacrylamide-urea gel. The same primer that was used for labeling of the probe was used for dideoxy sequencing with a Thermo Sequenase cycle sequencing kit (U.S. Biochemicals), and the sequencing reactions were performed together with the footprinting reactions. The gel

was electrophoresed at 60 W and dried, and then it was analyzed by using a PhosphorImager (Molecular Dynamics).

### 3.4.4 In vitro runoff transcription

The linear templates used for in vitro transcription assays were amplified by PCR by using primers oMN99-89 and oMN99-90 (positions -185 to 79 of *hmp*), primers oHG-7 and oHG-1 (positions -138 to 96 of *nasD*), and primers oMN98-24 and oMN98-25 (positions -169 to 96 of *fnr*). In order to determine the start sites of transcripts, different *hmp* (positions -185 to 61) and *nasD* (positions -138 to 66) templates were used, which were amplified with primers oMN99-89 and oMN98-22 and primers oHG-7 and oMN98-20, respectively. PCR products were purified with a QIA PCR purification kit (Qiagen). The in vitro transcription buffer contained 25 mM Tris-HCl (pH 7.5), 100 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 5 mM $MgCl_2$, 0.25 mM ATP, 50 μg of bovine serum albumin per ml, 10% glycerol, and 0.4 U of RNasin RNase inhibitor (Promega) per μl. ResD or ResE or both were incubated in 20 μl of transcription buffer at room temperature for 10 min. RNAP and templates were added at final concentrations of 25 and 5 nM, respectively, and the reaction mixtures were incubated for 10 min at room temperature. ATP, GTP, and CTP (each at a concentration of 100 μM), UTP (25 μM), and 5 μCi of [α-$^{32}$P]UTP (800 Ci/mmol) were added to start transcription. After incubation at 37°C for 20 min, 10 μl of stop solution (1 M ammonium acetate, 100 μg of yeast RNA per ml, 30 mM EDTA) was added. The reaction mixture was precipitated with ethanol, and the pellet was dissolved in 3.5 μl of loading dye solution (7 M urea, 100 mM EDTA, 5% glycerol, 0.05% bromophenol blue). Transcripts were analyzed on an 8% polyacrylamide-urea gel. RNA markers were prepared by using the Decade marker system protocol (Ambion Inc.).

### 3.4.5 Phosphorylation of ResD by ResE

ResE (1 μM) with or without 1 μM ResD (wild-type or the D57A mutant protein) was incubated in 50 μl of transcription buffer containing 0.22 μM [γ-$^{32}$P]ATP at room temperature. After incubation for 5, 10, and 15 min, 10 μl of the reaction mixture was added to 4.5 μl of 5x sodium dodecyl sulfate (SDS) loading buffer (60 mM Tris-HCl [pH 6.8], 25% glycerol, 2% SDS, 0.3 M DTT, 0.1% bromophenol blue). The protein samples

were separated on an SDS—12% polyacrylamide gel, and the dried gel was analyzed by using a phosphorimager.

### 3.4.6 Measurement of β-galactosidase activity

*B. subtilis* cells were grown aerobically and anaerobically in 2xYT supplemented with 1% glucose, 0.2% potassium nitrate, and appropriate antibiotics in the absence or presence of 1 mM IPTG. The anaerobic cultures were prepared by filling tubes with a cell suspension as described previously (Nakano *et al.*, 1996). Cells were inoculated (starting optical density at 600 nm, 0.02) from cultures grown overnight on DS agar medium (Nakano *et al.*, 1988). Samples were withdrawn at time intervals, and β-galactosidase activity was determined as previously described (Nakano *et al.*, 1988) and was expressed in Miller units (Miller, 1972).

**TABLE 3.1 *B. subtilis* strains and plasmids used in this study**

| Strain or plasmid | Description | Reference or source |
|---|---|---|
| Strains | | |
| JH642 | *trpC2 pheA1* | J. A. Hoch |
| LAB2135 | *trpC2 pheA1* Δ*resDE*::*tet* | (Nakano *et al.*, 1996) |
| ORB4599 | *trpC2 pheA1 amyE*::*Pspank-hy resD* (pMMN546) | This study |
| ORB4600 | *trpC2 pheA1 amyE*::*Pspank-hy resD* (D57A) (pMMN547) | This study |
| ORB4605 | *trpC2 pheA1* Δ*resDE*::*tet amyE*::*Pspank-hy resD* (pMMN546) | This study |
| ORB4606 | *trpC2 pheA1* Δ*resDE*::*tet amyE*::*Pspank-hy resD* (D57A) (pMMN547) | This study |
| ORB4612 | *trpC2 pheA1* Δ*resDE*::*tet amyE*::*Pspank-hy resD* (pMMN546) SPβc2del2::Tn*917*::pMMN288 (*fnr-lacZ*) | This study |
| ORB4613 | *trpC2 pheA1* Δ*resDE*::*tet amyE*::*Pspank-hy resD* (pMMN546) SPβc2del2::Tn*917*::pMMN392 (*nasD-lacZ*) | This study |
| ORB4614 | *trpC2 pheA1* Δ*resDE*::*tet amyE*::*Pspank-hy resD* (pMMN546) SPβc2del2::Tn*917*::pML107 (*hmp-lacZ*) | This study |
| ORB4616 | *trpC2 pheA1* Δ*resDE*::*tet amyE*::*Pspank-hy resD* (D57A) (pMMN547) SPβc2del2::Tn*917*::pMMN288 (*fnr-lacZ*) | This study |
| ORB4617 | *trpC2 pheA1* Δ*resDE*::*tet amyE*::*Pspank-hy resD* (D57A) (pMMN547) SPβc2del2::Tn*917*::pMMN392 (*nasD-lacZ*) | This study |
| ORB4618 | *trpC2 pheA1* Δ*resDE*::*tet amyE*::*Pspank-hy resD* (D57A) (pMMN547) SPβc2del2::Tn*917*::pML107 (*hmp-lacZ*) | This study |
| Plasmids | | |

| | | |
|---|---|---|
| PDR111 | Integration plasmid with P*spank-hy,* $Amp^r$ $Spc^r$ | (Britton *et al.*, 2002) |
| PTYB4 | Expression vector with self-cleavable intein tag | New England Biolabs |
| pXH22 | pTYB2 carrying *resD* | (Zhang and Hulett, 2000) |
| pMMN424 | pTYB4 carrying *resE* | (Nakano *et al.*, 2000b) |
| pMMN539 | pTYB4 carrying *resD* (D57A) | This study |
| pMMN546 | pDR111 carrying *resD* | This study |
| pMMN547 | pDR111 carrying *resD* (D57A) | This study |

**TABLE 3.2 Oligonucleotide primers used in this study**

| Primer | Sequence (5' to 3')[a] |
|---|---|
| oMN98-19 | GGAATTCAAAATGTGAATGA |
| oMN98-20 | CGGGATCCATTACCAACAA |
| oMN98-22 | CGGGATCCGATTGTTTTGTT |
| oMN98-24 | GGAATTCAGAGGTGGCGTTA |
| oMN98-25 | CGGGATCCAGCAATTCATAC |
| oMN99-89 | GGAATTCCCAAAACATAAGT |
| oMN99-90 | CGGGATCCAGTGCTTTTAAT |
| oMN00-102 | CAGGGGGAAACCATGGACCAAA |
| oMN00-103 | GCTTTTCCAAAATTTCACCCGGGTTCAGCGCCGACCT |
| oMN02-205 | AGATAAGTCGACAGAAGGAAAGCAGGG |
| oMN02-206 | GCATAAGCATGCCTACTACGCTTTTCC |
| oMN03-220 | ATTTTGCTTGCTCTGATGATGCC |
| oMN03-221 | GGCATCATCAGAGCAAGCAAAAT |
| oHG-1 | TATCTCTTCAATGGCCCTTA |
| oHG-5 | GTTAGTCCGTTTTTGCTA |
| oHG-6 | CCTTTCGAAAAGATGTAT |
| oHG-7 | AAATGCCCGGTTTTAAGG |

[a] Restriction enzyme sites used for cloning are underlined.

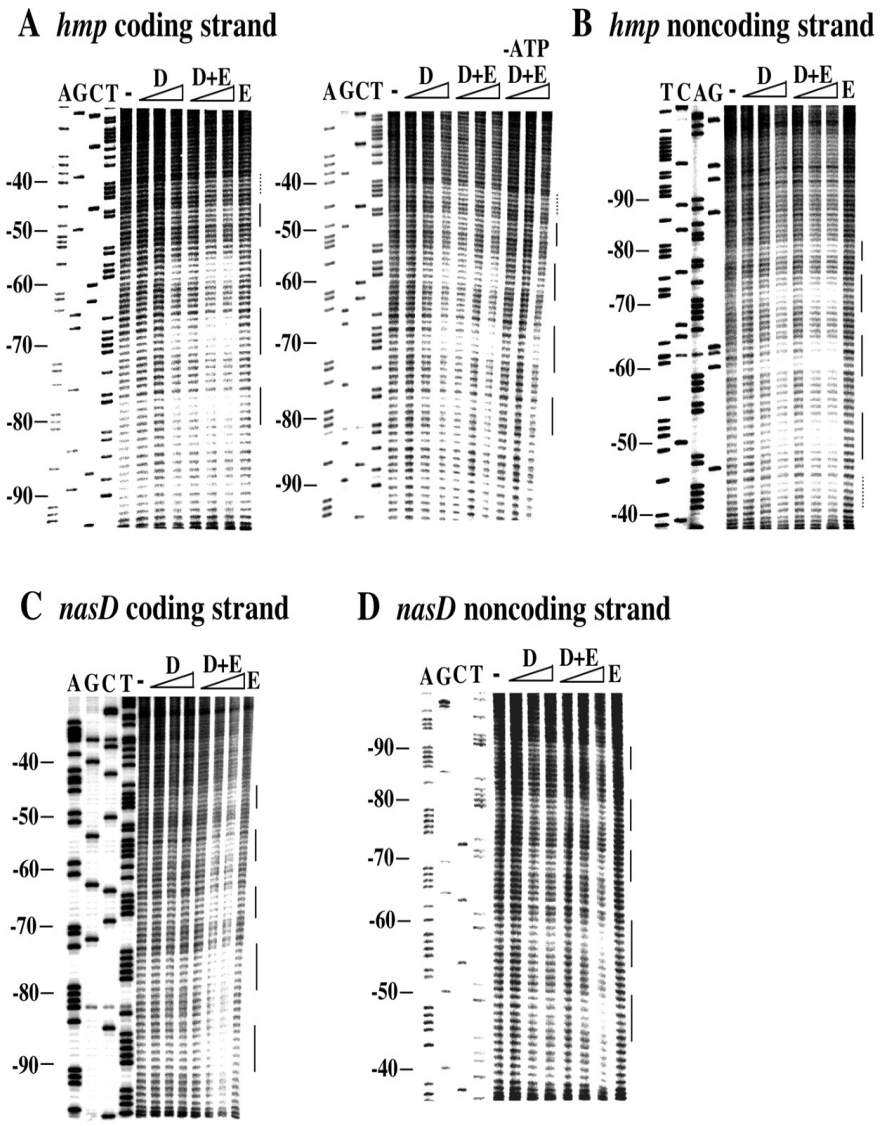



FIG. 3.1. Hydroxyl radical footprinting of the *hmp* promoter. A DNA fragment carrying *hmp* (positions -133 to 27) or *nasD* (positions -185 to 66) was obtained by PCR by using 32P-end-labled primer and unlabeled primer as described in Materials and Methods. The DNA fragment, labeled at the 5' end of either the coding or noncoding strand, was incubated with different amounts of ResD (D) (1, 2, and 4 μM), with 4 μM ResE (E), or with both ResD and ResE before hydroxyl radical treatment. The labeled *hmp* coding strand was also incubated with ResD and ResE in the absence of ATP. The numbers on the left indicate the nucleotide positions relative to the transcriptional start site. Sequence reactions (lanes A, G, C, and T) were carried out by using the labeled primer that was used for PCR. The protected regions indicated by solid lines on the right are separated by approximately 10 bp. The protected region in *hmp* indicated by a dotted line is separated by approximately 5 bp from the adjacent protected region.

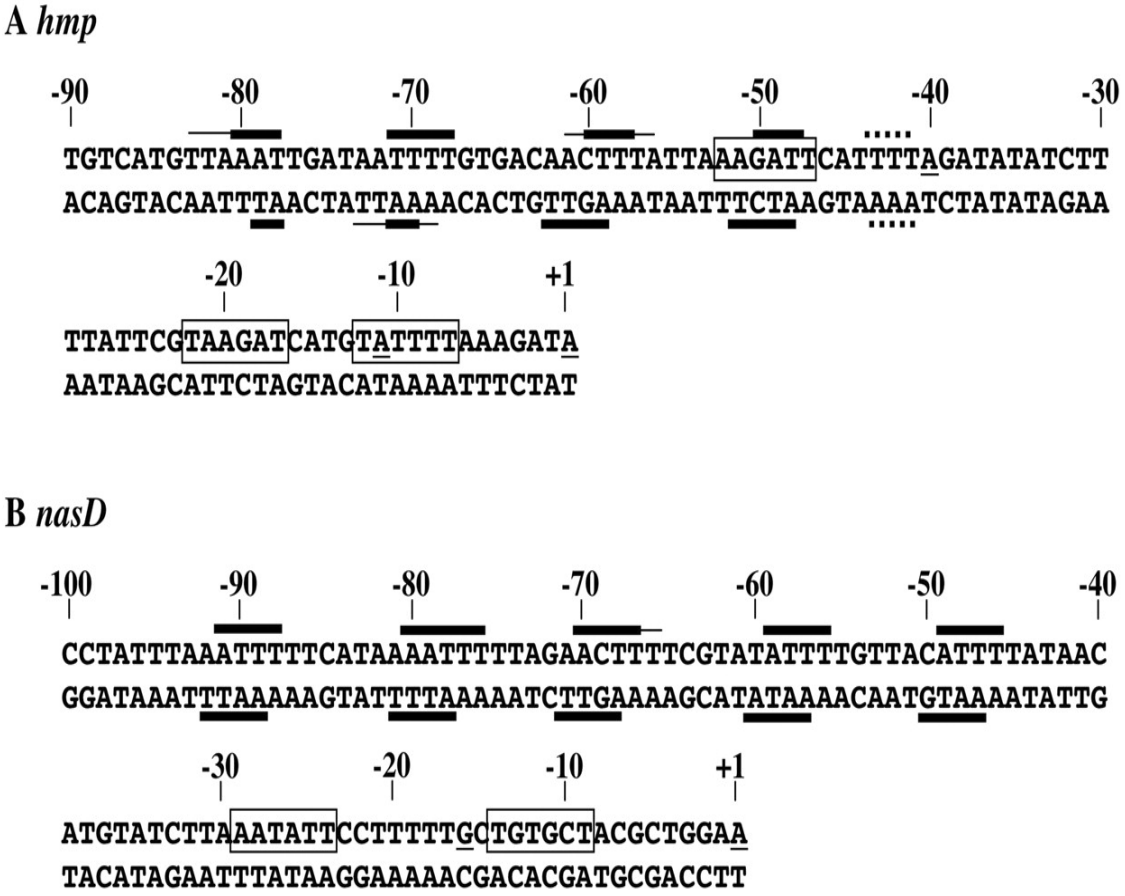



FIG. 3.2. ResD binding regions upstream of *hmp* (A) and *nasD* (B). The nucleotide sequences of the *hmp* and *nasD* regulatory regions relative to the transcription start site are shown for both coding (top) and noncoding (bottom) strands. The thick lines indicate the nucleotides protected from attack by hydroxyl radicals; the thin lines indicate the regions that were partially protected; and the dotted lines indicate the ResD binding regions that are located on different faces of the DNA helix than the rest of the binding regions. Transcription start sites utilized in vitro are underlined, and each -10 sequence is enclosed in a box.

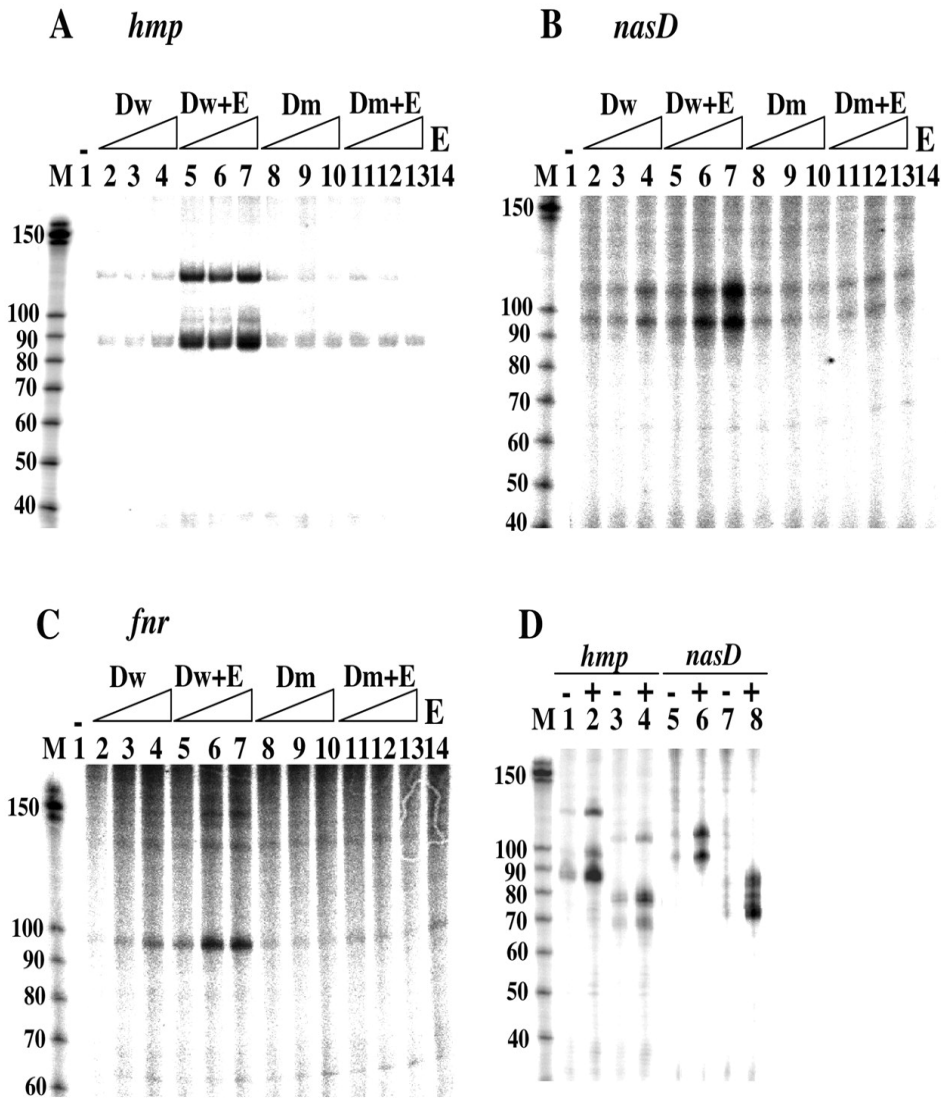



FIG. 3.3. (A to C) In vitro transcription analysis of the *hmp* (A), *nasD* (B), and *fnr* (C) promoters. Transcription was carried out with 25 nM purified RNAP and 5 nM template without ResD and ResE (lane 1), with increased amounts of wild-type ResD (Dw) (lanes 2 to 4), with wild-type ResD and ResE (lanes 5 to 7), with the D57A ResD mutant (Dm) (lanes 8 to 10), with the D57A ResD mutant and ResE (lanes 11 to 13), and with only ResE (E) (lane 14). The amounts of ResD and ResE used were 0.1, 0.2, and 0.4 μM for *hmp* transcription and 0.25, 0.5, and 1.0 μM for *nasD* and *fnr* transcription. Equal amounts of ResD and ResE were used, and the highest concentration of ResE was used in the reaction mixture containing only ResE. (D) Transcription analysis of the *hmp* and *nasD* promoters with different templates. Transcription was carried out as described above for panels A and B in the absence (-) or in the presence (+) of 0.5 μM ResD and ResE (for *hmp*) or 1.0 μM ResD and ResE (for *nasD*). The templates were as follows: lanes 1 and 2, *hmp* (positions -185 to 79); lanes 3 and 4, *hmp* (positions -185 to 61); lanes 5 and 6, *nasD* (positions -138 to 96); and lanes 7 and 8, *nasD* (positions -138 to 66). The positions of RNA size markers (in nucleotides) are shown in lane M.

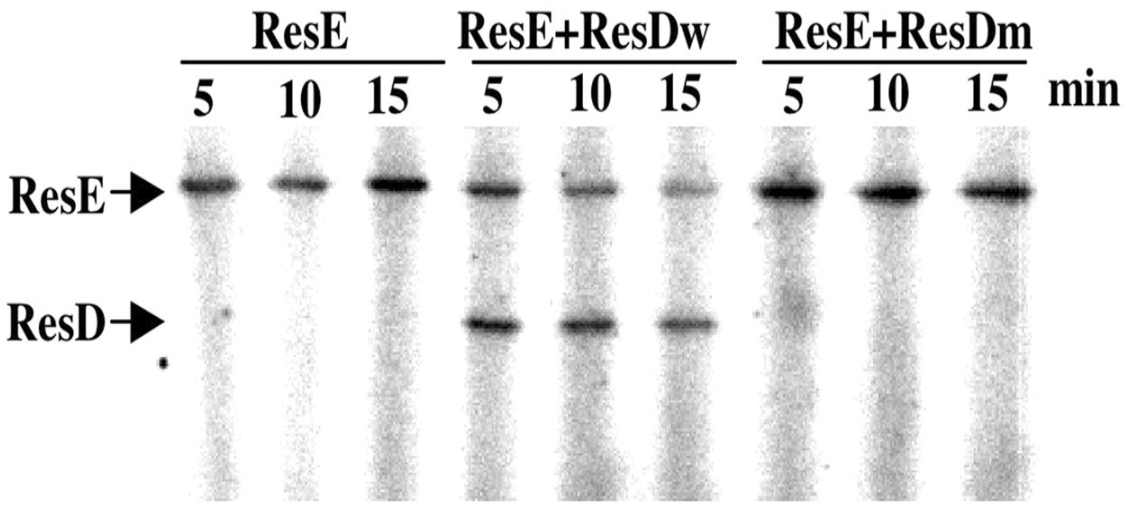



FIG. 3.4. Phosphorylation assay of wild-type and mutant ResD. ResE, in the absence and presence of wild-type ResD (ResDw) or the D57A mutant ResD (ResDm), was incubated with [$\gamma$-$^{32}$P]ATP at room temperature for the times indicated. The samples were separated on an SDS-polyacrylamide gel before autoradiography.

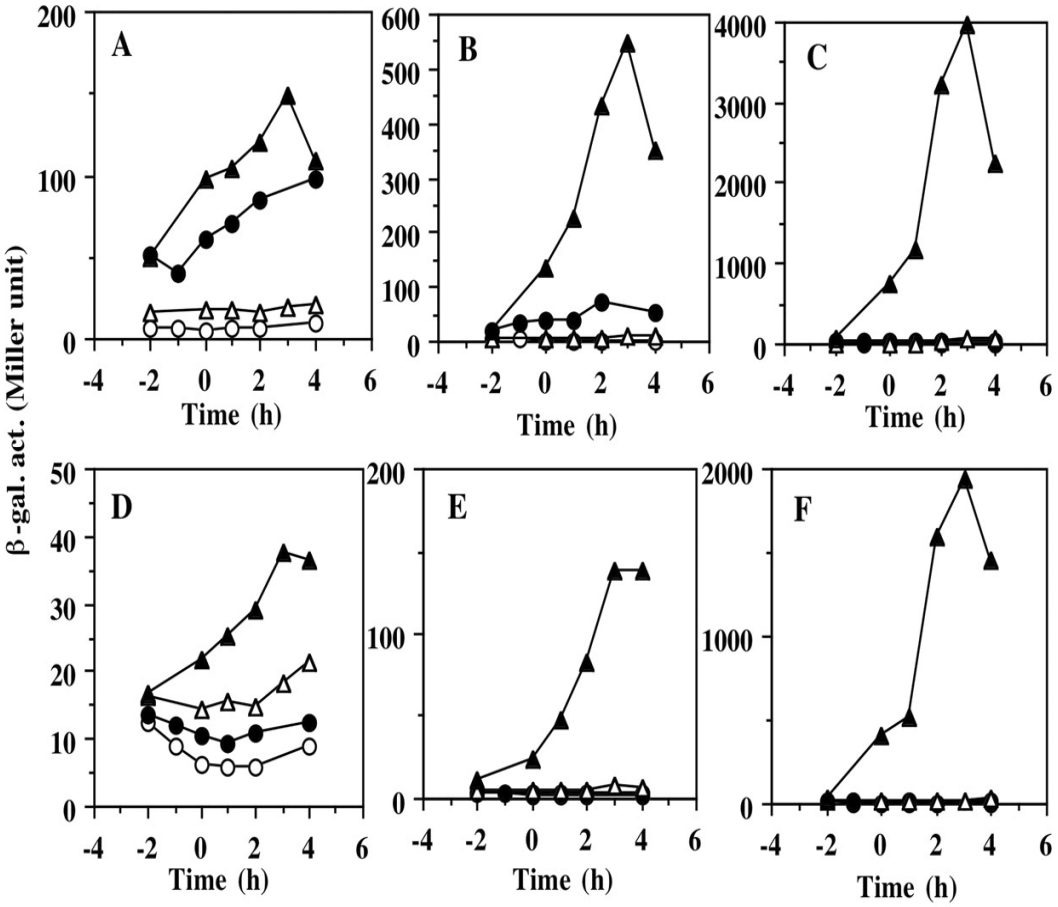



FIG. 3.5. Expression of *fnr-lacZ*, *nasD-lacZ*, and *hmp-lacZ* in cells grown under aerobic and anaerobic conditions. Δ*resDE* strains carrying *fnr-lacZ* (A and D), *nasD-lacZ* (B and E), and *hmp-lacZ* (C and F) were grown in 2xYT supplemented with 1% glucose and 0.2% potassium nitrate and in the absence or presence of IPTG. The strains carried IPTG-inducible wild-type *resD* (A to C) or mutant *resD* (D57A) (D to E) at the *amyE* locus. Symbols:○, aerobic growth in the absence of IPTG; ●, aerobic growth in the presence of 1 mM IPTG; Δ, anaerobic growth in the absence of IPTG:▲, anaerobic growth in the presence of 1 mM IPTG. Time zero was the end of the exponential growth phase. β-gal. act., β-galactosidase activity.

# CHAPTER 4
# INTERACTION BETWEEN RESD AND RNAP DURING TRANSCRIPTION INITIATION

## 4.1 INTRODUCTION

Bacteria adopt regulation of transcription initiation to express or repress appropriate genes under certain conditions. The transcription initiation is controlled by either transcription activators or repressors. Genes controlled by transcription activators often lack the consensus -35 sequence, therefore σ is unable to form stable interaction with the core promoter. One class of activators (class I) bind upstream of the -35 region and recruit RNAP by direct interaction with αCTD. The best-studied class I activators is CRP (cyclic AMP receptor protein), which binds the *lac* or *galP1* promoter (Ebright, 1993). There are two classes CRP-dependent promoters. At class I promoters such as the *lac* promoter, CRP binds at the site centered -61.5 (or further upstream) and transcription activation involves three protein-protein and protein-DNA interactions –CRP-αCTD (287 determinant), αCTD (265 determinant)-DNA that locates immediately downstream of the CRP-binding site, and αCTD (261 determinant)-$\sigma^{70}$ region 4 (Busby and Ebright, 1999). At the class II promoters such as the *galP1* promoter, the DNA site for CRP is centered at -41.5 and overlaps the -35 sequence. Transcription activation at the class II promoter involves four interactions – CRP- αCTD (287 determinant), αCTD (265 determinant)-DNA immediately upstream of the CRP-binding site, CRP-$\sigma^{70}$ region 4, and CRP-αNTD (Rhodius and Busby, 2000). The variability of the location where class I activators bind is due to the flexibility of the linker that connects αNTD and αCTD (Jeon *et al.*, 1997). The crystal structure of the CRP- αCTD-DNA complex revealed no large-scale conformational changes associated with CRP and αCTD, supporting the hypothesis that the CRP-dependent activation is exerted by a simple recruitment mechanism (Benoff *et al.*, 2002).

In addition to its important role in the interaction with activators, αCTD is critical to interact with UP elements that are present in the rRNA promoters (Ross *et al.*, 1993). Similarly, αCTD of *B. subtilis* enhanced the transcription of promoters carrying UP elements in the absence of activators (Fredrick *et al.*, 1995; Meijer and Salas, 2004).

Class II activators interact with the σ subunit. For example, the bacteriophage λcI protein interacts with region 4 of $\sigma^{70}$. The side chain of R588 in $\sigma^{70}$ (corresponding to R347 in $\sigma^{A}$) is involved in the direct interaction with an acidic patch on the surface of λcI and the interaction facilitates the binding of region 4 to the promoter -35 element (Nickels *et al.*, 2002). The structure of the λcI-region 4 of $\sigma^{70}$ -DNA terbary ccomplex suggested that the cooperative binding of λcI and $\sigma^{70}$ by accelerating the isomerization step (Jain *et al.*, 2004).

As mentioned in 1.4.7, the transactivation loop between helix2 and helix3 of OmpR and PhoB has been implicated in the interaction of αCTD and σ, respectively (Fig 4.1) (Kato *et al.*, 1996; Makino *et al.*, 1996). Alanine scanning mutagenesis of the transactivation loop of *B. subtilis* PhoP, the OmpR/PhoB subfamily RR, also showed that residues in the loop are critical for PhoP interaction with RNAP (Chen *et al.*, 2004). The transactivation loop of OmpR is three residues longer than one of PhoB (Blanco *et al.*, 2002; Martinez-Hackert and Stock, 1997b). The functional analysis of PhoP transactivation revealed that PhoP, like OmpR has a long transactivation loop (Chen *et al.*, 2004). The structure information indicates that the loop exposes to the surface when PhoB and OmpR bind to DNA (Blanco *et al.*, 2002; Martinez-Hackert and Stock, 1997b). The α3 helix adjacent to the transactivation loop recognizes target DNA. In addition to the α3 helix, mutagenesis or structure prediction analysis of PhoB, OmpR and PhoP showed that certain residues of the transactivation loop are also critical for DNA binding (Blanco *et al.*, 2002; Chen *et al.*, 2003a; Makino *et al.*, 1996; Russo *et al.*, 1993).

In this chapter, we investigated whether the ResD transactivation loop is critical for transcription of *hmp*, *nasD* and *fnr*. Using alanine scanning mutant library, we determined RNAP subunits that likely interact with ResD during transcriptional initiation and identified probable key residues involved in the interaction with ResD.

## 4.2 RESULTS

### 4.2.1 Critical residues of ResD for transcription activation of *fnr*, *nasD* and *hmp*

The transactivation loop between helix2 and helix3 of RRs of the OmpR/PhoB family has been implicated in the interaction of RNAP (Fig 4.1) (Chen *et al.*, 2004; Kato *et al.*, 1996; Makino *et al.*, 1996), we carried out mutagenesis of amino acid residues in the putative transactivation loop (Val 191→Arg 201) of ResD to determine whether the transactivation loop of ResD is important for transcription activation (Fig. 4.1). In addition to the transactivation loop, Pro 179 and Ser 181 in OmpR β5 were reported to be involved in the interaction between OmpR and the α subunit (Kato *et al.*, 1996). Therefore, Val 181 and Glu 183 of ResD, which correspond to Pro 179 and Ser 181 in OmpR, were substituted with alanine. Because Arg 211 is well conserved in the OmpR/PhoB subfamily and is believed to be critical for the DNA binding activity of RRs (Kato *et al.*, 1996), Arg 211 was also substituted with alanine as a control. The mutant *resD* genes were generated by two-step PCR reaction and were integrated into the *amyE* locus of *B. subtilis* chromosome as described in Materials and Methods. In this construct, the native *resDE* genes were disrupted by a *tet* cassette, and the *resDE* gene (with the wild-type or mutant *resD*) was expressed under the IPTG-inducible promoter. The mutations of this region do not affect the ResD protein stability (data not shown). Transcriptional *lacZ* fusions to the *fnr*, *nasD* and *hmp* promoter were used to monitor ResD-dependent transcription as previously described (Geng *et al.*, 2004). Transcription of these genes was activated under anaerobic conditions but not aerobic conditions (data not shown) in the presence of IPTG, when wild-type *resDE* was expressed at the *amyE* locus (Fig. 4.2). The result demonstrated that ResD expressed from the IPTG-dependent promoter is activated in response to oxygen limitation. Figure 4.3 shows the effect of ResD alanine substitutions on the expression of *hmp*, *nasD* and *fnr*, which is summarized in Figure 4.1. Transcription of *hmp*, *nasD*, and *fnr* was hardly activated in the R211A mutant, suggesting that R211 of ResD, like the corresponding residue in OmpR and PhoB, is involved in DNA binding. The alanine substitution in F197, G198, D199, and R201 in the putative transactivation loop severely affected *fnr* expression. Among these mutations,

G198A and R201A highly reduced *nasD* expression. In contrast, neither mutation except R201A significantly affected *hmp* expression.

Two types of mutations in ResD could reduce its ability to activate transcription. One type is the mutation that affects DNA binding affinity, defined as binding mutations; the other type is the mutation that affects the interaction of ResD with RNAP, defined as interaction mutations. In order to discriminate these mutations, DNA-binding ability of ResD mutants G198A, D199A and R201A were checked by DNase I footprinting analysis of the *hmp* and *nasD* promoters. Note that as described earlier (Geng *et al.*, 2004; Geng *et al.*, 2007), ResD itself is unable to bind the *fnr* promoter, therefore we were unable to determine the effect of these mutations on the binding activity to the *fnr* promoter. The wild-type and the mutant ResD proteins were purified as described in Materials and Methods (Fig. 4.4). The result in Figure 4.5 shows that G198A and R201A ResD mutants completely lost the binding activity to the *nasD* promoter, which explains why G198A and R201A severely affect the transcription of *nasD*, and likely of *fnr*. The G198A mutant was also unable to bind the *hmp* promoter, but the transcription of *hmp* was only slightly reduced in the strain producing ResD (G198A) (Fig. 4.3). This inconsistent result of G198A on the effect of *hmp* will be discussed later (see Discussion). The binding affinity of ResD (D199A) mutant protein to the *hmp* promoter is slightly higher to that of the wild-type ResD protein, and the mutant protein has much higher binding affinity to the *nasD* promoter than the wild-type ResD. The result is in good agreement with the in vivo result that *hmp* and *nasD* expression is significantly increased in the ResD (D199A) strain (Fig. 4.3).

### 4.2.2 Alanine scanning mutagenesis of αCTD revealed key residues for *nasD* expression activated by ResD

As mentioned in Introduction, the interaction between αCTD and OmpR plays a critical role in transcription activation (Kato *et al.*, 1996; Kondo *et al.*, 1997; Slauch *et al.*, 1991). Our previous result using an αCTD mutant library (Zhang *et al.*, 2006) and DNase I fooprinting analysis also indicated that the interaction between ResD and αCTD is important for *fnr* transcriptional initiation (Chapter 2.2). We used the αCTD mutant library to determine whether αCTD is required for activation of *hmp* and *nasD*

transcription, and if so, to identify residues that likely interact with ResD at these promoters. Effects of αCTD mutations on *hmp* and *nasD* expression are shown in Figure 4.6. The mutations that severely affect *fnr* expression (see Fig. 2.6) –E254A, V260A, Y263A, K267A, A269I and N290A– also reduced *nasD* expression; in contrast, none of the single alanine substitutions significantly affected *hmp* expression, although Y263A and A269I weakly reduced the expression. A structure model of αCTD indicates that all residues except N290 constitute the same surface. N290, which is separated from the other five residues (Newberry *et al.*, 2005), is probably involved in binding of αCTD to the DNA backbone like *E coli* N294 (Gaal *et al.*, 1996).

Y263 of αCTD has been reported to interact with Spx, a transcriptional regulator in *B. subtilis* (Nakano *et al.*, 2003b). Mutations in Y263 and K267, which showed the most severe effect on *nasD* and *fnr* expression, are adjacently located in the tertiary structure of αCTD (Newberry *et al.*, 2005) (Fig. 4.7). In order to determine the role of the two residues in transcriptional activation, a RNAP double mutant containing Y263A and K267A αCTD was constructed. The double mutant nearly abolished *nasD* and *fnr* expression, indicating that the effect of the two substitutions is additive (Fig. 4.7). The expression of *hmp* was only slightly affected by the Y263A and K267A single mutations and the Y263A K267A double mutation showed a moderate additive effect (Fig. 4.7).

We previously proposed that E256, V260, Y263, K267 and A269 form a surface-exposed patch on αCTD, which is likely to interact with the ResD at the *fnr* promoter (Fig. 2.8) (Geng *et al.*, 2007). Similarly, this surface-exposed patch may interact with ResD at the *nasD* promoter and the side chains of residues Y263 and K267 might be components involved in the direct interaction with ResD. Although E254, together with V260, Y263, K267, and A269, may constitute the patch that interacts with ResD during *nasD* and *fnr* transcription initiation, an alternative role of E254 could be envisioned. E254 is located in a region (residues 254 to 257) with a net negative charge, which corresponds to the 258-261 region in *E. coli* αCTD (Fig. 4.8). The 258-261 region of *E. coli* αCTD was reported to interact with region 4.2 of $\sigma^{70}$ (Chen *et al.*, 2003a; Ross *et al.*, 2003). Therefore, E254 of αCTD may interact with the σ subunit rather than interacting with ResD during *nasD* and *fnr* transcriptional initiation. This possibility will be examined in 4.2.4.

### 4.2.3 Interaction between ResD and αCTD at the *hmp* and *nasD* promoters

Previous results of in vivo mutagenesis and in vitro analysis of *fnr* transcriptional activation by ResD indicate that the interaction of ResD with αCTD results in cooperative binding of ResD and RNAP at the *fnr* promoter, which facilitates transcriptional initiation (Geng *et al.*, 2007). Transcription of *nasD* requires the same residues of ResD and αCTD that are essential for *fnr* expression, suggesting that the interaction of ResD with αCTD is also involved in the transcription activation of *nasD*. Potential interactions between ResD and αCTD at the *hmp* and *nasD* promoter region were further examined by DNase I footprinting experiment (Fig. 4.9). In this experiment, we used the wild-type and the K267A α proteins because the K267A mutation in αCTD highly reduced *nasD* expression, but had a minor effect on *hmp* expression in vivo (Fig. 4.6).

Either wild-type or mutant α binds to a fairly large region of the *hmp* and *nasD* promoter and the protected region extended from -28 to -59 and from -9 to +6 in the *hmp* promoter and from -19 to -62 in the *nasD* promoter (Fig. 4.9 and Fig. 4.10). When ResD was present at a concentration suboptimal for binding, wild-type α enhanced the binding of ResD to the *nasD* and *hmp* promoter (Fig. 4.10A and B, lane 3). Similarly, the K267A mutant stimulated the binding of ResD to the *hmp* promoter as well as wild-type α (Fig. 4.10.A, lane 5). In contrast, the K267A mutation abolished the stimulatory effect on ResD binding to the *nasD* promoter (Fig. 4.10.B, lane 5). These results are consistent with the in vivo result that *nasD,* but not *hmp,* expression was greatly reduced in the K267A mutant and the results strongly suggest that the interaction of K267 with an unidentified ResD residue is critical for transcription activation of *nasD*.

Both wild-type and K267A α extended the region (between -42 and -67) of the *hmp* promoter protected by ResD to further downstream regions, -42 ~ -38, -22 ~ -19 and -9 ~ +6. Among these regions, -42 ~ -38 was not protected by α alone or ResD alone. In addition, two hypersensitive sites at -45 and -43 were created by ResD and α (either wild-type or K267A) co-incubation with *hmp*. In the *nasD* promoter, the cleavage at -42, -39 and -37 is almost equal in the free probe, α alone, and ResD alone reactions.

However, in the presence of both ResD and wild-type α, -42 site became hypersensitive to DNase I and -39 site was slightly more protected. Unlike the wild-type α, α (K267A) did not enhance the protection at -39 in presence of ResD. These hypersensitive sties caused by the co-incubation of ResD and α suggest that ResD may interact with α resulting in DNA helix bending during transcription initiation of *hmp* and *nasD*.

### 4.2.4 Effects of single amino acid substitution of the $\sigma^A$ subunit on ResDE-controlled genes expression

As described earlier (section 1.4.6), the σ subunit is also involved in activator-dependent transcription initiation. Because *hmp* expression was only weakly affected by the αCTD mutations, we examined the possibility that the $\sigma^A$ subunit of RNAP is involved in the interaction between ResD and RNAP at the *hmp* promoter. Figure 4.11 shows the effect of single amino acid substitution in $\sigma^A$ region 4.2 [provided by C. Moran, Emory University and (Baldus *et al.*, 1995)] on *hmp*, *nasD* and *fnr* expression. K356A, H359A and R362A reduced *hmp*, *nasD* and *fnr* expression, and K356E and H359R almost completely impaired *hmp* expression, In contrast to the αCTD mutations, the mutations of $\sigma^A$ overall showed more severe effect on *hmp* transcription than *nasD* and *fnr* transcription. The three basic residues –K356, H359 and R362 – are located in the same helix (Fig. 4.8). Region 4.2, which is highly conserved among the primary σ family, has been reported to interact with Spo0A, a transcriptional regulator in *B. subtilis*. The K356E and H359R mutations, which most severely affected *hmp* expression, were shown to abolish Spo0A-dependent transcription activation by impairing interaction between $\sigma^A$ and Spo0A (Baldus *et al.*, 1995). Because the residue of Spo0A that interacts with $\sigma^A$ region 4.2 is E221 carrying negatively charge (Kumar *et al.*, 2004), the conserved acidic residues around the transactivation loop in ResD might interact with $\sigma^A$ region 4.2 to activate *hmp* transcription. However, substitutions of the acidic residues in ResD, namely D179, E185, E190, and D204, did not affect *hmp* expression (Fig. 4.12), suggesting that amino acid residues in ResD that interact with $\sigma^A$ region 4.2 reside outside of the region shown in Fig. 4.1. On the contrary, the D179A, E190A and D204A mutations affected *fnr* expression and the D204A mutation also reduced *nasD* expression. D204 locates in α3,

which is known to be involved in recognition of the DNA helix, as shown in OmpR (Pratt and Silhavy, 1994) and PhoB (Makino *et al.*, 1996) in *E. coli*, and PhoP in *B. subtilis* (Chen *et al.*, 2004), the D204A mutation might lead to decreased *nasD* and *fnr* transcription by affecting the DNA binding affinity of ResD. The D179 residue is close to the end of α1, and E190 locates in the end of α2. How the mutations of D179 and E190 affect *fnr* transcription is unknown.

### 4.2.5 Putative interaction between αCTD and region 4.2 of $\sigma^A$

In *E. coli*, the 594-604 region of $\sigma^{70}$ (which has net positive charge and corresponds to 352-362 in *B. subtilis* $\sigma^A$) interacts with the 258-261 region of αCTD (which has net negative charge and corresponds to 254-257 in *B. subtilis* αCTD) (Paul *et al.*, 2004). The interaction between the 594-604 region of $\sigma^{70}$ and 258-261 of αCTD is involved in activation of the *rrnB* P1, an UP element-dependent promoter (Ross *et al.*, 2003) as well as in activator-dependent transcription (Chen *et al.*, 2003a). The residues involving in the interaction between αCTD and $\sigma^{70}$ in *E. coli* are well conserved in *B. subtilis* (Fig. 4.8). Because none of the αCTD mutations does not affect *hmp* expression, it is unlikely that the interaction of $\sigma^A$ and αCTD is required for transcription activation of *hmp*. In contrast, the residues in *B. subtilis* corresponding to those involved in $\sigma^{70}$ - αCTD interaction –E254 in αCTD and K356, H359 and R362 in $\sigma^{70}$– are also critical to *nasD* and *fnr* expression activated by ResD. Therefore, we examined whether *nasD* and *fnr* expression requires the interaction between $\sigma^A$ and αCTD.

The direct evidence for this interaction was explored by suppressor analysis. Because the interaction between αCTD and σ is likely electrostatic interaction, *rpoA* E254K was generated to switch the negative charge into positive charge at a side-chain of residue 254. αCTD mutation of E254K, like E254A, reduced *nasD* and *fnr* expression (Fig. 4.13). *rpoA* E255A could not be isolated in the wild-type *sigA* background probably because it is essential for transcription of indispensable housekeeping genes; however the E255A mutation can be obtained in certain *sigA* single mutant backgrounds, for example, in *sigA* K356E or *sigA* H359R mutant, suggesting the possible interaction of E225 with K356 and/or H359. Attempt to construct *rpoA* E255K was unsuccessful even in the *sigA* mutant backgrounds. We examined *nasD* and *fnr* expression in the strains carrying

different combination of *rpoA* and *sigA* mutations (Fig. 4.13), except in *rpoA* E254K *sigA* R362A mutant, which could not be transduced by SPβ phage carrying promoter-*lacZ* fusions. The results showed that most of the combined mutations affected *fnr* and *nasD* expression more severely than the corresponding single mutations except that effects of $\sigma^A$ K356E and H359R on *fnr* expression are slightly relieved by *rpoA* E255A. The suppressor effect of K356E and H359R on the E255A mutation could not be addressed since the E255A single mutant could not be obtained as described above. We speculate that E255 of αCTD may interact with K356 and/or H359 of $\sigma^A$, although this possibility remains to be further investigated.

## 4.3 DISCUSSION

We identified critical residues of ResD for *fnr*, *nasD* and *hmp* expression by alanine substitution mutagenesis of amino acids in and near the transactivation loop (Fig. 4.1). R201A and R211A mutations reduced the expression of all three genes. F197A, G198A and D199A affected *fnr* transcription, while only G198A reduced *nasD* expression. It remains to be determined why F197A, G198A and D199A mutations have differential effects on the expression of *hmp*, *nasD* and *fnr*. One possibility is that ResD has different binding patterns on *hmp*, *nasD* and *fnr* promoters, therefore the roles of these residues are different during ResD binding to each promoter. The DNA-binding ability of G198A, D199A and R201A ResD mutants was examined by DNase I footprinting assay of *nasD* and *hmp* (Fig. 4.5). The result showed that both R201A and G198A mutations were binding mutations. The complete loss of DNA binding activity of the G198 mutant is in good agreement with the in vivo result that indicates that G198A highly reduces *nasD* expression (Fig. 4.2). However, the G198 mutation impairs the binding of ResD to the *hmp* promoter as well, although the mutation has only a minor effect on the *hmp* expression in vivo. This contradictory result may suggest that the defect of G198A can be compensated by unknown mechanism in vivo. This in vivo compensatory effect could be caused by another protein that interacts with ResD or the *hm*p promoter. For example, NsrR that binds immediately downstream of the ResD-binding site might participates in stabilizing binding of G198 to DNA. Alternatively, the template DNA topology might

affect the binding of G198A to *hmp.* Anaerobiosis leads to a decrease in negative supercoiling of plasmid DNA in *B. subtilis*, (Krispin and Allmansberger, 1995), which might result in increased binding of G198A compared to the linear template DNA used for DNase I footprinting analysis. In addition, non-specific DNA binding proteins such as HBSu may alter DNA topology in vivo (Kohler and Marahiel, 1997), which is absent in in viro experiments. Contrary to R201A and G198A, the D199A mutation increased ResD binding to the promoter DNA, *nasD* in particular. Taken together, these results indicate that all of ResD transactivation loop mutations that reduced ResD activation exhibit altered DNA binding ability at least to one promoter. This result is unexpected given that previous studies have shown the transactivation loop of RRs in the OmpR/PhoB subfamily to interact with a subunit of RNAP. As shown in Fig. 4.1, the transactivation loop of *B. subtilis* PhoP shares a high similarity in amino acid sequence to the loop of ResD. Seven residues of transactivation loop are involved in PhoP interaction with RNAP and none of the residues in the loop is important for DNA-binding activity (Chen *et al.*, 2004). The residues G199 and R202 of PhoP, corresponding to G198 and R201 of ResD, are also critical for transcription of the Pho regulon. However the electrophoresis mobility shift assay showed that G199A and R202A mutations of PhoP did not affect DNA-binding ability, unlike the corresponding residues of ResD. We propose that the transactivation loop of ResD is not involved in the interaction with a subunit of RNAP, and rather affects DNA-binding affinity. Although residues involved in the interaction with RNAP remain to be uncovered, it is intriguing that recent study indicated that mutations in the transactivation loop of OmpR, which were once thought to be activation mutations, are likely to be binding mutations (Maris *et al.*, 2005).

Spx, a transcriptional regulator, outputs positive and negative transcriptional control via interacting with αCTD of RNAP (Nakano *et al.*, 2003b; Nakano *et al.*, 2005). The amino acid sequence of region 32-67 of Spx is similar to that of 141-171 of ResD, although the secondary structure of the region differes between Spx and ResD (Fig. 4.14). Genetic experiments and structural analysis revealed the residues in the region 32-67 of Spx are involved in the interaction with αCTD, such as R47, G52, T53 and D54 (Nakano *et al.*, 2003b; Newberry *et al.*, 2005). Notably, ResD has nearly identical residues between the residue 153 to 160 – R157, G158, T159, and E160 (Fig. 4.14). In addition,

Y263 and K267 of αCTD that interacts with Spx are also critical to ResD activation of *nasD* and *fnr* [(Nakano *et al.*, 2000c; Zhang *et al.*, 2006) (Fig. 2.6 and Fig. 4.6)]. Therefore, the residues of ResD involved in interaction with αCTD might reside in the region 141-171, particularly 153-160. To examine the possibility, the G158 will be substituted with arginine, because G52R disrupts the interaction between Spx and αCTD (Nakano *et al.*, 2000c). The effect of the G158R mutation of ResD on *nasD* and *fnr* transcription will be examined.

The mutational analysis of αCTD (Fig. 4.6) and $\sigma^A$ (Fig. 4.11) showed that expression of *fnr* and *nasD*, but not of *hmp*, was highly reduced by certain αCTD mutations, whereas $\sigma^A$ mutations affected *hmp* expression more severely than *nasD* and *fnr* expression. Based on these results, we concluded that ResD interacts with αCTD at the *fnr* and *nasD* promoter and that ResD interacts with $\sigma^A$ at the *hmp* promoter. However, DNase I footprinting assay showed that α enhanced ResD binding to *hmp* and changed the cleavage pattern around region -45 ~ -38 (Fig. 4.10). Therefore, ResD likely interacts with α at the *hmp* promoter in vitro, although the αCTD mutations hardly affect *hmp* expression in vivo. One possibility is that *hmp* can interact with αNTD instead of αCTD at the *hmp* promoter during transcriptional initiation, the possibility that can be examined by using αCTD instead of the full-length α subunit for co-footprinting assay. Another possibility is that ResD interacts with αCTD, but the impaired ResD-α interaction resulted from the αCTD mutations such as K267A is compensated by ResD-σ interaction in vivo. In addition, we cannot exclude the possibility that the in vitro interaction between α and ResD is an artifact caused by the conditions used in the footprinting experiment, which include the use of a liner PCR product instead of supercoiled DNA or the amount of α that might be higher than the cellular concentration.

αCTD of *E. coli* (Benoff *et al.*, 2002) and *B. subtilis* (Newberry *et al.*, 2005) are similar in secondary structure; however, the residue Y263 of *B. subtilis* αCTD is conserved in gram-positive bacteria, while gram-negative bacteria have a conserved alanine residue in the corresponding position 267 (Zuber, 2004). Genetic and structural analysis showed that Y263 interacts with the G52 residue of Spx, a transcriptional repressor that was only found in gram-positive bacteria (Nakano *et al.*, 2003b; Newberry *et al.*, 2005). Y263 is the most critical residue for *fnr* and *nasD* transcription as shown

here and in our previous paper (Geng *et al.*, 2007). Spx, when overproduced, interacts with αCTD, thus interfering ResD-dependent transcription activation (Nakano *et al.*, 2003b). This Spx-dependent inhibition of *hmp* transcription is relieved either by the Spx G52R mutation or by the αCTD Y263C mutation, as either mutation results in the loss of Spx-αCTD interaction. In contrast, *nasD* and *fnr* transcription is restored by the Spx G52R mutation, but not by the αCTD Y263C mutation (Ying Zhang, Peter Zuber, and Michiko Nakano, unpublished result). This observation is explained by the current finding that the Y263 residue is essential for transcription of *nasD* and *fnr*, but not of *hmp.*

αCTD and region 4.2 of $\sigma^{70}$ in *E. coli* not only interact with transcription activators [see reviews (Gourse *et al.*, 2000; Gross *et al.*, 1998), but also interact each other (Chen *et al.*, 2003a; Ross *et al.*, 2003). We speculated that a similar interaction might occur between αCTD and $\sigma^{A}$ region 4.2 during *fnr* and *nasD* transcriptional initiation. We tried to obtain direct evidence for the interaction by suppressor analysis. The rationale of the approach is that the effect resulted from the charge change of a residue in αCTD could be rescued by the opposite charge change of a residue in $\sigma^{A}$ region 4.2, if the residues are involved in electrostatic interaction. We did not obtain any clear suppression effect, however. For example, E254K did not rescue the reduced *nasD* and *fnr* transcription in the *sigA* mutant carrying K356E, on the contrary, the transcription is more severely reduced in the E254K K356E double mutant strain than in the E254K or K356E single mutant strain (Fig. 4.13). Therefore, it is more likely that E254, together with V260, Y263, K267, and A269 in αCTD, is involved in interaction with ResD than with $\sigma^{A}$. In addition, the E255A mutant is isolated only in the $\sigma^{A}$ mutant background carrying the K356E or H359R allele, suggesting that E255 of αCTD and K356 or H359 of $\sigma^{A}$ are involved in direct interaction at certain promoters.

## 4.4 MATERIALS AND METHODS

### 4.4.1 Strains and plasmids

All *B. subtilis* strains used in this study are derivatives of JH642 (*trpC2 pheA1*) (Table 1). *E. coil* DH5α was used as the host for plasmid construction. *E. coil* ER2566 was used as the host for overproducing ResD and ResE proteins. RNAP α subunit and

αCTD proteins were overproduced in *E. coil* BL21 (DE3)/pLysS. Plasmids and primers used in this study are listed in Table 1 and 2, respectively.

*resDE* was first amplified by PCR using the two primers oMMN02-205 and oMMN02-214 and chromosome DNA isolated from JH642 as a template. The purified PCR product was digested with SalI and SphI and cloned into pDG148 (Stragier *et al.*, 1988) to generate pMMN543. The inserted *resD* and res*E* genes were verified by nucleotide sequencing. To express wild-type *resDE* from isopropyl-β-thiogalactopyranoside (IPTG)-inducible P*hyperspank* (P*spank-hy*) promoter, *resDE* was subcloned into pDR111 (Britton *et al.*, 2002). The *resDE* fragment recovered from pMMN543 by digestion with SalI and SphI was cloned into pDR111 digested with the same enzymes to generate pMMN553. A NheI site was introduced into *resE* by a site-directed silent mutagenesis to generate pHG17. pHG17 was constructed by two-step PCR. Partially overlapped PCR products were generated with oMMN205-oHG14 and oHG-13-oMMN214 primer pairs in which pMMN553 was used as a template. oHG13 and oHG14 are mutagenetic primers to generate the substitution of codon CTT with CTA in Leu49, which also creates a NheI site. The resultant PCR products were used as templates for a second PCR in which primers oMMN02-205 and oMMN02-214 were used. The PCR product of the second-step PCR was digested with SalI and SphI and cloned into PDR111 digested with the same enzymes to generate pHG17. The *resE* fragment, recovered from pHG17 digested with NheI and SphI, was cloned into pDR111 to generate pHG12. pHG12 was used to construct the plasmids which are similar to pHG17 but harboring mutant *resD*. For each construction, oMMN02-205-reverse and oHG14-forward primers pairs were used for the first-step PCR together with pMMN553 as a template. A set of forward and reverse primers specify a single mutation and an additional restriction enzyme site was also introduced to diagnose the mutation. The resultant PCR products were used as templates for second-step PCR in which oMMN02-205 and oHG14 primer pairs were used. The second-step PCR products were digested by SalI and NdeI and cloned into pHG12. pHG17 and its derivatives carrying mutant *resD* genes were used to transform *B. subtilis* JH642 strain with selection for spectinomycin resistance ($Spc^r$ 75 μg/ml) and transformants were screened for amylase-negative phenotype that is indicative of a double recombination event at the *amyE* locus. The chromosome DNA

isolated from *B. subtilis*, which is carrying IPTG-inducible *resDE* at the *amyE* locus, was used to transform LAB2135 (Δ*resDE*). The resulting strains contain the intact wild-type or mutant *resDE* genes under control of the P*spank-hy* promoter. These strains were transduced with SPβ lysate carrying *hmp-lacZ* (Nakano *et al.*, 2000b), *nasD-lacZ* (Nakano *et al.*, 1995), or *fnr-lacZ* (Nakano *et al.*, 1996). The transductants were selected for chloramphenicol resistance ($Cm^r$ 5 μg/ml).

To overproduce ResD mutant proteins in *E. coli*, the mutant *resD* genes were cloned into intein self-cleavable expression vector (New England Biolabs). Mutant *resD* genes were amplified by PCR using primers pair oMMN00-102 and oMMN00-103. The pHG17 derivatives harboring mutant *resD* genes were used as templates. The PCR products were digested with NcoI and SmaI and inserted into pTYB4 (New England Biolabs) . The resulting plasmid was used to transform *E. coli* ER2566 with selection for ampicillin resistance ($Amp^r$ 25 μg/ml).

*B. subtilis* αCTD library was constructed in Dr. Peter Zuber's laboratory (Zhang *et al.*, 2006). *B. subtilis rpoA* mutations –E254K, Y263A, Y263A and K267A – were constructed in a similar way to previously published (Nakano *et al.*, 2000c).

To construct *B. subtilis* carrying *rpoA sigA* double mutants, the chromosome DNA isolated from *sigA* mutants was used to transform *B. subtilis* carrying desired *rpoA* mutations with selection for neomycin resistance (25 μg/ml). The presence of the *rpoA* mutation in the transformant was confirmed by PCR of the *rpoA* allele, followed by cleavage with the restriction enzyme that recognizes the mutated sequence. The *B. subtilis sigA* mutants were provided by Dr. Charles Moran, Emory University (Baldus *et al.*, 1995).

### 4.4.2 Measurement of beta-galactosidase activities

*B. subtilis* cells were cultured in 2xYT supplemented with 1% glucose and 0.2% $KNO_3$ with appropriate antibiotics and 1mM IPTG was added into cell cultures to express *resDE*. The anaerobic cell cultures and sample collection were described elsewhere (Nakano *et al.*, 1996). β-galactosidase activities were determined as previously described (Nakano *et al.*, 1988) and shown as Miller units (Miller, 1972).

### 4.4.3 Purification of proteins

ResD and ResE were purified as described elsewhere (Geng *et al.*, 2004). Mutant ResD proteins were overproduced in *E. coli* ER2566 carrying plasmid pHG34, pHG35, pHG36, pHG37 and pHG38, and purified by the procedure used for the wild-type ResD purification (Geng *et al.*, 2004). The wild-type and K267A α proteins were overproduced in *E. coli* BL21 (DE3) (pLysS) carrying plasmid pSN28 and pHG73, respectively, and purified as described in previous paper (Nakano *et al.*, 2005). Construction of pHG73 is similar to the one of pSN28 (Nakano *et al.*, 2005), except that chromosome DNA isolated from ORB5362 (*rpoA*K267A) was used as the template for PCR reaction.

### 4.4.4 DNase I footprinting analysis

A fragment carrying *hmp* (from -133 to +27) and *nasD* (from -138 to +22) promoters were used for DNase I footprinting. The probe labeling, purification and reaction conditions were described in the previous papers (Geng *et al.*, 2007; Nakano *et al.*, 2000b).

**TABLE 4.1 Bacteria strains and plasmids**

| Strain or plasmid | Description | Reference or source |
|---|---|---|
| *E. coli* strains | | |
| DH5α | | Lab stock |
| ER2566 | | New England Biolabs |
| BL21 (DE3) (pLysS) | | Lab stock |
| *B. subtilis* strains | | |
| JH642 | Parental strain (*trpC2 pheA1*) | J. A. Hoch |
| LAB2135 | *resDE*::*tet* | (Nakano *et al.*, 1996) |
| LAB2252 | SPβc2Δ2::Tn*917*::pMMN288 (*fnr-lacZ*) | (Nakano *et al.*, 1996) |
| LAB2518 | SPβc2Δ2::Tn*917*::pML107 (*hmp-lacZ*) | (Nakano *et al.*, 2000b) |
| LAB2854 | SPβc2Δ2::Tn*917*::pMMN392 (*nasD-lacZ*) | (Nakano *et al.*, 1998) |
| MH5636 | $His_{10}$-*rpoC* | (Qi and Hulett, 1998) |
| ORB4874 | *rpoA*(*Y263A*) | (Geng *et al.*, 2007) |
| ORB4884 | *rpoA*(*Y263A*) SPβc2Δ2::Tn*917*::pMMN288 (*fnr-lacZ*) | (Geng *et al.*, 2007) |
| ORB4885 | *rpoA*(*Y263A*) SPβc2Δ2::Tn*917*::pMMN392 (*nasD-lacZ*) | (Geng *et al.*, 2007) |
| ORB4886 | *rpoA*(*Y263A*) SPβc2Δ2::Tn*917*::pML107 (*hmp-lacZ*) | (Geng *et al.*, 2007) |
| ORB6116 | *rpoA*(*K267A*) $His_{10}$ *rpoC* | (Zhang *et al.*, 2006) |
| ORB5769 | *rpoA*(*Y263A K267A*) | This study |
| ORB5952 | *rpoA*(*Y263A K267A*) SPβc2Δ2::Tn*917*::pML107 (*hmp-lacZ*) | This study |
| ORB5954 | *rpoA*(*Y263A K267A*) SPβc2Δ2::Tn*917*::pMMN392 (*nasD-lacZ*) | This study |
| ORB5953 | *rpoA*(*Y263A K267A*) SPβc2Δ2::Tn*917*::pMMN288 (*fnr-lacZ*) | This study |
| ORB4884 | *trpC2 pheA1 rpoA* Y263A SPβ*c2del2*::Tn*917*::pMMN288 | This study |
| ORB4885 | *trpC2 pheA1 rpoA* Y263A SPβ*c2del2*::Tn*917*::pMMN392 | This study |
| ORB4886 | *trpC2 pheA1 rpoA* Y263A SPβ*c2del2*::Tn*917*::pML107 | This study |
| ORB5048 | trpC2 pheA *amyE*::pHG16(*amyE*::*Hyper-spank resDE resD* Q193A) | This study |
| ORB5049 | *trpC2 pheA1amyE*::pHG18(*amyE*::*Hyper-spank resDE resD* D183A) | This study |
| ORB5054 | *trpC2 pheA1amyE*::pHG13(*amyE*::*Hyper-spank resDE resD* V181A) | This study |
| ORB5055 | *trpC2 pheA1amyE*::pHG14(*amyE*::*Hyper-spank resDE resD* V191A) | This study |

| | | |
|---|---|---|
| ORB5056 | *trpC2 pheA1amyE*::pHG15(*amyE*::*Hyper-spank resDE resD* W192A) | This study |
| ORB5057 | *trpC2 pheA1amyE*::pHG17(*amyE*::*Hyper-spank resDE resD* NheI) | This study |
| ORB5059 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG17(*amyE*::*Hyper-spank resDE resD* NheI) | This study |
| ORB5060 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG13(*amyE*::*Hyper-spank resDE resD* V181A) | This study |
| ORB5061 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG18(*amyE*::*Hyper-spank resDE resD* D183A) | This study |
| ORB5062 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG14(*amyE*::*Hyper-spank resDE resD* V191A) | This study |
| ORB5063 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG15(*amyE*::*Hyper-spank resDE resD* W192A) | This study |
| ORB5064 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG16(*amyE*::*Hyper-spank resDE resD* Q193A) | This study |
| ORB5065 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG17(*amyE*::*Hyper-spank resDE resD* NheI) SPβ*c2del2*::Tn*917*::pML107 (*hmp-lacZ*) | This study |
| ORB5066 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG17(*amyE*::*Hyper-spank resDE resD* NheI) SPβ*c2del2*::Tn*917*::pMMN392 (*nasD-lacZ*) | This study |
| ORB5067 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG17(*amyE*::*Hyper-spank resDE resD* NheI) SPβ*c2del2*::Tn*917*::pMMN288 (*fnr-lacZ*) | This study |
| ORB5068 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG13(*amyE*::*Hyper-spank resDE resD* V181A) SPβ*c2del2*::Tn*917*::pML107 (*hmp-lacZ*) | This study |
| ORB5069 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG13(*amyE*::*Hyper-spank resDE resD* V181A) SPβ*c2del2*::Tn*917*::pMMN392 (*nasD-lacZ*) | This study |
| ORB5070 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG13(*amyE*::*Hyper-spank resDE resD* V181A) SPβ*c2del2*::Tn*917*::pMMN288 (*fnr-lacZ*) | This study |

| | | |
|---|---|---|
| ORB5071 | *trpC2 pheA1* Δ*resDE::tet* *amyE*::pHG18(*amyE*::*Hyper-spank resDE resD* D183A) SPβ*c2del2*::Tn*917*::pML107 (*hmp-lacZ*) | This study |
| ORB5072 | *trpC2 pheA1* Δ*resDE::tet* *amyE*::pHG18(*amyE*::*Hyper-spank resDE resD* D183A) SPβ*c2del2*::Tn*917*::pMMN392 (*nasD-lacZ*) | This study |
| ORB5073 | *trpC2 pheA1* Δ*resDE::tet* *amyE*::pHG18(*amyE*::*Hyper-spank resDE resD* D183A) SPβ*c2del2*::Tn*917*::pMMN288 (*fnr-lacZ*) | This study |
| ORB5074 | *trpC2 pheA1* Δ*resDE::tet* *amyE*::pHG15(*amyE*::*Hyper-spank resDE resD* W192A) SPβ*c2del2*::Tn*917*::pML107 (*hmp-lacZ*) | This study |
| ORB5075 | *trpC2 pheA1* Δ*resDE::tet* *amyE*::pHG15(*amyE*::*Hyper-spank resDE resD* W192A) SPβ*c2del2*::Tn*917*::pMMN392 (*nasD-lacZ*) | This study |
| ORB5076 | *trpC2 pheA1* Δ*resDE::tet* *amyE*::pHG15(*amyE*::*Hyper-spank resDE resD* W192A) SPβ*c2del2*::Tn*917*::pMMN288 (*fnr-lacZ*) | This study |
| ORB5101 | *trpC2 pheA1* Δ*resDE::tet* *amyE*::pHG14(*amyE*::*Hyper-spank resDE resD* V191A) SPβ*c2del2*::Tn*917*::pML107 (*hmp-lacZ*) | This study |
| ORB5102 | *trpC2 pheA1* Δ*resDE::tet* *amyE*::pHG14(*amyE*::*Hyper-spank resDE resD* V191A) SPβ*c2del2*::Tn*917*::pMMN392 (*nasD-lacZ*) | This study |
| ORB5103 | *trpC2 pheA1* Δ*resDE::tet* *amyE*::pHG14(*amyE*::*Hyper-spank resDE resD* V191A) SPβ*c2del2*::Tn*917*::pMMN288 (*fnr-lacZ*) | This study |
| ORB5104 | *trpC2 pheA1* Δ*resDE::tet* *amyE*::pHG16(*amyE*::*Hyper-spank resDE resD* Q193A) SPβ*c2del2*::Tn*917*::pML107 (*hmp-lacZ*) | This study |
| ORB5105 | *trpC2 pheA1* Δ*resDE::tet* *amyE*::pHG16(*amyE*::*Hyper-spank resDE resD* Q193A) SPβ*c2del2*::Tn*917*::pMMN392 (*nasD-lacZ*) | This study |
| ORB5106 | *trpC2 pheA1* Δ*resDE::tet* *amyE*::pHG16(*amyE*::*Hyper-spank resDE* | This study |

| | | |
|---|---|---|
| | *resD* Q193A) | |
| | SPβ*c2del2*::Tn*917*::pMMN288 (*fnr-lacZ*) | |
| ORB5108 | *trpC2 pheA1amyE*::pHG19(*amyE*::*Hyper-spank resDE resD* Y194A) | This study |
| ORB5109 | *trpC2 pheA1amyE*::pHG20(*amyE*::*Hyper-spank resDE resD* E195A) | This study |
| ORB5111 | *trpC2 pheA1amyE*::pHG22(*amyE*::*Hyper-spank resDE resD* F197A) | This study |
| ORB5112 | *trpC2 pheA1amyE*::pHG23(*amyE*::*Hyper-spank resDE resD* G198A) | This study |
| ORB5113 | *trpC2 pheA1amyE*::pHG24(*amyE*::*Hyper-spank resDE resD* D199A) | This study |
| ORB5114 | *trpC2 pheA1amyE*::pHG25(*amyE*::*Hyper-spank resDE resD* L200A) | This study |
| ORB5121 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG19(*amyE*::*Hyper-spank resDE resD* Y194A) | This study |
| ORB5122 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG20(*amyE*::*Hyper-spank resDE resD* E195A) | This study |
| ORB5124 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG22(*amyE*::*Hyper-spank resDE resD* F197A) | This study |
| ORB5125 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG23(*amyE*::*Hyper-spank resDE resD* G198A) | This study |
| ORB5126 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG24(*amyE*::*Hyper-spank resDE resD* D199A) | This study |
| ORB5127 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG25(*amyE*::*Hyper-spank resDE resD* L200A) | This study |
| ORB5140 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG19(*amyE*::*Hyper-spank resDE resD* Y194A) | This study |
| | SPβ*c2del2*::Tn*917*::pML107 (*hmp-lacZ*) | |
| ORB5141 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG19(*amyE*::*Hyper-spank resDE resD* Y194A) | This study |
| | SPβ*c2del2*::Tn*917*::pMMN392 (*nasD-lacZ*) | |
| ORB5142 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG19(*amyE*::*Hyper-spank resDE resD* Y194A) | This study |
| | SPβ*c2del2*::Tn*917*::pMMN288 (*fnr-lacZ*) | |
| ORB5143 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG20(*amyE*::*Hyper-spank resDE* | This study |

| | | |
|---|---|---|
| | *resD* E195A)<br>SPβ*c2del2*::Tn*917*::pML107 (*hmp-lacZ*) | |
| ORB5144 | *trpC2 pheA1* Δ*resDE::tet*<br>*amyE*::pHG20(*amyE*::*Hyper-spank resDE*<br>*resD* E195A)<br>SPβ*c2del2*::Tn*917*::pMMN392 (*nasD-lacZ*) | This study |
| ORB5145 | *trpC2 pheA1* Δ*resDE::tet*<br>*amyE*::pHG20(*amyE*::*Hyper-spank resDE*<br>*resD* E195A)<br>SPβ*c2del2*::Tn*917*::pMMN288 (*fnr-lacZ*) | This study |
| ORB5149 | *trpC2 pheA1* Δ*resDE::tet*<br>*amyE*::pHG22(*amyE*::*Hyper-spank resDE*<br>*resD* F197A)<br>SPβ*c2del2*::Tn*917*::pML107 (*hmp-lacZ*) | This study |
| ORB5150 | *trpC2 pheA1* Δ*resDE::tet*<br>*amyE*::pHG22(*amyE*::*Hyper-spank resDE*<br>*resD* F197A)<br>SPβ*c2del2*::Tn*917*::pMMN392 (*nasD-lacZ*) | This study |
| ORB5151 | *trpC2 pheA1* Δ*resDE::tet*<br>*amyE*::pHG22(*amyE*::*Hyper-spank resDE*<br>*resD* F197A)<br>SPβ*c2del2*::Tn*917*::pMMN288 (*fnr-lacZ*) | This study |
| ORB5152 | *trpC2 pheA1* Δ*resDE::tet*<br>*amyE*::pHG23(*amyE*::*Hyper-spank resDE*<br>*resD* G198A)<br>SPβ*c2del2*::Tn*917*::pML107 (*hmp-lacZ*) | This study |
| ORB5153 | *trpC2 pheA1* Δ*resDE::tet*<br>*amyE*::pHG23(*amyE*::*Hyper-spank resDE*<br>*resD* G198A)<br>SPβ*c2del2*::Tn*917*::pMMN392 (*nasD-lacZ*) | This study |
| ORB5154 | *trpC2 pheA1* Δ*resDE::tet*<br>*amyE*::pHG23(*amyE*::*Hyper-spank resDE*<br>*resD* G198A)<br>SPβ*c2del2*::Tn*917*::pMMN288 (*fnr-lacZ*) | This study |
| ORB5155 | *trpC2 pheA1* Δ*resDE::tet*<br>*amyE*::pHG24(*amyE*::*Hyper-spank resDE*<br>*resD* D199A)<br>SPβ*c2del2*::Tn*917*::pML107 (*hmp-lacZ*) | This study |
| ORB5156 | *trpC2 pheA1* Δ*resDE::tet*<br>*amyE*::pHG24(*amyE*::*Hyper-spank resDE*<br>*resD* D199A)<br>SPβ*c2del2*::Tn*917*::pMMN392 (*nasD-lacZ*) | This study |
| ORB5157 | *trpC2 pheA1* Δ*resDE::tet*<br>*amyE*::pHG24(*amyE*::*Hyper-spank resDE*<br>*resD* D199A)<br>SPβ*c2del2*::Tn*917*::pMMN288 (*fnr-lacZ*) | This study |

| | | |
|---|---|---|
| ORB5158 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG25(*amyE*::*Hyper-spank resDE resD* L200A) SPβ*c2del2*::Tn*917*::pML107 (*hmp-lacZ*) | This study |
| ORB5159 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG25(*amyE*::*Hyper-spank resDE resD* L200A) SPβ*c2del2*::Tn*917*::pMMN392 (*nasD-lacZ*) | This study |
| ORB5160 | *trpC2 pheA1* Δ*resDE::tet amyE*::pHG25(*amyE*::*Hyper-spank resDE resD* L200A) SPβ*c2del2*::Tn*917*::pMMN288 (*fnr-lacZ*) | This study |
| ORB 5616 | *trpC2 pheA1*sigA::pJB2 wt Spβ*c2del2*::Tn*917*::pML107(*hmp-lacZ*) | This study |
| ORB 5618 | *trpC2 pheA1*sigA::pJB2 wt Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | This study |
| ORB 5619 | *trpC2 pheA1*sigA::pJB2 wt Spβ*c2del2*::Tn*917*::pMNN288(*fnr-lacZ*) | This study |
| ORB 5620 | *trpC2 pheA1*sigA R355A Spβ*c2del2*::Tn*917*::pML107(*hmp-lacZ*) | This study |
| ORB 5622 | *trpC2 pheA1*sigA R355A wt Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | This study |
| ORB 5623 | *trpC2 pheA1*sigA R355A wt Spβ*c2del2*::Tn*917*::pMNN288(*fnr-lacZ*) | This study |
| ORB 5624 | *trpC2 pheA1*sigA R358A Spβ*c2del2*::Tn*917*::pML107(*hmp-lacZ*) | This study |
| ORB 5626 | *trpC2 pheA1*sigA R358A wt Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | This study |
| ORB 5627 | *trpC2 pheA1*sigA R358A wt Spβ*c2del2*::Tn*917*::pMNN288(*fnr-lacZ*) | This study |
| ORB 5628 | *trpC2 pheA1*sigA S361A Spβ*c2del2*::Tn*917*::pML107(*hmp-lacZ*) | This study |
| ORB 5630 | *trpC2 pheA1*sigA S361A wt Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | This study |
| ORB 5631 | *trpC2 pheA1*sigA S361A wt Spβ*c2del2*::Tn*917*::pMNN288(*fnr-lacZ*) | This study |
| ORB 5632 | *trpC2 pheA1*sigA R362A Spβ*c2del2*::Tn*917*::pML107(*hmp-lacZ*) | This study |
| ORB 5634 | *trpC2 pheA1*sigA R362A wt Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | This study |
| ORB 5635 | *trpC2 pheA1*sigA R362A wt Spβ*c2del2*::Tn*917*::pMNN288(*fnr-lacZ*) | This study |
| ORB 5636 | *trpC2 pheA1*sigA K364A Spβ*c2del2*::Tn*917*::pML107(*hmp-lacZ*) | This study |
| ORB 5638 | *trpC2 pheA1*sigA K364A wt Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | This study |

| | | |
|---|---|---|
| ORB 5639 | *trpC2 pheA1*sigA K364A wt Spβ*c2del2*::Tn*917*::pMNN288(*fnr-lacZ*) | This study |
| ORB 5640 | *trpC2 pheA1*sigA L366A Spβ*c2del2*::Tn*917*::pML107(*hmp-lacZ*) | This study |
| ORB 5642 | *trpC2 pheA1*sigA L366A wt Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | This study |
| ORB 5643 | *trpC2 pheA1*sigA L366A wt Spβ*c2del2*::Tn*917*::pMNN288(*fnr-lacZ*) | This study |
| ORB 5644 | *trpC2 pheA1*sigA D368A Spβ*c2del2*::Tn*917*::pML107(*hmp-lacZ*) | This study |
| ORB 5646 | *trpC2 pheA1*sigA D368A wt Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | This study |
| ORB 5647 | *trpC2 pheA1*sigA D368A wt Spβ*c2del2*::Tn*917*::pMNN288(*fnr-lacZ*) | This study |
| ORB5709 | *trpC2 pheA1amyE*::pHG27(*amyE*::*Hyper-spank resDE resD* R201A) | This study |
| ORB5710 | *trpC2 pheA1amyE*::pHG28(*amyE*::*Hyper-spank resDE resD* R211A) | This study |
| ORB 5713 | *trpC2 pheA1*Δ*resDE::tet amyE*::pHG27(*amyE*::*Hyper-spank resDE resD* R201A) | This study |
| ORB 5714 | *trpC2 pheA1*Δ*resDE::tet amyE*::pHG28(*amyE*::*Hyper-spank resDE resD* R211A) | This study |
| ORB 5716 | *trpC2 pheA1*Δ*resDE::tet amyE*::pHG27(*amyE*::*Hyper-spank resDE resD* R201A) Spβ*c2del2*::Tn*917*::pML107(*hmp-lacZ*) | This study |
| ORB 5718 | *trpC2 pheA1*Δ*resDE::tet amyE*::pHG27(*amyE*::*Hyper-spank resDE resD* R201A) Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | This study |
| ORB 5719 | *trpC2 pheA1*Δ*resDE::tet amyE*::pHG27(*amyE*::*Hyper-spank resDE resD* R201A) Spβ*c2del2*::Tn*917*::pMNN288(*fnr-lacZ*) | This study |
| ORB 5720 | *trpC2 pheA1*Δ*resDE::tet amyE*::pHG28(*amyE*::*Hyper-spank resDE resD* R211A) Spβ*c2del2*::Tn*917*::pML107(*hmp-lacZ*) | This study |
| ORB 5722 | *trpC2 pheA1*Δ*resDE::tet amyE*::pHG28(*amyE*::*Hyper-spank resDE resD* R211A) Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | This study |
| ORB 5723 | *trpC2 pheA1*Δ*resDE::tet amyE*::pHG28(*amyE*::*Hyper-spank resDE* | This study |

| | | |
|---|---|---|
| | *resD* R211A)<br>Spβ*c2del2*::Tn*917*::pMNN288(*fnr-lacZ*) | |
| ORB 5725 | *trpC2 pheA1amyE*::pHG29(*amyE*::*Hyper-spank resDE resD* D179A) | This study |
| ORB 5726 | *trpC2 pheA1amyE*::pHG30(*amyE*::*Hyper-spank resDE resD* E185A) | This study |
| ORB 5727 | *trpC2 pheA1amyE*::pHG31(*amyE*::*Hyper-spank resDE resD* E190A) | This study |
| ORB 5728 | *trpC2 pheA1amyE*::pHG32(*amyE*::*Hyper-spank resDE resD* D204A) | This study |
| ORB 5769 | *trpC2 pheA1 rpoA* Y263A K267A | This study |
| ORB 5774 | *trpC2 pheA1*Δ*resDE::tet amyE*::pHG29(*amyE*::*Hyper-spank resDE resD* D179A) | This study |
| ORB 5775 | *trpC2 pheA1*Δ*resDE::tet amyE*::pHG30(*amyE*::*Hyper-spank resDE resD* E185A) | This study |
| ORB 5776 | *trpC2 pheA1*Δ*resDE::tet amyE*::pHG31(*amyE*::*Hyper-spank resDE resD* E190A) | This study |
| ORB 5777 | *trpC2 pheA1*Δ*resDE::tet amyE*::pHG32(*amyE*::*Hyper-spank resDE resD* D204A) | This study |
| ORB 5790 | *trpC2 pheA1*Δ*resDE::tet amyE*::pHG29(*amyE*::*Hyper-spank resDE resD* D179A)<br>Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | This study |
| ORB 5791 | *trpC2 pheA1*Δ*resDE::tet amyE*::pHG29(*amyE*::*Hyper-spank resDE resD* D179A)<br>Spβ*c2del2*::Tn*917*::pMNN288(*fnr-lacZ*) | This study |
| ORB 5793 | *trpC2 pheA1*Δ*resDE::tet amyE*::pHG30(*amyE*::*Hyper-spank resDE resD* E185A)<br>Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | This study |
| ORB 5794 | *trpC2 pheA1*Δ*resDE::tet amyE*::pHG30(*amyE*::*Hyper-spank resDE resD* E185A)<br>Spβ*c2del2*::Tn*917*::pMNN288(*fnr-lacZ*) | This study |
| ORB 5796 | *trpC2 pheA1*Δ*resDE::tet amyE*::pHG31(*amyE*::*Hyper-spank resDE resD* E190A)<br>Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | This study |
| ORB 5797 | *trpC2 pheA1*Δ*resDE::tet amyE*::pHG31(*amyE*::*Hyper-spank resDE resD* E190A) | This study |

| | | |
|---|---|---|
| | Spβ*c2del2*::Tn*917*::pMNN288(*fnr-lacZ*) | |
| ORB 5799 | *trpC2 pheA1*Δ*resDE::tet amyE*::pHG32(*amyE*::*Hyper-spank resDE resD* D204A) | This study |
| | Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | |
| ORB 5800 | *trpC2 pheA1*Δ*resDE::tet amyE*::pHG32(*amyE*::*Hyper-spank resDE resD* D204A) | This study |
| | Spβ*c2del2*::Tn*917*::pMNN288(*fnr-lacZ*) | This study |
| ORB 5842 | *trpC2 pheA1* sig A K356E | This study |
| | Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | |
| ORB 5843 | *trpC2 pheA1* sig A H359A | This study |
| | Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | |
| ORB 5844 | *trpC2 pheA1* sig A K356A | This study |
| | Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | |
| ORB 5845 | *trpC2 pheA1* sig A H359R | This study |
| | Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | |
| ORB 5846 | *trpC2 pheA1* sig A K356E | This study |
| | Spβ*c2del2*::Tn*917*::pMNN288(*fnr-lacZ*) | |
| ORB 5847 | *trpC2 pheA1* sig A H359A | This study |
| | Spβ*c2del2*::Tn*917*::pMNN288(*fnr-lacZ*) | |
| ORB 5848 | *trpC2 pheA1* sig A K356A | This study |
| | Spβ*c2del2*::Tn*917*::pMNN288(*fnr-lacZ*) | |
| ORB 5849 | *trpC2 pheA1* sig A H359R | This study |
| | Spβ*c2del2*::Tn*917*::pMNN288(*fnr-lacZ*) | |
| ORB 5923 | *trpC2 pheA1* Δ*resDE::tet rpoA* K267A | This study |
| ORB 5952 | *trpC2 pheA1 rpoA* Y263A K267A | This study |
| | Spβ*c2del2*::Tn*917*::pML107(*hmp-lacZ*) | |
| ORB 5954 | *trpC2 pheA1 rpoA* Y263A K267A | This study |
| | Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | |
| ORB5955 | *trpC2 pheA1 rpoA* Y263A K267A | This study |
| | Spβ*c2del2*::Tn*917*::pMNN288(*fnr-lacZ*) | |
| ORB6023 | *trpC2 pheA1 rpoA* E254A sig A K356E | This study |
| ORB6024 | *trpC2 pheA1 rpoA* E254A sigA H359R | This study |
| ORB6116 | *rpoA*(*K267A*) $His_{10}$ *rpoC* | This study |
| ORB6148 | *trpC2 pheA1 rpoA* E254A sig A K356E | This study |
| | Spβ*c2del2*::Tn*917*::pMNN288(*fnr-lacZ*) | |
| ORB6151 | *trpC2 pheA1 rpoA* E254A sigA H359R | This study |
| | Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | |
| ORB6152 | *trpC2 pheA1 rpoA* E254A sigA H359R | This study |
| | Spβ*c2del2*::Tn*917*::pMNN288(*fnr-lacZ*) | |
| ORB6169 | *trpC2 pheA1 rpoA* E254K | This study |
| ORB6172 | *trpC2 pheA1 rpoA* E255A sigA K356E | This study |
| ORB6173 | *trpC2 pheA1 rpoA* E255A sigA H359R | This study |
| ORB6201 | *trpC2 pheA1amyE*::pHG47(*amyE*::*Hyper-spank resDE resD* D199R) | This study |

| | | |
|---|---|---|
| ORB6474 | *trpC2 pheA1 rpoA* E254K Spβ*c2del2*::Tn*917*::pML107(*hmp-lacZ*) | This study |
| ORB6475 | *trpC2 pheA1 rpoA* E254K Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | This study |
| ORB6476 | *trpC2 pheA1 rpoA* E254K Spβ*c2del2*::Tn*917*::pMMN288(*fnr-lacZ*) | This study |
| ORB6478 | *trpC2 pheA1 rpoA* E255A sigA K356E Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | This study |
| ORB6480 | *trpC2 pheA1 rpoA* E255A sigA H359R Spβ*c2del2*::Tn*917*::pML107(*hmp-lacZ*) | This study |
| ORB6481 | *trpC2 pheA1 rpoA* E255A sigA H359R Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | This study |
| ORB6482 | *trpC2 pheA1 rpoA* E255A sigA H359R Spβ*c2del2*::Tn*917*::pMMN288(*fnr-lacZ*) | This study |
| ORB6486 | *trpC2 pheA1 rpoA* E255K sigA K356E Spβ*c2del2*::Tn*917*::pML107(*hmp-lacZ*) | This study |
| ORB6487 | *trpC2 pheA1 rpoA* E255K sigA K356E Spβ*c2del2*::Tn*917*::pMNN392(*nasD-lacZ*) | This study |
| ORB6488 | *trpC2 pheA1 rpoA* E255K sigA K356E Spβ*c2del2*::Tn*917*::pMMN288(*fnr-lacZ*) | This study |
| Plasmids | | |
| pDG148 | *E. coli*/*B. subtilis* shuttle plasmid; $Amp^r$ $neo^r$ | (Stragier *et al.*, 1988) |
| PDR111 | Integration plasmid with P*spank-hy*, $Amp^r$ $Spc^r$ | (Britton *et al.*, 2002) |
| pTYB2 | Expression vector with self-cleavable intein tag | New England Biolabs |
| pTYB4 | Expression vector with self-cleavable intein tag | New England Biolabs |
| pAG58 | Integration plasmid with P*spac*, $Amp^r$ $Cm^r$ | (Youngman *et al.*, 1989) |
| pMMN543 | pDG148 carrying *resDresE** | This study |
| pMMN553 | pDR111 carrying *resDresE** | This study |
| pHG9 | pAG58 carrying *rpoA* (Y263A) | This study |
| pHG12 | pDR111 carrying *resE'* | This study |
| pHG13 | pDR111 carrying *resD*(V181A)*resE* | This study |
| pHG14 | pDR111 carrying *resD*(V191A)*resE* | This study |
| pHG15 | pDR111 carrying *resD*(W192A)*resE* | This study |
| pHG16 | pDR111 carrying *resD*(Q193A)*resE* | This study |
| pHG17 | pDR111 carrying *resDresE* | This study |
| pHG18 | pDR111 carrying *resD*(D183A)*resE* | This study |
| pHG19 | pDR111 carrying *resD*(Y194A)*resE* | This study |
| pHG20 | pDR111 carrying *resD*(E195A)*resE* | This study |
| pHG22 | pDR111 carrying *resD*(F197A)*resE* | This study |
| pHG23 | pDR111 carrying *resD*(G198A)*resE* | This study |
| pHG24 | pDR111 carrying *resD*(D199A)*resE* | This study |
| pHG25 | pDR111 carrying *resD*(L200A)*resE* | This study |
| pHG26 | pDR111 carrying *resD*(F196A)*resE* | This study |

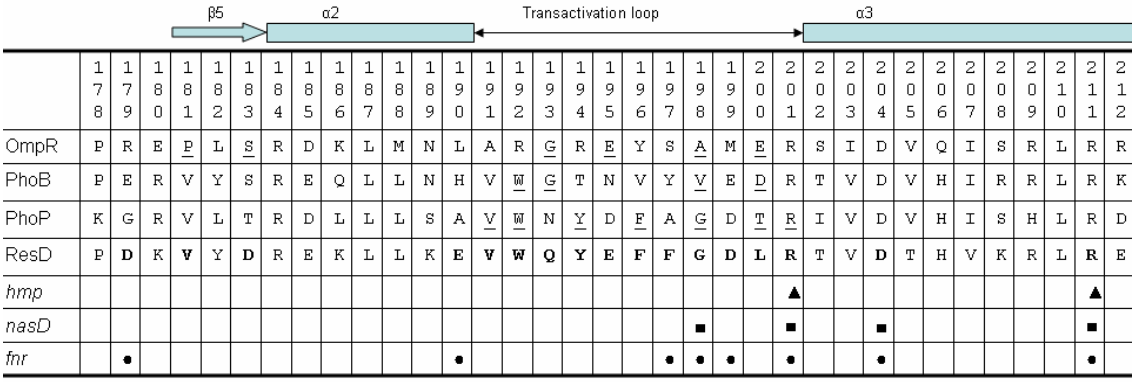
| | | |
|---|---|---|
| pHG27 | pDR111 carrying *resD*(R201A)*resE* | This study |
| pHG28 | pDR111 carrying *resD*(R211A)*resE* | This study |
| pHG29 | pDR111 carrying *resD*(D179A)*resE* | This study |
| pHG30 | pDR111 carrying *resD*(E185A)*resE* | This study |
| pHG31 | pDR111 carrying *resD*(E190A)*resE* | This study |
| pHG32 | pDR111 carrying *resD*(D204A)*resE* | This study |
| pHG33 | pAG58 carrying *rpoA* (Y263A K267A) | This study |
| pHG34 | pTYB4 carrying *resD*(F197A) | This study |
| pHG35 | pTYB4 carrying *resD*(R201A) | This study |
| pHG36 | pTYB4 carrying *resD*(G198A) | This study |
| pHG37 | pTYB4 carrying *resD*(D199A) | This study |
| pHG38 | pTYB4 carrying *resD*(R211A) | This study |
| pHG44 | pAG58 carrying *rpoA* (E254K) | This study |
| pHG47 | pDR111 carrying *resD*(D199R)*resE* | This study |
| pHG73 | pTYB2 carrying *rpoA* K267A | This study |

***There is no additional NheI site in *resE*.**

**TABLE 4.2 Oligonucleotides**

| Oligonucleotide | Sequence (5' to 3') | purpose |
|---|---|---|
| oHG-1 | GGCATCATCAGAGCAAGCAAAAT | *nasD* template |
| oHG-2 | CTCGAAGAAAGTCACGTTGT | *fnr* DNase I footpring |
| oHG-3 | TCTGTTCGTTCTGCCAACTGCTTAAA | pHG9 |
| oHG-4 | TTTAAGCAGTTGGCAGAACGAACAGA | pHG9 |
| oHG-5 | GTTAGTCCGTTTTTGCTA | *hmp* DNase I footpring |
| oHG-6 | CCTTTCGAAAAGATGTAT | *hmp* DNase I footpring |
| oHG-7 | AAATGCCCGGTTTTAAGG | *nasD* template and DNase I footpring |
| oHG-9 | GGATGTATTGGCAGGAAAG | *fnr* DNase I footpring |
| oHG-10 | CCGCTCCTTATCAAATGT | *nasD* DNase I footpring |
| oHG-13 | CACAGCTAGCGAATAAAG | pHG17 |
| oHG-14 | CTTTATTCGCTAGCTGTG | pHG17 |
| oHG-15 | TGACAAGGCATATGATCGGGAA | pHG13 |
| oHG-16 | TTCCCGATCATATGCCTTGTCA | pHG13 |
| oHG-17 | AAGGTGTATGCTCGCGAAAAGCTT | pHG18 |
| oHG-18 | AAGCTTTTCGCGAGCATACACCTT | pHG18 |
| oHG-19 | CTTCTGAAAGAGGCCTGGCAATAT | pHG14 |
| oHG-20 | ATATTGCCAGGCCTCTTTCAGAAG | pHG14 |
| oHG-21 | GAAAGAAGTGGCGCAATATGAG | pHG15 |
| oHG-22 | CTCATATTGCGCCACTTCTTTC | pHG15 |
| oHG-23 | GAAGTGTGGGCATATGAGTTT | pHG16 |
| oHG-24 | AAACTCATATGCCCACACTTC | pHG16 |
| oHG-25 | GTGTGGCAAGCTGAATTCTTTGGTGAT | pHG19 |
| oHG-26 | ATCACCAAAGAATTCAGCTTGCCACAC | pHG19 |
| oHG-27 | GTGTGGCAATACGCGTTTTTTGGTGA | pHG20 |
| oHG-28 | TCACCAAAAAACGCGTATTGCCACAC | pHG20 |
| oHG-29 | GGCAATATGAGGCCTTTGGTGATTTA | pHG26 |
| oHG-30 | TAAATCACCAAAGGCCTCATATTGCC | pHG26 |
| oHG-31 | AATATGAGTTTGCCGGCGATTTACGGACA | pHG22 |
| oHG-32 | TGTCCGTAAATCGCCGGCAAACTCATATT | pHG22 |

| | | |
|---|---|---|
| oHG-33 | GAGTTTTTTGCAGATCTACGGAC AGTG | pHG23 |
| oHG-34 | CACTGTCCGTAGATCTGCAAAAA ACTC | pHG23 |
| oHG-35 | GAGTTTTTTGGAGCTCTACGGAC AGTG | pHG24 |
| oHG-36 | CACTGTCCGTAGAGCTCCAAAAA ACTC | pHG24 |
| oHG-37 | TTTTTTGGTGATGCACGGACAGTG GA | pHG25 |
| oHG-38 | TCCACTGTCCGTGCATCACCAAA AAA | pHG25 |
| oHG-39 | TTTTGGTGATCTCGCGACAGTG | pHG27 |
| oHG-40 | CACTGTCGCGAGATCACCAAAA | pHG27 |
| oHG-41 | AAACGCTTGGCGGAGAAG | pHG28 |
| oHG-42 | CTTCTCCGCCAAGCGTTT | pHG28 |
| oHG-43 | AAAACGCCGGCCAAGGTGTAT | pHG29 |
| oHG-44 | ATACACCTTGGCCGGCGTTTT | pHG29 |
| oHG-45 | TGATCGGGCAAAACTTCTG | pHG30 |
| oHG-46 | CAGAAGTTTTGCCCGATCA | pHG30 |
| oHG-47 | CTTCTGAAAGCAGTGTGGC | pHG31 |
| oHG-48 | GCCACACTGCTTTCAGAAG | pHG31 |
| oHG-49 | ACAGTGGCAACTCACGT | pHG32 |
| oHG-50 | ACGTGAGTTGCCACTGT | pHG32 |
| oHG-51 | TCTGTTCGTTCTGCCAACTGCTTA GC | pHG33 |
| oHG-52 | GCTAAGCAGTTGGCAGAACGAAC AGA | pHG33 |
| oHG-53 | CCTCTTATTCCATGGCCAATACA | pHG44 |
| oHG-64 | CAGAAAGATCTAATTCTTTAATTG TC | pHG44 |
| oHG-69 | TTTTTTGGTAGACTACGGACATTG | pHG47 |
| oHG-70 | CACTGTCCGTAGTCTACCAAAAA A | pHG47 |




| | 178 | 179 | 180 | 181 | 182 | 183 | 184 | 185 | 186 | 187 | 188 | 189 | 190 | 191 | 192 | 193 | 194 | 195 | 196 | 197 | 198 | 199 | 200 | 201 | 202 | 203 | 204 | 205 | 206 | 207 | 208 | 209 | 210 | 211 | 212 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OmpR | P | R | E | P | L | S | R | D | K | L | M | N | L | A | R | G | R | E | Y | S | A | M | E | R | S | I | D | V | Q | I | S | R | L | R | R |
| PhoB | P | E | R | V | Y | S | R | E | Q | L | L | N | H | V | W | G | T | N | V | Y | V | E | D | R | T | V | D | V | H | I | R | R | L | R | K |
| PhoP | K | G | R | V | L | T | R | D | L | L | L | S | A | V | W | N | Y | D | F | A | G | D | T | R | I | V | D | V | H | I | S | H | L | R | D |
| ResD | P | **D** | K | **V** | Y | **D** | R | E | K | L | L | K | **E** | **V** | **W** | **Q** | **Y** | **E** | **F** | **F** | **G** | **D** | **L** | **R** | T | V | **D** | T | H | V | K | R | L | **R** | E |
| *hmp* | | | | | | | | | | | | | | | | | | | | | | | | ▲ | | | | | | | | | | ▲ | |
| *nasD* | | | | | | | | | | | | | | | | | | | | | ■ | | | ■ | | | ■ | | | | | | | ■ | |
| *fnr* | | ● | | | | | | | | | | | ● | | | | | | | ● | ● | ● | | ● | | | ● | | | | | | | ● | |

FIG. 4.1. Alignment of ResD sequence with PhoB, OmpR and PhoP sequence. Possible secondary structure of ResD is assigned at the top of the figure. The residues substituted with alanine are indicated in bold letters. The numbers refer to the residue of ResD. Amino acid residues in PhoB, OmpR and PhoP that affect transcriptional activation likely through interaction with RNAP are indicated by underlines (Kato *et al.*, 1996; Makino *et al.*, 1996; Chen *et al.*, 2004) . Residues in ResD that reduce expression more than 50% are marked by ▲(*hmp* expression); ■(*nasD* expression); ●(*fnr* expression).

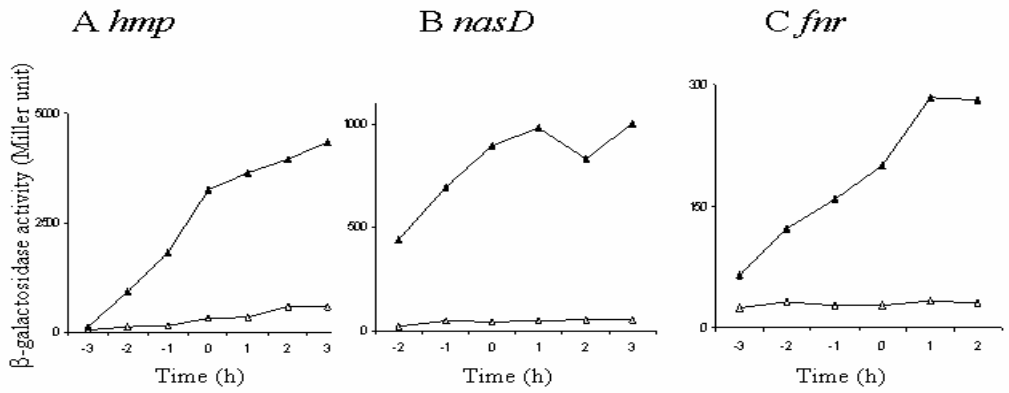



FIG. 4.2. Expression of *hmp-lacZ* (A), *nasD-lacZ* (B) and *fnr-lacZ* (C). Δ*resDE* strains carrying the IPTG-inducible wild-type *resDE* genes at the *amyE* locus were grown under anaerobic conditions in 2xYT +1% glucose + 0.2% $KNO_3$ in the absence of IPTG (Δ) or in the presence of 1mM IPTG (▲). Time zero indicates the end of exponential growth phase.

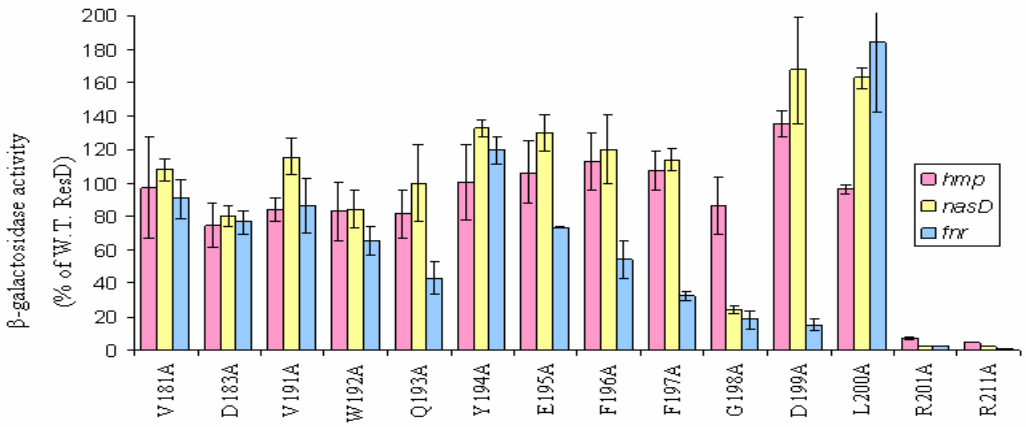



FIG. 4.3. Effect of single alanine substitutions of ResD on expression of *hmp*, *nasD*, and *fnr*. Cells were grown under anaerobic conditions in 2xYT+1% glucose+0.2% $KNO_3$ +1mM IPTG. Experiments were repeated two to three times, and the average of maximal activities around T1 (1 h after the end of exponential growth) is expressed as percentage of the activity in cells where wild-type ResD was expressed from the IPTG-inducible promoter.

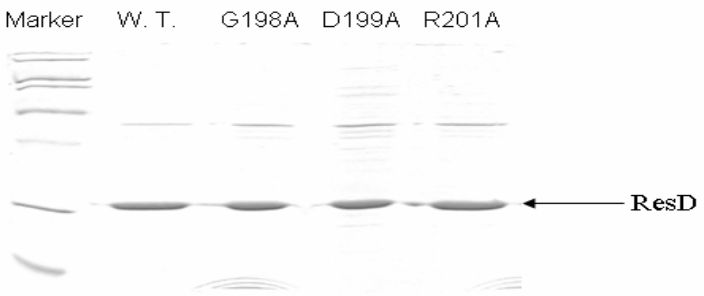



FIG. 4.4. ResD overexpressed and purified from *E. coli*. 5 μg protein samples were loaded onto 12% SDS-polyacrylamide gel. Sizes of molecular mass marker are 200, 116.25, 97.4, 66.2, 45, 31, and 21.5 KD.

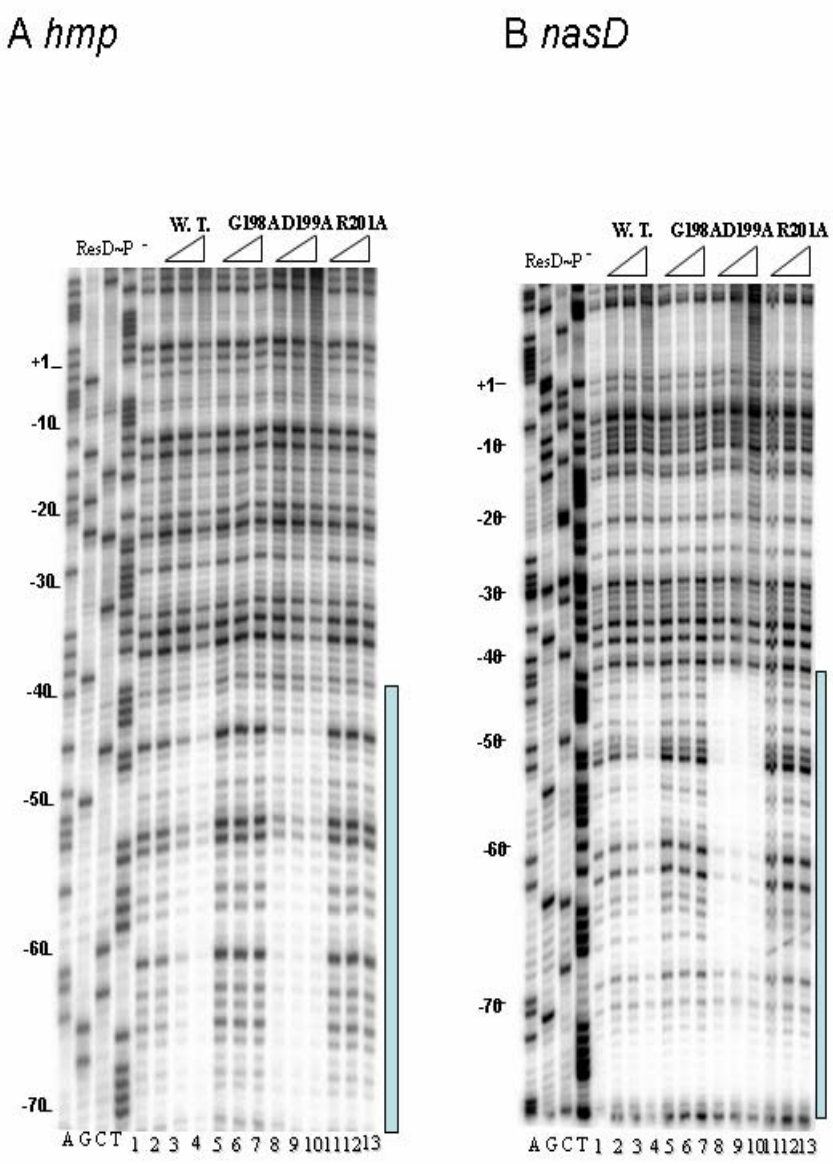



FIG. 4.5. DNase I footprinting analysis of the *hmp* (A) and *nasD* (B) promoters. The labeled coding strands of each promoter fragment were used for binding reactions with ResD phosphorylated with ResE. ResD~P concentration (μM) used in the each reaction was, 1, 2, 4 μM. Protected regions from DNase I digestion are marked by solid lines. Dideoxynucleotide sequencing reactions using the same primer are also shown and nucleotide positions are marked relative to the transcription start site.

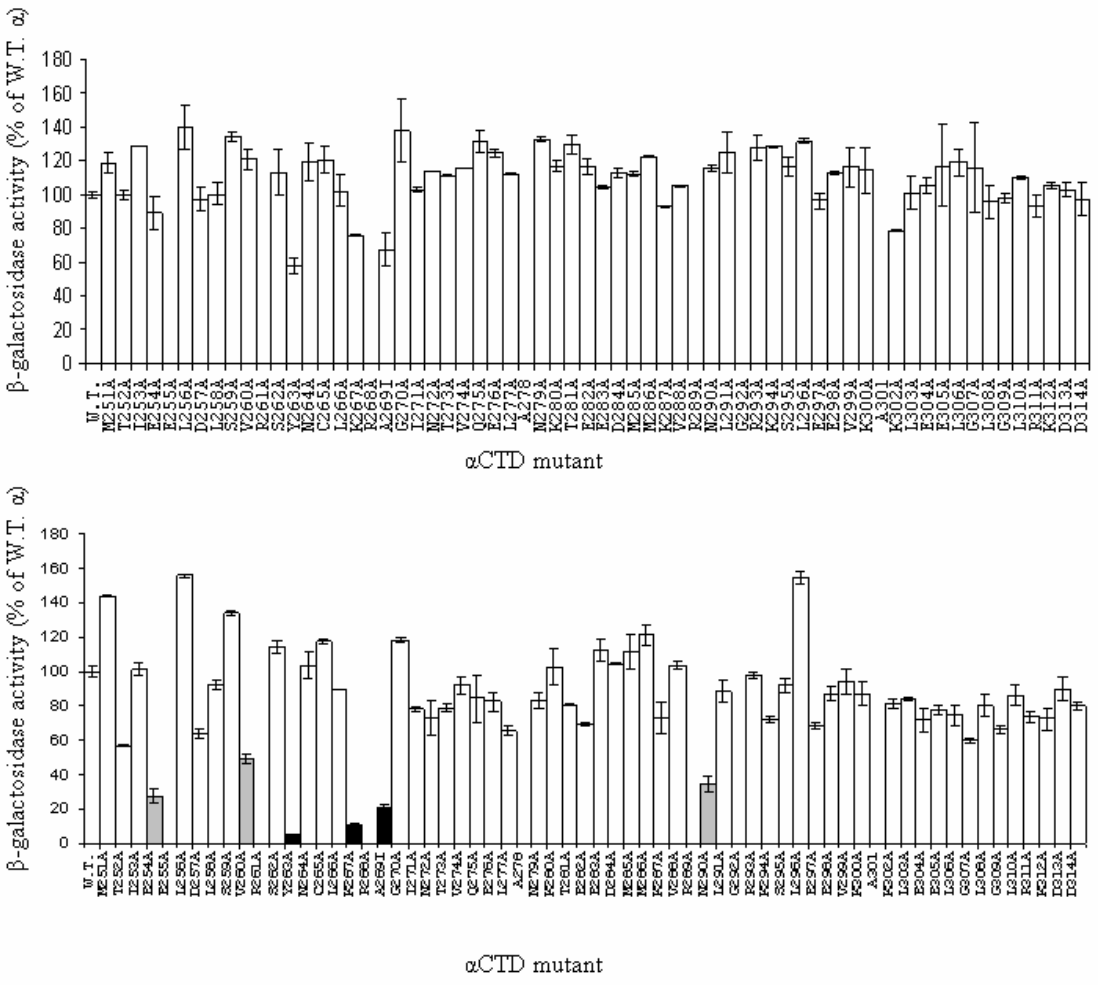



FIG. 4.6. Effects of amino acid substitutions of αCTD on ResDE-dependent gene expression. Cells were grown in 2xYT +1%glucose +0.2%$KNO_3$ under anaerobic conditions. Black bars indicate substitutions that reduced gene expression to less than 20% of wild-type level, and grey bars indicate substitutions that reduced gene expression to 30-50%. Experiments were repeated two or three times, and the average of maximal activities around T1(1 h after the end of exponential growth) is expressed as percentage of the activity in cells carrying the wild-type *rpoA*.

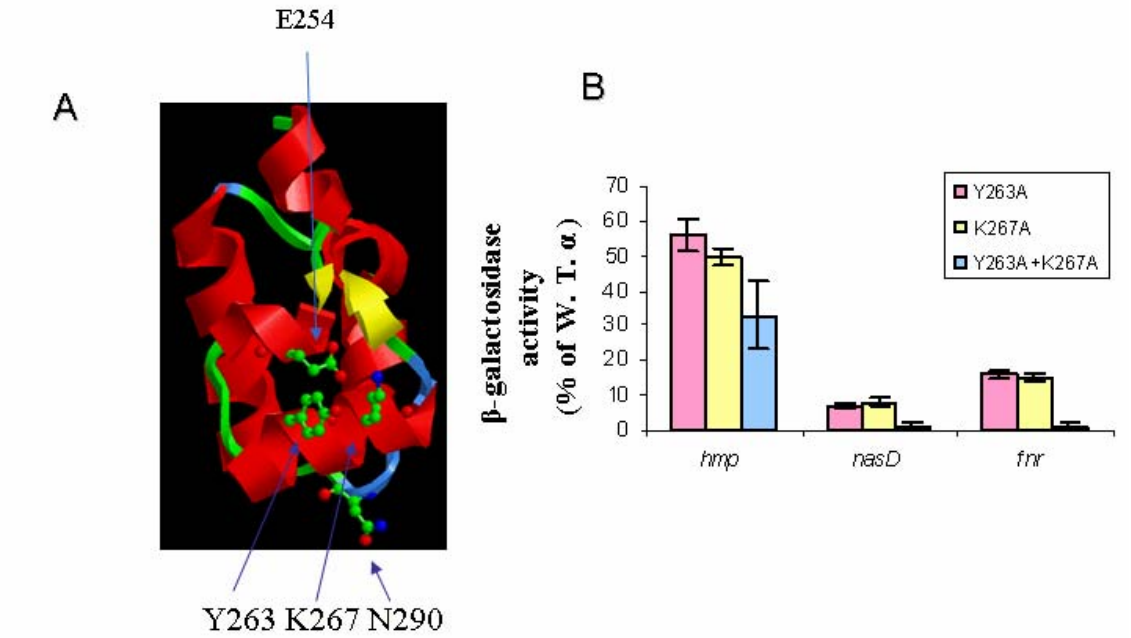



FIG. 4.7. (A) The structure of *B. subtilis* αCTD. (B) Effects of amino acid substitutions of αCTD on ResD-dependent gene expression under anaerobic conditions. β-Galactosidase activity was determined from anaerobic cultures as shown in Fig. 4.3.

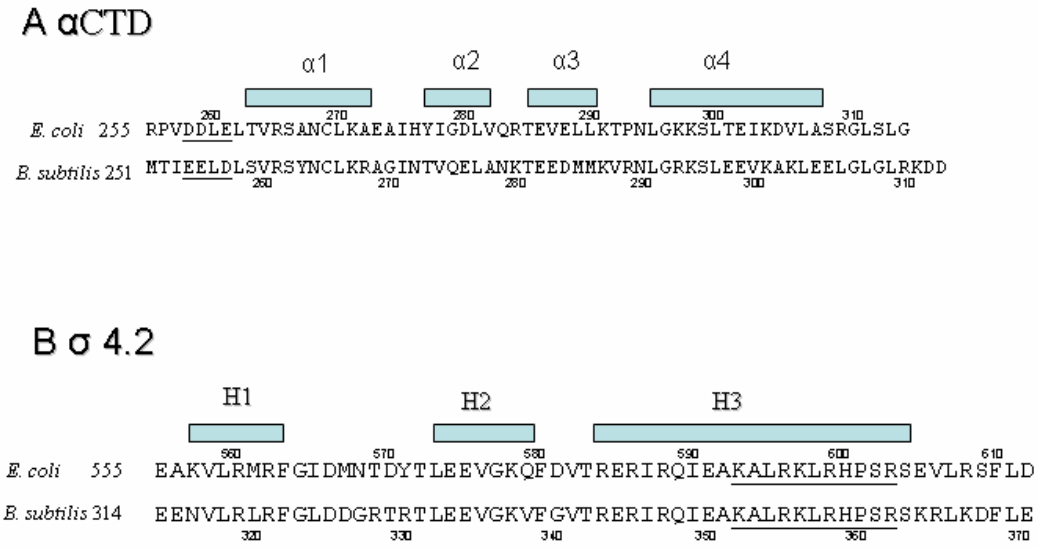



FIG. 4.8. Alignments of αCTD (A) and σ region 4.2 (B) sequences from *E. coli* and *B. subtilis* and their secondary structure assignment. The αCTD residues 258-261 in *E. coli* and 254-257 in *B. subtilis*, and the σ residues 593-603 in *E. coli* and 352-362 in *B. subtilis* are indicated by underlines.

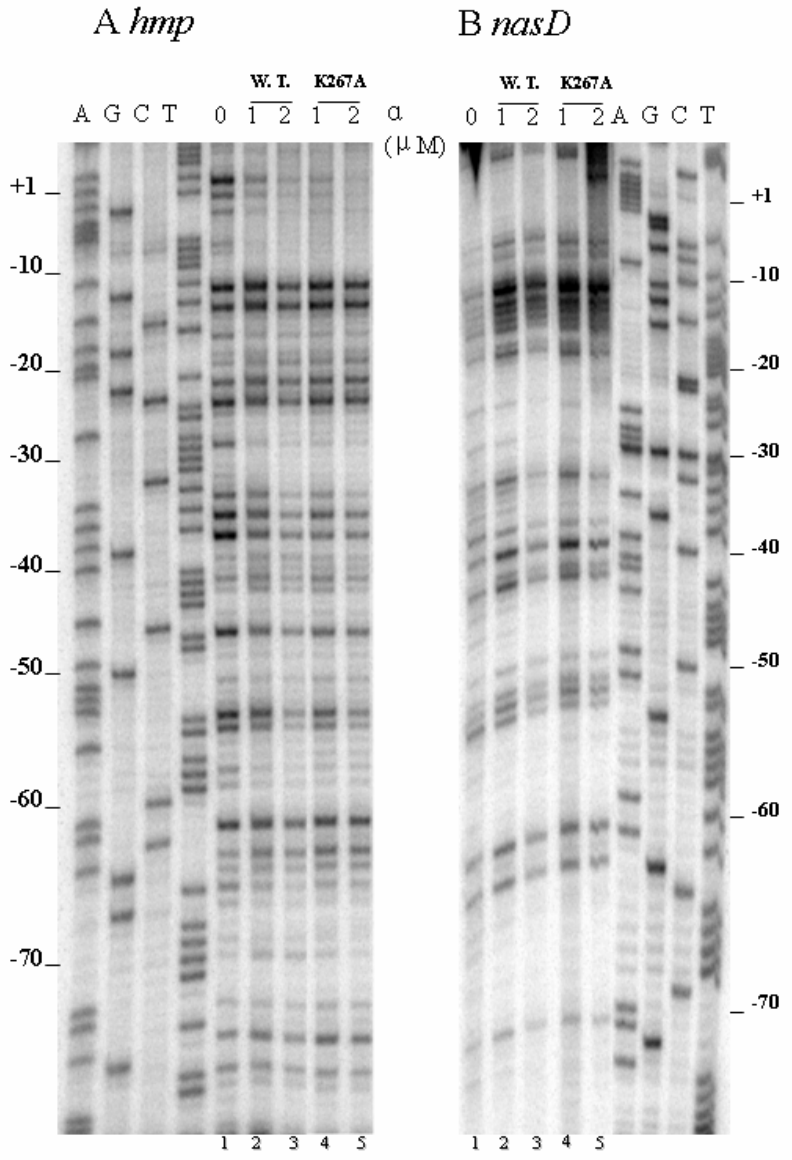



FIG. 4.9. DNase I footprinting analysis of *hmp* (A) and *nasD* (B) promoters. The labeled coding strands of each promoter fragment were used for binding reactions with α. Dideoxynucleotide sequencing reactions using the same primer are also shown and nucleotide positions are marked relative to the transcription start site.

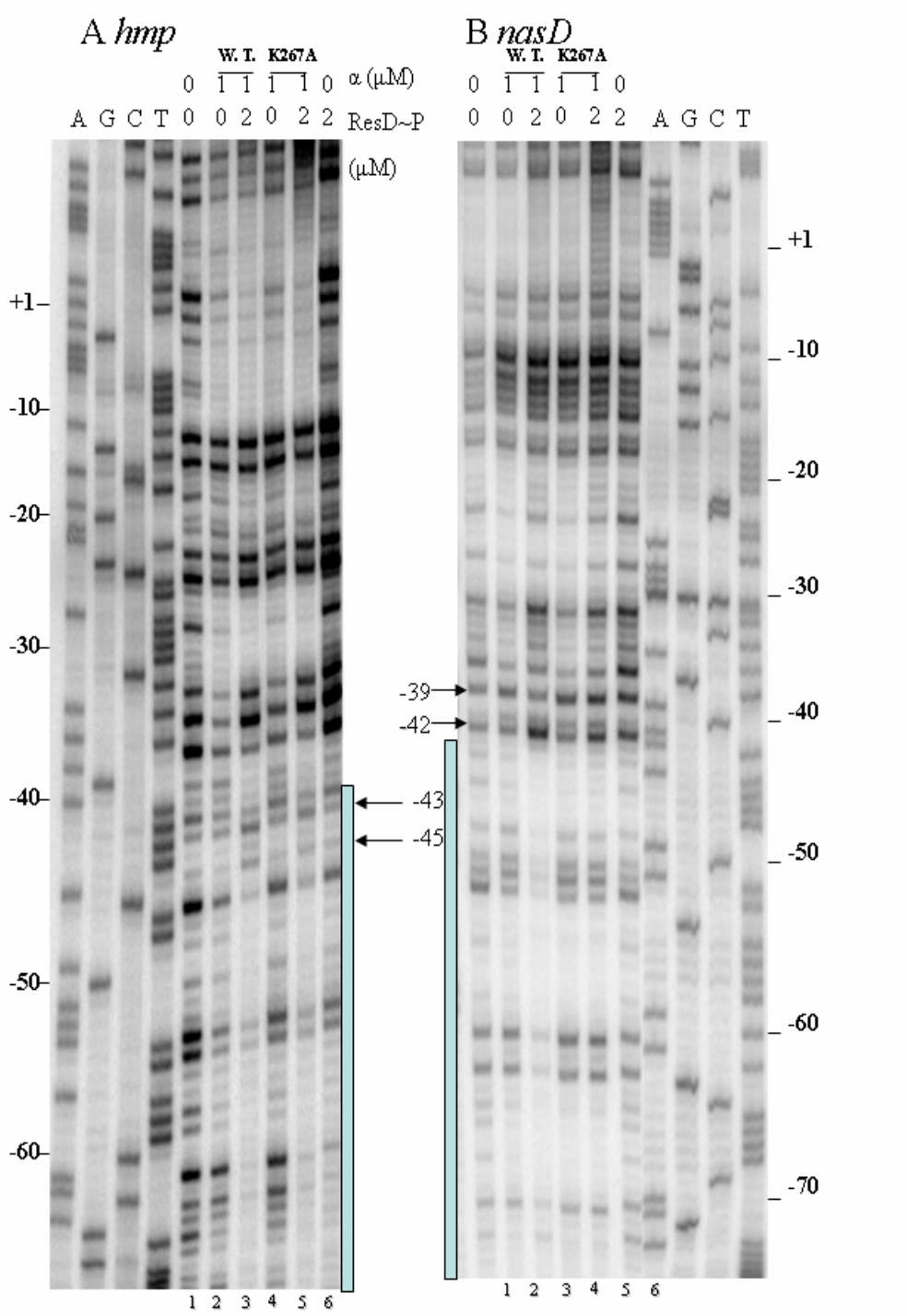



FIG. 4.10. DNase I footprinting analysis of *hmp* (A) and *nasD* (B) promoters. The labeled coding strands of each promoter fragment were used for binding reactions with ResD phosphorylated with ResE. α was added to reaction. Regions protected by ResD~P from DNase I digestion are marked by solid lines. Dideoxynucleotide sequencing reactions using the same primer are also shown and nucleotide positions are marked relative to the transcription start site.

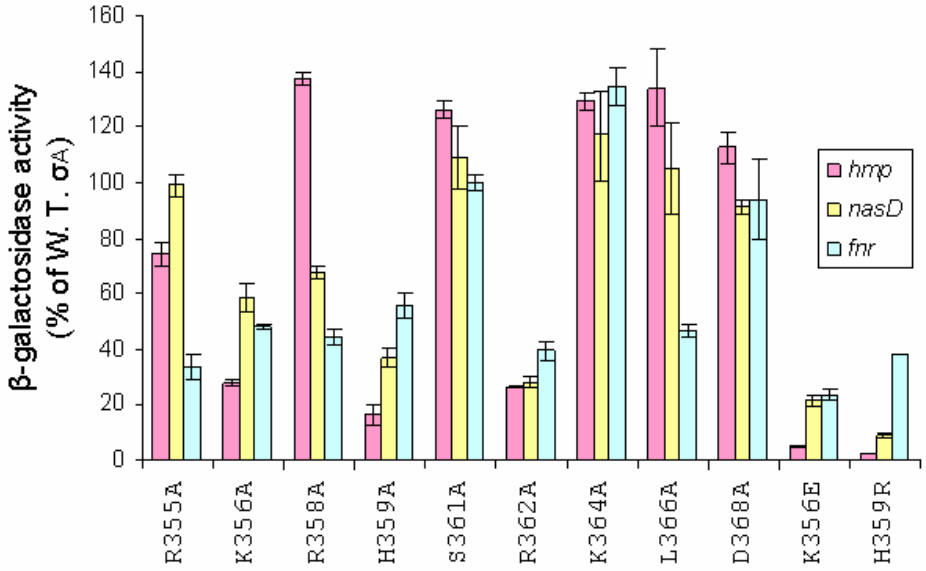



FIG. 4.11. The effect of amino acid substitution of $\sigma^A$ on ResD-dependent gene expression. Cells were grown under anaerobic conditions in 2xYT+1%glucose +0.2%$KNO_3$. β-Galactosidase activity was determined from anaerobic cultures as shown in Fig. 4.3.

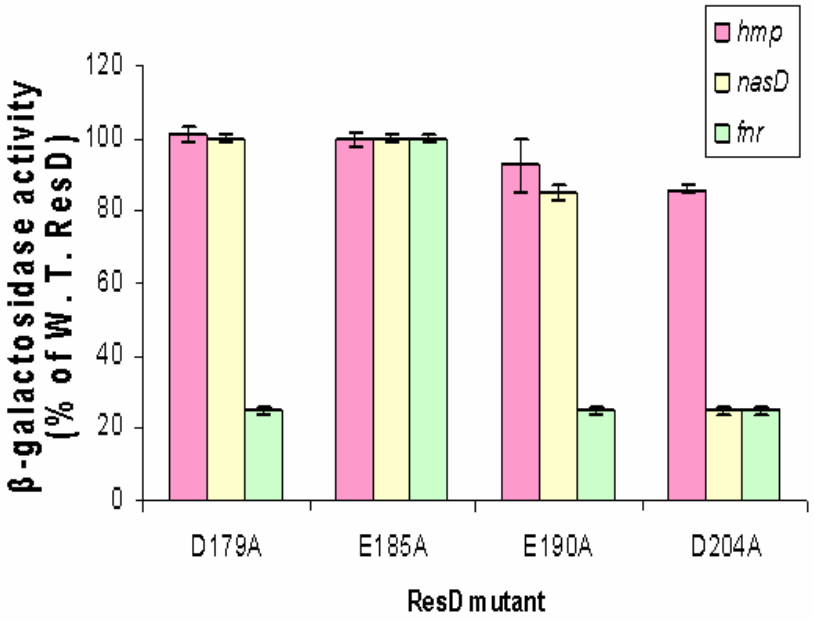



FIG. 4.12. The effect of single alanine substitutions of ResD on expression of *hmp*, *nasD*, and *fnr*. Cells were grown under anaerobic conditions in 2xYT +1% glucose +0.2% $KNO_3$ +1mM IPTG. β-Galactosidase activity was determined from anaerobic cultures as shown in Fig. 4.3.

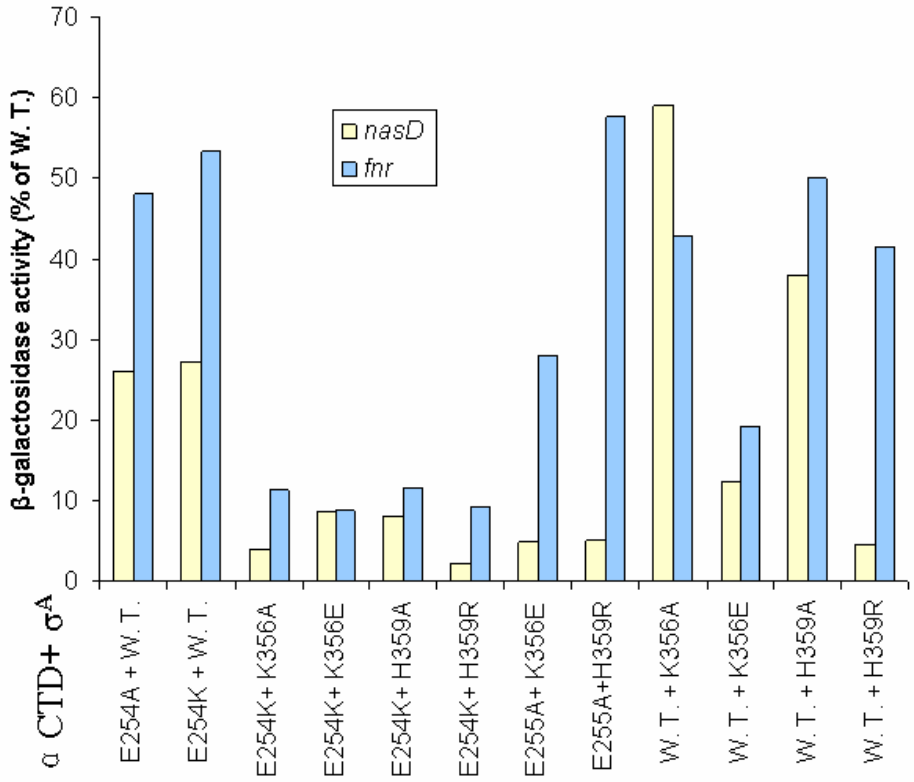



FIG. 4.13. Suppressor analysis of interaction between determinant of αCTD and determinant of $\sigma^A$. Cells were grown under anaerobic conditions in 2xYT +1% glucose +0.2% $KNO_3$. β-Galactosidase activity was determined from anaerobic cultures as shown in Fig. 4.3.

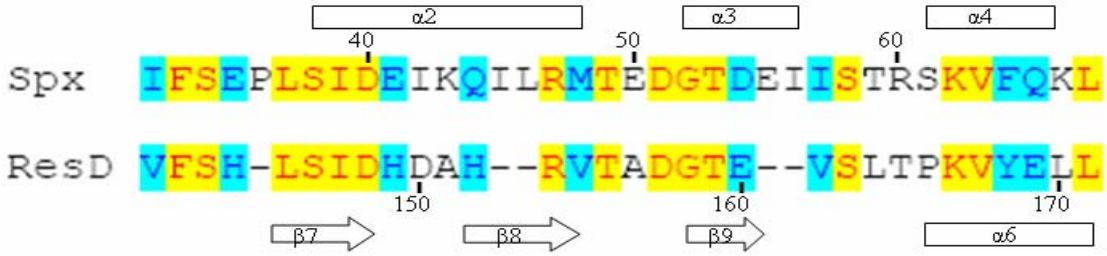



FIG. 4.14. Alignments of ResD with Spx. The secondary structure of Spx observed in the crystal structure is labeled above the sequence (Newberry et al., 2005), and the secondary structure of ResD predicted from the DrrB structure is labeled below the sequence. The identical residues are highlighted in yellow; the similar residues are highlighted in blue.

# CHAPTER 5

# THE NITRIC OXIDE-RESPONSIVE REGULATOR NSRR CONTROLS RESDE-DEPENDENT GENE EXPRESSION[3]

## 5.1 INTRODUCTION

Nitric oxide (NO) is a most versatile signaling molecule whose effects are observed throughout all biological kingdoms. Because NO is highly reactive, it can interact with transition metals, cause S-nitrosylation, and react with superoxide to generate peroxynitrite, which is a strong oxidant. Nitrosylation of metals and cysteine is particularly suitable for controlling gene expression because it is a reversible modification [reviewed in references (Bogdan, 2001) and (Stamler *et al.*, 2001)]. Accumulated evidence indicates that NO controls bacterial gene expression by regulating the activities of transcription factors. Such factors include NnrR, NNR, DnrD, and DNR that activate denitrification genes in *Rhodobacter sphaeroides* (Kwiatkowski and Shapleigh, 1996), *Paracoccus denitrificans* (Van Spanning *et al.*, 1999), *Pseudomonas stutzeri* (Vollack and G., 2001), and *Pseudomonas aeruginosa* (Arai *et al.*, 2003), respectively. These transcription factors all belong to the cyclic AMP receptor protein/fumarate nitrate reductase regulator (FNR) family. In addition to DNR, *P. aeruginosa* has an NO-responsive, $\sigma^{54}$-dependent transcriptional regulator, FhpR, which activates the flavohemoglobin gene (Arai *et al.*, 2005). FhpR is homologous to NorR, a member of the NtrC family of response regulators, although phosphorylation does not play a role in activation of NorR, unlike many response regulators (Pohlmann *et al.*, 2000). NorR, by responding to NO, controls the NO reductase gene in *Ralstonia eutropha* (Pohlmann *et al.*, 2000) and *E. coli* (D'Autreaux *et al.*, 2005). Because deletion of the GAF (cyclic GMP-

[3] This material has been published in this or similar form in *Journal of Bacteriology* and is used here with permission of the American Society for Microbiology.

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

stimulated phosphodiesterases, adenylate cyclases, and FhlA) domain in the N-terminal region of NorR results in a constitutive (NO-independent) phenotype, it was proposed that the GAF domain inhibits the ATPase activity of the central $AAA^+$ domain, and the inhibition is released by receiving the NO-dependent signal (D'Autreaux *et al.*, 2005; Pohlmann *et al.*, 2000). This assumption was proven by the result that the NorR GAF domain contains unprecedented mono-iron center that is not coordinated to histidine (Klink *et al.*, 2007). NO binds iron to form mononitrosyl iron, which results in the stimulation of ATPase activity of NorR, thereby activating transcription of the genes encoding NO reductase (D'Autreaux *et al.*, 2005)

A similar nitrosylation of iron modulates activities of other transcription factors. For example, NO interacts with the [2Fe-2S] center in SoxR that controls gene expression in response to superoxide stress (Ding and Demple, 2000). Interaction of SoxR with NO leads to a dinitrosyl-iron formation in the [2Fe-2S] center. In vitro transcription experiments showed that the NO-modified SoxR is able to activate *soxS* at a level similar to that of oxidized SoxR. Similarly, NO reacts with the [4Fe-4S] cluster of FNR to generate a dinitrosyl-iron-cysteine complex (Cruz-Ramos *et al.*, 2002). FNR represses expression of *hmp* in *E. coli* grown under anaerobic conditions. In contrast to the effect of nitrosylation on SoxR, nitrosylation of FNR reduces binding to the *hmp* promoter, leading to induction of *hmp*. Another example of an NO-sensitive regulator is the ferric uptake regulation protein (Fur), which contains nonheme ferrous iron. When cellular iron levels become low, Fur loses iron, and the apo repressor protein is unable to bind to its target DNA; hence, genes involved in iron uptake are expressed. The exposure to NO results in iron nitrosylation of Fur, leading to loss of DNA-binding activity through an uncharacterized conformational change (D'Autreaux *et al.*, 2002).

We have previously shown that ResDE-dependent gene expression is stimulated by NO (Nakano, 2002). The ResD-ResE signal transduction system is involved in the control of aerobic and anaerobic respiration in *Bacillus subtilis*. NO-dependent stimulation of the ResDE regulon is more profound under anaerobic conditions than aerobic conditions. The effect of NO on gene expression requires both ResD and ResE, but expression of *hmp* also exhibits ResDE-independent transcriptional control. We proposed that the ResE sensor kinase likely perceives a signal, either NO itself or a signal

derived from NO, leading to phosphorylation of ResD (Nakano, 2002). As previously described, ResE does not contain cysteine, which eliminates the possibility of S-nitrosylation by NO, and there is no evidence that ResE binds a metal. Therefore, it has remained mysterious how NO up-regulates ResDE-dependent gene expression. In the manuscript, we will present evidence that the activity of NsrR, a recently identified nitrite (NO)-responsive transcriptional regulator (Rodionov *et al.*, 2005), is responsible for the up-regulation of the ResDE regulon in response to NO.

## 5.2 RESULTS

### 5.2.1 A null mutation in *yhdE (nsrR)* results in aerobic derepression of *hmp*

Our previous results showed that NO up-regulates *hmp* expression under aerobic and anaerobic conditions, and it does so, albeit to a lesser extent, in the absence of ResDE (Nakano, 2002). In the hope of identifying a regulator involved in ResDE-independent activation of *hmp* by NO, a transcription factor (TF) array experiment was carried out. In this assay we first used ORB6126, a *resDE* mutant carrying *hmp-lacZ*. Two transformants exhibited a $Lac^+$ phenotype. One such transformant was shown to carry a *cat* insertion in the *lacR* gene. Since *lacR* encodes a repressor of the *B. subtilis* β-galactosidase gene, the observed $Lac^+$ phenotype was caused by activation of the endogenous β-galactosidase and not by activation of *hmp-lacZ*. The other $Lac^+$ clone was identified as the *yhdE* mutant (Fig. 5.1A and B).

The result suggested that YhdE is a transcriptional repressor of *hmp* and that *hmp* is expressed in the absence of ResDE if *yhdE* is mutated. In this TF array experiment around 10% of transformants were not recovered, probably because some mutations, when combined with the *resDE* mutation, caused adverse effects on growth. Therefore, we repeated the TF array using a wild-type strain (ORB6120) carrying *hmp-lacZ*. All transformants were obtained in this experiment, and again the *lacR* and *yhdE* mutants showed a $Lac^+$ phenotype (Fig. 5.1C). In addition, a *spo0A* mutant appeared pale blue on the X-Gal plate (data not shown), suggesting that Spo0A negatively regulates ResDE-dependent *hmp* expression, which is consistent with the previous observation that Spo0A exerts a negative role in *resDE* expression (Sun *et al.*, 1996a).

In order to confirm the TF array results, we used a transcriptional *hmp-lacZ* fusion to examine the effect of the *yhdE* mutation on aerobic *hmp* expression in the wild-type and *resDE* mutant strains. Figure 5.1D shows that *hmp* was expressed poorly in aerobic cultures of the wild-type strain as expected, whereas the expression was substantially increased in the *yhdE* mutant. It also shows that the aerobic derepression of *hmp* caused by the *yhdE* mutation was partially independent of the ResDE two-component regulatory system. These results confirmed the TF array results indicating that YhdE is likely a transcriptional repressor of *hmp*. Similar *lacZ* assay experiments showed that the *yhdE* mutation does not bring about aerobic derepression of *fnr* and *nasD*, unlike *hmp* (data not shown).

Since anaerobic *hmp* expression is highly dependent on ResDE (LaCelle *et al.*, 1996), we wondered if ResDE-independent transcription of *hmp* in the *yhdE* mutant under aerobic conditions utilizes a transcription start site different from that activated by ResD in anaerobic cultures. Primer extension analysis of RNA isolated from wild type cultured under anaerobic conditions confirmed the previously identified transcription start site (LaCelle *et al.*, 1996) (Fig. 5.2). The experiment also showed that *hmp* is transcribed from the same start site in the aerobically grown *resDE yhdE* mutant cells.

A protein-protein BLAST search showed that YhdE belongs to a newly identified NsrR subfamily of the Rrf2 family (Rodionov *et al.*, 2005) (see details in Discussion). NsrR from *Nitrosomonas europaea* is a nitrite-responsive repressor of *nirK* encoding nitrite reductase (Beaumont *et al.*, 2004). In addition, a recent work showed that *E. coli nsrR* (*yjeB*) encodes a negative regulator of genes that are up-regulated by nitrosative stress (Bodenmiller and Spiro, 2006). In light of these studies, we hypothesized that the previously observed ResDE-independent induction of *hmp* upon nitrosative stress during aerobic growth is likely the result of inactivation of YhdE repressor activity. Hence, YhdE was renamed NsrR.

### 5.2.2 NsrR plays an important role in ResDE-dependent transcription during anaerobic growth

The result described above indicated that NsrR represses *hmp* expression under aerobic conditions. We have previously shown that oxygen limitation is required but not

sufficient for anaerobic induction of *hmp* as well as other genes of the ResDE regulon. To attain full induction of ResDE-controlled genes, nitrate, nitrite, or NO is also needed. During nitrate respiration the membrane-bound respiratory nitrate reductase (NarGHI enzyme) catalyzes reduction of nitrate to nitrite. Nitrate reductase is required for nitrate-dependent up-regulation but not for nitrite- and NO-dependent activation (LaCelle *et al.*, 1996; Nakano *et al.*, 1998; Nakano *et al.*, 2000a; Nakano, 2002). Since the final product of nitrate respiration in *B. subtilis* is ammonium and NO is not an obligatory product of nitrate respiration, if and how NO is produced during nitrate respiration remains unknown. The previous observation of nitrite- and NO-dependent activation of ResDE-controlled genes is particularly intriguing, given that NsrR from *N. europaea* and *E. coli* was proposed to be a nitrite- and NO-sensitive repressor, respectively (Beaumont *et al.*, 2004; Bodenmiller and Spiro, 2006; Rodionov *et al.*, 2005), although it has not been shown whether nitrite and NO directly regulates the repressor activity of NsrR. Therefore, we next examined whether NsrR has any role in *hmp* expression under anaerobic conditions.

As shown previously, *hmp* expression was low when wild-type cells were grown anaerobically under fermentative conditions (in the presence of glucose and pyruvate), and the expression was highly induced during growth by anaerobic respiration (in the presence of glucose and nitrate) (Fig. 5.3A). The *nsrR* mutation caused a dramatic increase in *hmp* expression in the absence of nitrate but resulted in decreased expression in the presence of nitrate. Importantly, the *nsrR* mutant becomes insensitive to nitrate (Fig. 5.3A). A similar effect by the *nsrR* mutation was observed during examination of anaerobic *nasD* and *fnr* expression (Fig. 5.3B and C). These results indicated that NsrR serves as a negative regulator for ResDE-dependent transcription in anaerobic cultures in the absence of nitrate. The results also suggested that full induction of ResDE-dependent gene expression observed in the presence of nitrate requires NsrR. Anaerobic expression of *hmp*, *nasD*, and *fnr* in the *nsrR* mutant still required ResDE, although ResDE-independent *hmp* transcription at the level similar to aerobic expression was detected in the *nsrR* mutant (data not shown).

### 5.2.3 NO modulates NsrR activity

Unlike the wild-type strain, ResDE-dependent gene expression is not stimulated by nitrate in the *narG* mutant, but stimulation can be restored in the *narG* mutant by the addition of nitrite (LaCelle *et al.*, 1996; Nakano *et al.*, 1998; Nakano *et al.*, 2000a). Since NO had a stimulatory effect similar to nitrite, we hypothesized that NO is an inducer of the ResDE regulon, and nitrate is required for full induction because nitrate reductase reduces it to nitrite, which is then converted to NO (Nakano, 2002). One could envisage that NsrR senses NO and regulates expression of the ResDE-controlled genes. In fact, *E. coli* NsrR, a repressor of genes that are up-regulated by nitrosative stress, was shown to be sensitive to NO (Bodenmiller and Spiro, 2006). On the other hand, NsrR of *N. europaea* was reported to be sensitive to nitrite and not to NO (Beaumont *et al.*, 2004). Therefore, we next examined whether NsrR-dependent gene regulation in *B. subtilis* is sensitive to NO. The *narG* and *narG nsrR* strains carrying *hmp-lacZ* were cultured in 2xYT supplemented with 0.5% glucose and 0.5% pyruvate. The *narG* mutant is unable to grow anaerobically by nitrate respiration, but it grows well in the presence of glucose and pyruvate by generating ATP through fermentation (Nakano *et al.*, 1997). Although the *narG* strain grew anaerobically in the glucose-pyruvate medium, *hmp* expression was very low (Fig. 5.4A). Consistent with the result shown in Fig. 5.3A, introduction of the *nsrR* mutation into the *narG* mutant caused a dramatic increase in *hmp* expression before the addition of spermine NONOate (Fig. 5.4A, time zero). We then investigated the effect of NO by using spermine NONOate as a source of NO. Spermine NONOate dissociates at neutral pH with a half-life of 39 min at 37°C, releasing 2 mol of NO per mole. Addition of 100 μM spermine NONOate to the *narG* strain at an $OD_{600}$ of 0.2 resulted in a large increase in *hmp* expression. The *hmp* expression in the *narG nsrR* mutant was increased over time, which is likely due to accumulation of phosphorylated ResD. Unlike the *narG* strain, the expression in the *narG nsrR* mutant was hardly affected by exposure to spermine NONOate, although the expression began to increase 1 h after the addition (Fig. 5.4A). A similar stimulatory effect of NO on *nasD* and *fnr* expression was observed in the *narG* mutant but not in the *narG nsrR* mutant (Fig. 5.4B and C).

The observed effect of spermine NONOate strongly suggested that NO eliminates NsrR negative control, but we could not completely exclude the possibility that NO is

oxidized to nitrite using a small amount of oxygen likely present in our oxygen-limited conditions. In attempts to test this possibility, we used an NO scavenger, carboxy-PTIO. Carboxy-PTIO interacts with NO and generates nitrite (Akaike *et al.*, 1993; Goldstein *et al.*, 2003); thus, it is useful for distinguishing whether nitrite or NO causes observed effects on gene control. Induction of *nasD* expression in the *narG* mutant at 0.5 and 1 h after the addition of 100 μM spermine NONOate was eliminated by simultaneously adding 1 mM carboxy-PTIO to the cultures (Fig. 5.5). Carboxy-PTIO is known to interact with NO by a 1:1 stoichiometry (Akaike *et al.*, 1993); however, we found under our culture conditions that carboxy-PTIO was consumed, probably by radicals produced by *B. subtilis*, and that equimolar amounts were not sufficient to scavenge NO. Thus, we used an excess amount of carboxy-PTIO over NO. Expression of *nasD* was not significantly affected by 1 mM carboxy-PTIO alone in the *narG* and *narG nsrR* strains, indicating that the effect of carboxy-PTIO in the NO-treated cultures was not caused by the scavenger itself. As shown before (Nakano *et al.*, 1998), nitrite (5 mM) also induced *nasD* expression in the *narG* mutant (Fig. 5.5). The increased *nasD* expression by nitrite was also abolished in the presence of carboxy-PTIO, demonstrating that NO, generated from nitrite, is the direct effector of NsrR.

How does NO affect regulation through NsrR? One possibility is that the intracellular concentration of NsrR is regulated by NO, while the other possibility, which is more likely, is that NO affects NsrR activity. In order to examine the former possibility, NsrR levels were examined in cells cultured under different conditions (Fig. 5.6A and B). Western blot analysis using anti-NsrR antibody indicated that NsrR was slightly more abundant in anaerobic cultures than aerobic cultures. It also showed that the addition of nitrate or NO to anaerobic cultures, which induces ResDE-dependent gene expression, did not decrease NsrR levels. These results argue that the effect of NO on expression of the ResDE-controlled genes is not caused by regulation of the NsrR concentration by NO; instead, NsrR activity is likely regulated.

Next, we examined whether NsrR affects *resD* expression; ResD amounts were compared between wild type and the *nsrR* mutant (Fig. 5.6C and D). Western blot analysis using anti-ResD antibody showed that the ResD level was similar between wild type and the *nsrR* mutant cultured anaerobically in the presence or absence of nitrate.

Therefore, it is unlikely that the effect of NsrR on ResDE-dependent gene expression is due to altered ResD expression.

### 5.2.4 NsrR directly represses *hmp* and *nasD* transcription

During aerobic purification of NsrR, we found that NsrR was brownish immediately after elution from an Ni-NTA column, and UV/visible light absorption spectra of the NsrR protein showed an absorbance at 440 nm and a shoulder at around 550 nm (Nakano, unpublished data), indicating that *B. subtilis* NsrR is an Fe-S protein. However, the absorbance of the purified protein quickly disappeared under aerobic conditions, which suggests that the Fe-S center is sensitive to oxygen, as previously observed with many Fe-S proteins. Concentrations of iron bound to NsrR and the free acid-labile sulfide were determined in aerobically purified NsrR protein as described in previous reports (Beinert, 1983; Hennessy *et al.*, 1984). Neither iron nor $S^{2-}$ was detectable using the methods (data not shown). Thus, NsrR purified under aerobic conditions is so-called apo-NsrR that lacks Fe-S center. Although purified NsrR protein lost the Fe-S center and, therefore, we were unable to assess the effect of NO on NsrR activity, we used in vitro transcription assays to examine whether the apo-NsrR protein retained repressor activity (Fig. 5.7). We used *rpsD* encoding ribosomal protein S4 as a control because the *nsrR* mutation does not affect *rpsD* expression in vivo (data not shown). At a concentration of 0.5 μM, NsrR repressed ResDE-dependent *hmp* and *nasD* transcription to 30 to 40%, indicating that NsrR directly represses transcription probably by binding to *hmp* and *nasD*. In contrast, *fnr* transcription was repressed only at a higher amount of NsrR. Since NsrR hardly affects *rpsD* transcription at the concentration we used, *fnr* expression could also be directly repressed by NsrR but at the higher concentration.

### 5.25 Apo-NsrR directly binds to the *hmp* and *nasD* promoters

Apo-NsrR repressed ResDE-dependent transcription activated by phosphorylated ResD in vitro. One possibility is that apo-NsrR represses transcription by affecting ResD phosphorylation. Phosphorylation assay showed that apo-NsrR did not alter the level of ResD phosphorylation by ResE in vitro (data not shown). Another possibility is that NsrR,

as a repressor, can bind to the promoters and inhibits transcriptional initiation. As described earlier, NsrR belongs to the Rrf2 family. The Rrf2 family transcription regulators have HTH DNA binding motif (http://www.ncbi.nlm.nih.gov/BLAST/). The DNase I footprinting analysis was carried out to examine whether apo-NsrR binds directly to the promoter regions (Fig. 5.8). The region from -10 to -1 of *hmp* was strongly protected by 2 μM apo-NsrR (Fig. 5.8A). A similar protection was observed on the region from -15 to -36 of *nasD* promoter (Fig. 5.8B). In contrast, NrsR, at up to 8 μM concentration, did not bind to the *fnr* promoter, although 2 μM apo-NsrR showed repression of *fnr* transcription. Apo-NsrR at high concentration inhibits *fnr* transcription in vitro, although NsrR binding to the *fnr* promoter can not be detected by DNase I footprinting. One explanation is that apo-NsrR might interact with ResD or RNAP to affect the interaction between ResD and RNAP, which inhibits ResD activation of *fnr* transcription in vitro. These results indicate that apo-NsrR likely inhibits transcription by binding to the *hmp* and *nasD* promoter regions. A putative NsrR binding site was proposed by a comparative genomic analysis (Rodionov *et al.*, 2005). Sequences similar to the putative NsrR binding site are detected in the *hmp* and *nasD* promoter regions but not present in the *fnr* promoter (see Discussion).

## 5.3 DISCUSSION

Our previous study showed that expression of ResDE-controlled genes, *nasD* and *hmp*, in particular, is activated by NO (Moore *et al.*, 2004; Nakano, 2002). This NO-dependent stimulation is much stronger under anaerobic conditions than aerobic conditions. Since *B. subtilis* is not a denitrifier, reduction of nitrite generates ammonium, not NO. Therefore, it is not obvious if and how NO is endogenously generated during nitrate respiration. However, recent studies of nondenitrifying *E. coli* indicated that NO is produced from nitrite during nitrate respiration, and production requires periplasmic cytochrome *c* nitrite reductase (Nrf) and cytoplasmic siroheme-dependent nitrite reductase (Nir) (Corker and Poole, 2003). *B. subtilis* has only the siroheme-dependent nitrite reductase encoded by *nasDE*. To determine whether NasDE nitrite reductase catalyzes the reduction of nitrite to NO in *B. subtilis*, we need to directly messure NO

generation in the *nasDE* mutant and the wild-type strains using a NO electrode (Corker and Poole, 2003). *B. subtilis* NO synthase (bsNOS) generates NO from L-arginine with redox partner YkuN in vitro (Wang *et al.*, 2007) ; however, the function of bsNOS as a source of NO in vivo has not been reported. Although it remains to be elucidated how *B. subtilis* generates NO, this work showed that NO, which is produced from nitrite under anaerobic conditions, plays a pivotal role in ResDE-dependent gene activation through NsrR.

*B. subtilis* NsrR is a protein of 16.5 kDa that belongs to the NsrR subfamily of the Rrf2 family of putative transcriptional regulators found in widely divergent bacteria (Rodionov *et al.*, 2005). Rrf2 family proteins have a predicted helix-turn-helix motif in the N terminus, which is likely involved in DNA binding. The NsrR subfamily is distinguished from other Rrf2 proteins because of conserved cysteines at the C terminus. *B. subtilis* has three Rrf2 family proteins, NsrR, CymR (YrzC), a master regulator of sulfur metabolism (Even *et al.*, 2006), and YwgB. Among the three *B. subtilis* proteins only NsrR has the conserved cysteines and is therefore classified into the NsrR subfamily. Several studies reported on gene regulation by the NsrR subfamily proteins. Rrf2 of *Desulfovibrio vulgaris* negatively regulates the *hmc* operon that encodes the high-molecular-mass cytochrome redox protein complex (Keon *et al.*, 1997). *N. europaea* NsrR represses *nirK*, which encodes nitrite reductase, and the repression is reversed by nitrite (Beaumont *et al.*, 2004). RirA of *Rhizobium leguminosarum* is an iron-responsive regulator. Mutations in the *rirA* gene lead to constitutive expression of genes that are normally repressed by iron (Todd *et al.*, 2002). In *Neisseria gonorrhoeae* and *Neisseria meningitides*, NsrR represses the expression of *aniA* (nitrite reductase) and *norB* (NO reductase) (Overton *et al.*, 2006; Rock *et al.*, 2007). In addition to genes involved in denitrification pathway, expression of *dnrN* (encoding a putative reactive nitrogen species response protein) is also repressed by NsrR in *N. gonorrhoeae* (Overton *et al.*, 2006).

Although some circumstantial evidence was presented that NsrR homologs are transcriptional regulators (Beaumont *et al.*, 2004; Yeoman *et al.*, 2004), direct evidence came from the in vitro transcription study that showed that IscR of *E. coli* directly represses the *iscSUA* operon encoding Fe-S cluster assembly proteins (Schwartz *et al.*,

2001). Electron paramagnetic resonance analysis indicated that IscR contains a [2Fe-2S] cluster and that its full repressor activity requires the Fe-S cluster assembly proteins. This suggested that the [2Fe-2S] cluster functions in regulation of IscR repressor activity (Schwartz *et al.*, 2001). A recent study of *E. coli* NsrR indicated that NsrR is a negative regulator of genes that are up-regulated by NO, and the *nsrR* mutation resulted in constitutive expression (Bodenmiller and Spiro, 2006). In addition, NO can relieve the NsrR-dependent repression of *aniA*, *norB*, *dnrN* in *N. gonorrhoeae* and *norB* in *N. meningitides* (Overton *et al.*, 2006; Rock *et al.*, 2007). Notably, NsrR repression of *aniA* is NO-independent in *N. meningitides* (Rock *et al.*, 2007). Because NsrR-dependent repression requires iron, it was proposed that NsrR is either directly or indirectly inactivated by iron limitation. Taken together, these studies suggest that NsrR carries an Fe-S cluster, and apo-NsrR, caused by iron limitation or lack of the Fe-S cluster assembly protein, is unable to bind to DNA. Interaction of NO with the Fe-S cluster, which could bring about iron-nitrosylation or removal of iron, likely results in weakened DNA-binding activity. We have shown that aerobically purified NsrR directly binds to the *hmp* and *nasD* promoters and represses *hmp* and *nasD* transcription. The NsrR protein used in this experiment lacks an Fe-S cluster, as judged by the UV/visible light absorption spectra (Michiko M. Nakano and Pierre Moënne-Loccoz, unpublished results) and determination of iron and free acid-labile sulfide. Therefore, apo-NsrR may bind to *hmp* and *nasD* and function as a transcriptional repressor. Although NsrR loses the Fe-S cluster during aerobic purification, we can not exclude the possibility that Fe-S cluster is intact in intact cells grown under aerobic conditions. In fact, the notion that NsrR represses aerobic *hmp* transcription in vivo (Fig. 5.1) suggests otherwise. Another possible reason that the Fe-S cluster in the purified protein is sensitive to oxygen is that the $His_6$-NsrR is not able to properly assemble the Fe-S cluster, which may cause the instability of Fe-S. To address the role of the Fe-S canter in NsrR activity, the effect of an iron chelator on NsrR repression will be investigated.

All of the NsrR homologs so far identified were shown to be repressors except that IscR directly activates and represses a different set of genes (Giel *et al.*, 2006). Consistently, putative NsrR-binding sites, identified through comparative genomic analysis, are mostly located near the transcription start site (Bodenmiller and Spiro, 2006;

Rodionov *et al.*, 2005). We have shown that the NsrR plays a negative role as a direct repressor for *hmp* and *nasD* and possibly for *fnr*.

In the hope of uncovering a clue to understanding the transcriptional control by NsrR, we examined whether there might be a putative NsrR-binding sequence in the regulatory region of *hmp*, *nasD*, and *fnr*. Rodionov and coworkers applied comparative genomic approaches to predict DNA binding motifs for various NsrR orthologs (Rodionov *et al.*, 2005). The predicted NsrR-binding motif is well conserved in gram-positive bacteria including most *Bacillus* and *Streptomyces* species, and candidate sites were observed only upstream of *hmp* (Rodionov *et al.*, 2005). Sequences similar to the proposed NsrR-binding site for the gram-positive bacteria (gATGyAT-N3-ArATryat, where y is C or T and r is G or A, and highly conserved nucleotides are shown in uppercase letters) (Rodionov *et al.*, 2005) were found in the *hmp* and *nasD* promoter regions (Fig. 5.9A). Two putative NsrR-binding sites were detected in the region overlapping the *hmp* transcription start site (sites 1 and 2), suggesting that these sites are targets for NsrR-dependent negative regulation. We detected two half-sites upstream of the *hmp* promoter, one of which is located on the noncoding strand. DNase I footprinting experiments showed that NsrR binds site 2 located close to -10 region, a core promoter element, possibly resulting in steric hindrance of RNAP binding to the promoter DNA. NsrR binds to site 1, half site 3A and 3B more weakly than to site 2. One possibility is that apo-NsrR has a weak affinity to these three sites and efficient binding of NsrR to these sites requires the Fe-S center. Another explanation is that NsrR may act as an anti-activator through direct interaction with ResD to inhibit ResDE-dependent transcription. Interaction with ResD may be required for NsrR binding to half site 3A and 3B. Our previous hydroxyl radical footprinting experiments showed that phosphorylated ResD binds in tandem to the same face of the DNA helix in the *hmp* promoter except for the most promoter-proximal ResD-binding site (Fig. 5.9, ResD-B) (Geng *et al.*, 2004). Interestingly, this ResD-binding site is likely on the same helical face as one of the putative NsrR-binding half-sites (Fig. 5.9, shown as 5.3B). The other half-site (3A) and the ResD-binding site (Fig. 5.9, ResD-A) adjacent to the most promoter-proximal site appear to be situated on the same face of the helix. It is tempting to speculate that one monomer of ResD interacts with NsrR bound to 3A and that the other ResD monomer

interacts with NsrR occupying 3B. In the *nasD* promoter, a putative NsrR-binding site was detected immediately downstream of the most promoter-proximal ResD-binding site. Apo-NsrR binding to the site was confirmed by DNase I footprinting. In contrast to the *hmp* and *nasD* promoters, we could not detect a putative NsrR-binding site in the *fnr* promoter. In this context it is worth mentioning that a recent study with IscR indicated that there are at least two different classes of IscR binding sites (Giel *et al.*, 2006). Our study also suggested that NsrR may play a positive role in ResDE-dependent transcription. The positive effect NsrR on *nasD* and *hmp* was observed in the presence of NO under anaerobic conditions (Fig. 5.3). Notably putative NsrR-binding sites (site 1 of *nasD* and halfsite 3A and 3B of *hmp*) are next to the ResD-binding sites (Fig. 5.9). In the absence of NO NsrR might block the interaction between ResD and RNAP, while NO-modified NsrR might switch to a co-activator to facilitate the interaction between ResD and RNAP. Although future studies are needed to elucidate in more detail the mechanism by which NsrR regulates ResDE-dependent gene regulation, this study has uncovered the link between oxygen limitation and NO, both of which are required for transcriptional activation of the ResDE regulon.

## 5.4 MATERIALS AND METHODS

### 5.4.1 Strains and plasmids

All *B. subtilis* strains used in this study are derivatives of JH642 except TF274, which is a derivative of the strain 168 (Table 5.1). Plasmids and oligonucleotides are listed in Table 5.1 and Table 5.2, respectively. *E. coli* DH5α [$\lambda^-$Φ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*($r_K^-$ $m_K^-$) *supE44 thi-1 gyrA relA1*] was used for plasmid construction. The *nsrR* mutant (TF274) was constructed as follows. Upstream and downstream regions of *nsrR* were amplified by PCR with the primer sets yhdE-F1/yhdE-R1 and yhdE-F2/yhdE-R2, respectively. The *cat* (chloramphenicol acetyltransferase) gene was amplified by PCR from plasmid pCBB31 using primers PUC-F and PUC-R. The 5' ends of yhdE-F2 and yhdE-R1 are complementary to the sequence of PUC-F and PUC-R, respectively. Three PCR products were mixed and used as templates for the second PCR with primers yhdE-F1 and yhdE-R2. The resultant PCR

fragment amplified via overlap extension was used for transformation of *B. subtilis* 168. The region amplified by PCR was sequenced, and one conserved mutation (GCA to GCG for Ala at residue 479) in the *ygxB* gene was identified. The *nsrR* mutation was moved by transformation to the JH642 background to construct isogenic strains.

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

### 5.4.2 TF array analysis

Transcription factor (TF) array analysis was carried out similarly to a previously described method (Hayashi *et al.*, 2006). In short, 285 genes encoding known and putative transcription factors were mutated by insertion of the *cat* gene. Chromosomal DNA isolated from *B. subtilis* carrying each mutation was spotted into 96-microwell plates. Competent cells of ORB 6120 (wild type) and ORB6126 (*resDE*) carrying *hmp-lacZ* (spectinomycin-resistance) were added into each well, and after transformation each culture was spotted onto Luria-Bertani (LB) agar plates supplemented with chloramphenicol. After overnight incubation at 37°C, colonies of the transformants were replica plated with a 96-pick replicator to LB agar containing chloramphenicol, spectinomycin, and 40 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal).

### 5.4.3 Primer extension analysis

The wild-type strain (JH642) was grown anaerobically in 2x yeast extract-tryptone (YT) medium (Nakano *et al.*, 1996) supplemented with 1% glucose and 0.2% $KNO_3$. Cells were harvested at an optical density at 600 nm ($OD_{600}$) of around 0.3. The *resDE nsrR* mutant (ORB6199) was grown aerobically in the same medium supplemented with tetracycline and chloramphenicol until an $OD_{600}$ of 0.5 was reached. RNA was purified using the RNeasy kit (QIAGEN) according to the manufacturer's instructions. Primer extension analysis was carried out as previously described (Nakano *et al.*, 2005) except that 4 μg of RNA was used with the primer oMMN99-90 that hybridizes with the *hmp* RNA between nucleotides 60 and 79 from the 5' end of the

transcript. Sequencing reactions were performed using a Thermo Sequenase Cycle Sequencing Kit (United States Biochemical) with the primer oMMN99-90 and pMMN448 as template.

### 5.4.4 Measurement of β-galactosidase activities

*B. subtilis* cells were cultured either in 2xYT supplemented with 1% glucose and 0.2% $KNO_3$ or in 2xYT supplemented with 0.5% glucose and 0.5% pyruvate. An NO donor, spermine NONOate (Cayman Chemical), was dissolved in 10 mM NaOH at a concentration of 100 mM. An NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, potassium salt (carboxy-PTIO) was purchased from Molecular Probes and dissolved in water to a concentration of 100 mM. Cells were harvested at 0.5- to 1-h intervals and β-galactosidase activity was measured as previously described (Miller, 1972).

### 5.4.5 Purification of proteins

In order to overproduce NsrR in *E. coli*, plasmid pMMN648 was constructed as follows. The *nsrR* coding region was amplified by PCR from JH642 chromosomal DNA using the primers oMN05-296 and oMN05-297. The resultant PCR product was digested with NdeI and SmaI, and the released *nsrR* fragment was inserted into PGBKT7 (Clontech) that was digested with the same enzymes to generate pMMN638. The *nsrR* gene recovered from pMMN638 by digesting with NdeI and BamHI was cloned into the NdeI-BamHI sites of a $His_6$-tagged expression vector pPROEX-1 (BRL) to construct pMMN648. *E. coli* BL21 (DE3)/pLysS [$F^-$ *ompT hsd5*$_B$($r_B^-m_B^-$) *gal dcm* (DE3) pLys5 ($Cm^r$)] was transformed with pMMN648 plasmid DNA. The resultant transformant was cultured at 37°C in 1 liter of LB medium supplemented with ampicillin and chloramphenicol. At an $OD_{600}$ of 0.5, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture, which was further incubated for 6 h at 30°C. Purification of NsrR was carried out under aerobic conditions. The harvested cells were broken by multiple passages through a French press, and a cleared lysate was recovered by centrifugation at 15,000 x *g* for 20 min. The lysate was mixed with 3 ml of $Ni^{2+}$-nitrilotriacetic acid (Ni-NTA) resin (QIAGEN) in buffer A (50 mM Tris-HCl, pH 8.0,

500 mM NaCl, 20 mM imidazole). The slurry was rotated slowly at 4°C for 1 h and applied to a column. The column was washed with 30 volumes of buffer A and then 10 volumes of buffer A containing 50 mM imidazole. $His_6$-NsrR was eluted with buffer A containing 300 mM imidazole. Fractions containing $His_6$-NsrR were pooled and dialyzed against buffer B (50 mM Tris-HCl, pH 7.6, 100 mM NaCl) and used for production of anti-NsrR antibody in rabbits (Josman, LLC). The iron and the $S^{2-}$ concentration of each preparation was measured as described in previous reports (Beinert, 1983; Hennessy *et al.*, 1984).

RNA polymerase (RNAP) was purified from *B. subtilis* MH5636 (Qi and Hulett, 1998), which produces an RNAP β' subunit fused to a $His_{10}$ tag. Purification of RNAP using Ni-NTA and HiQ chromatography was described elsewhere (Nakano *et al.*, 2005).

In order to purify $\sigma^A$ protein, plasmid pSN64 was constructed as follows. The *sigA* gene was amplified by PCR using primers oSN02-39 and oSN02-40 and JH642 chromosomal DNA as a template. The PCR product was digested with NcoI and cloned into pTYB4 digested with NcoI and SmaI. *E. coli* ER2566 (New England Biolabs) carrying pSN64 was grown at 37°C in LB medium supplemented with ampicillin. ER2566 genotype is F⁻λ⁻ *fhuA2* [*lon*] *ompT lacZ*::*T7 gene1 gal sulA11* Δ(*mcrC-mrr*)*114*::IS*10 R*(*mcr-73*::*mini*Tn*10*-Tet$^S$)*2 R*(*zgb-210*::Tn*10*)(Tet$^S$) *endA1* [*dcm*]. When the $OD_{600}$ reached approximately 0.5, IPTG was added to a final concentration of 0.5 mM, and the cultures were further incubated at 25°C for 3.5 h. Cells were harvested by centrifugation and resuspended with buffer C (25 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1 mM EDTA, 1 mM $MgCl_2$, 10% glycerol) containing 0.05% Triton X-100. Cells were broken by passages through a French press, and the lysate was centrifuged at 20,000 x *g* for 20 min. The supernatant was applied onto a chitin column equilibrated with buffer C. After being washed with buffer C, the column was washed with buffer D (25 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM $MgCl_2$, 10% glycerol). The intein tag was cleaved by incubating the column at 4°C overnight with buffer D containing 50 mM dithiothreitol. Proteins were eluted with buffer D, and fractions containing SigA protein were combined and applied onto a HiQ column equilibrated with buffer D and eluted with a 100 mM to 500 mM KCl linear gradient. Fractions containing SigA were pooled and dialyzed against buffer D before being frozen at –70°C.

### 5.4.6 In vitro runoff transcription

The linear templates used for in vitro transcription assays were amplified by PCR using the oligonucleotides listed in Table 5.2 as previously described (Geng *et al.*, 2004; Nakano *et al.*, 2005). In vitro transcription was carried out as described in a previous paper (Geng *et al.*, 2004) with minor modification. ResD and ResE (each, 1 μM) were incubated at room temperature for 15 min in 20 μl of transcription buffer [25 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 5 mM $MgCl_2$, 0.25 mM ATP, 50 μg of bovine serum albumin per ml, 10% glycerol, and 0.4 U of RNase inhibitor (RNasin; Promega)]. RNAP, $\sigma^A$ (both at 25 nM), various amounts of NsrR, and 5 nM template were added, and the reaction mixtures were incubated at room temperature for 10 min. ATP, GTP, CTP (each, 100 μM), UTP (25 μM), and 5 μCi of [α-$^{32}$P]UTP (800 Ci/mmol) were added to start transcription. After incubation at 37°C for 25 min, the reaction was stopped, and transcripts were analyzed on a 6% polyacrylamide-urea gel. The gel was analyzed with a PhosphorImager (Molecular Dynamics), and the intensity of bands was quantified using ImageQuant.

### 5.4.7 DNase I footprinting

The fragment carrying *hmp* (from -133 to 27) and *nasD* (from -138 to +22) promoters were used for DNase I footprinting experiments. The probe labeling, purification and reaction conditions were described in the previous paper (Geng *et al.*, 2007; Nakano *et al.*, 2000b). NsrR was incubated with labeled probe (50,000 cpm) in 20 μl of binding buffer (25 mM Tris-HCl [pH 7.5], 100 mM KCl, 1 mM EDTA, 4 mM DTT, 4 mM $MgCl_2$, 0.25 mM ATP) for 30 min at room temperature. The reaction was treated with 60 ng of DNase I at room temperature for 20 s for free probes and 40 s for reactions containing the protein(s). The same primers used for the labeling of DNA fragments were used for sequencing of the template DNAs with a Thermo Sequenase cycle sequencing kit (USB, Cleveland, Ohio). The sequencing reactions were run together with the footprinting reactions in 8% polyacrylamide-urea gels in Tris-borate buffer. The dried gels were analyzed by phosphorimaging.

### 5.4.8. Western blot analysis

To determine the NsrR amount, JH642 was aerobically and anaerobically cultured at 37°C in 2xYT supplemented with 1% glucose and 0.2% $KNO_3$. JH642 was also cultured in 2xYT with 0.5% glucose and 0.5% pyruvate under anaerobic conditions. At an $OD_{600}$ of 0.2, 100 μM spermine NONOate was added to the cultures grown in the presence of glucose and pyruvate, and the culture was further incubated for 1 h before harvest. As a negative control, ORB6179 (*nsrR*::*cat*) was used, which was cultured anaerobically in 2xYT supplemented with glucose and pyruvate. Cultures were harvested at an $OD_{600}$ of around 0.3 (for anaerobic cultures) or 0.5 (for aerobic cultures). Proteins were prepared from the harvested cells as described previously (Baruah *et al.*, 2004), and 15 μg of total protein was applied to a sodium dodecyl sulfate-polyacrylamide (15%) gel for electrophoresis. Western blot analysis was carried out using anti-NsrR antibody as previously described (Nakano *et al.*, 2000b).

To determine whether the *nsrR* mutation affects the ResD level, JH642 and ORB6179 were cultured anaerobically in 2xYT supplemented with 1% glucose and 0.2% $KNO_3$ or 2xYT supplemented with 0.5% glucose and 0.5% pyruvate. Cells were harvested at an $OD_{600}$ of around 0.6, when expression of ResDE-controlled genes is the highest. Ten micrograms of total protein was applied to a sodium dodecyl sulfate-polyacrylamide (12%) gel for electrophoresis, and Western blot analysis was carried out using anti-ResD antibody (Nakano *et al.*, 2000b).

**TABLE 5.3 B. subtilis strains and plasmids**

| Strain or plasmid | Characteristic or description | Reference or source |
|---|---|---|
| Strains | | |
| JH642 | Parental strain (*trpC2 pheA1*) | J. A. Hoch |
| LAB2135 | *resDE*::*tet* | (Nakano *et al.*, 1996) |
| LAB2252 | SPβc2Δ2::Tn*917*::pMMN288 (*fnr-lacZ*) | (Nakano *et al.*, 1996) |
| LAB2408 | *narG*::*phleo* | (Nakano *et al.*, 1998) |
| LAB2518 | SPβc2Δ2::Tn*917*::pML107 (*hmp-lacZ*) | (Nakano *et al.*, 2000b) |
| LAB2854 | SPβc2Δ2::Tn*917*::pMMN392 (*nasD-lacZ*) | (Nakano *et al.*, 1998) |
| LAB2966 | *narG*::*phleo* SPβc2Δ2::Tn*917*::pMMN392 (*nasD-lacZ*) | (Nakano *et al.*, 1998) |
| MH5636 | $His_{10}$-*rpoC* | (Qi and Hulett, 1998) |
| ORB3782 | *narG*::*phleo* SPβc2Δ2::Tn*917*::pML107 (*hmp-lacZ*) | This study |
| ORB3927 | *resDE*::*tet* SPβc2Δ2::Tn*917*::pML107 (*hmp-lacZ*) | This study |
| ORB4007 | *hmp*::pML67 (*hmp-lacZ*) $Cm^r$ | This study |
| ORB6120 | *hmp*::pML67 (*hmp-lacZ*) $Spc^r$ | This study |
| ORB6126 | *resDE*::*tet hmp*::pML67 $Spc^r$ | This study |
| ORB6179 | *nsrR*::*cat* | This study |
| ORB6187 | *nsrR*::*cat* SPβc2Δ2::Tn*917*::pMMN288 (*fnr-lacZ*) | This study |
| ORB6188 | *nsrR*::*cat* SPβc2Δ2::Tn*917*::pMMN392 (*nasD-lacZ*) | This study |
| ORB6189 | *nsrR*::*cat* SPβc2Δ2::Tn*917*::pML107 (*hmp-lacZ*) | This study |
| ORB6199 | *resDE*::*tet nsrR*::*cat* | This study |
| ORB6220 | *resDE*::*tet nsrR*::*cat* SPβc2Δ2::Tn*917*::pML107 (*hmp-lacZ*) | This study |
| ORB6436 | *narG*::*phleo nsrR*::*cat* | This study |

| | | |
|---|---|---|
| ORB6437 | *narG*::*phleo* SPβc2Δ2::Tn*917*::pMMN288 (*fnr-lacZ*) | This study |
| ORB6439 | *narG*::*phleo nsrR*::*cat* SPβc2Δ2::Tn*917*::pMMN288 (*fnr-lacZ*) | This study |
| ORB6440 | *narG*::*phleo nsrR*::*cat* SPβc2Δ2::Tn*917*::pMMN392 (*nasD-lacZ*) | This study |
| ORB6441 | *narG*::*phleo nsrR*::*cat* SPβc2Δ2::Tn*917*::pML107 (*hmp-lacZ*) | This study |
| TF274 | *nsrR*::*cat* | This study |
| Plasmids | | |
| pC194 | *Staphylococcus aureus* origin; $Cm^r$ | (Horinouchi and Weisblum, 1982) |
| pCBB31 | pUC19 with the *cat* gene from pC194 | This study |
| pGBKT7 | Gal4 DNA binding domain | Clontech |
| pMMN448 | pUC18 with *hmp* promoter (–191 to +79) | (Nakano *et al.*, 2000b) |
| pMMN638 | pGBKT7 with *nsrR* | This study |
| pMMN648 | pPROEX1 with *nsrR* | This study |
| PPROEX-1 | Expression vector with $His_6$ tag | BRL |
| pSN64 | pTYB4 with *sigA* | This study |
| pTK*lac* | Promoter-probe vector | (Kenny and Moran Jr., 1991) |

**TABLE 5.4 Oligonucleotides**

| Oligonucleotide | Sequence (5' to 3') | Purpose |
|---|---|---|
| oHG-1 | GGCATCATCAGAGCAAGCAAAAT | *nasD* template |
| oHG-7 | AAATGCCCGGTTTTAAGG | *nasD* template |
| oMN98-24 | GGAATTCAGAGGTGGCGTTA | *fnr* template |
| oMN98-25 | CGGGATCCAGCAATTCATAC | *fnr* template |
| oMN99-89 | GGAATTCCCAAAACATAAGT | *hmp* template |
| oMN99-90 | CGGGATCCAGTGCTTTTAAT | *hmp* template and primer extension |
| oMN05-296 | GGCGCGGGCATATGAAGTTAACCAATTATAC | pMMN638 |
| oMN05-297 | GTCCTCCCGGGCTATTCCTTCATTTTTAAAA | pMMN638 |
| oSN02-39 | CATGCCATGGCTGATAAACAAACCAC | PSN64 |
| oSN02-40 | TTCAAGGAAATCTTTCAAACGTTTAC | PSN64 |
| oSN03-86 | CATGTTTTTATCACCTAAAAGTTTACCAC | *rpsD* template |
| oSN03-87 | CGATACACCTTATTGATAAGGAACAAAATG | *rpsD* template |
| PUC-F | GTTTTCCCAGTCACGACG | *nsrR* mutation |
| PUC-R | GAATTGTGAGCGGATAAC | *nsrR* mutation |
| yhdE-F1 | TGTCCTTGTCTCAAGTTGTC | *nsrR* mutation |
| yhdE-R1 | GTTATCCGCTCACAATTCACTTCATAATAAGACCTCAG | *nsrR* mutation |

| | | |
|---|---|---|
| yhdE-F2 | CGTCGTGACTGGGAAAACCAAAGAAGATATCATGAAGC | *nsrR* mutation |
| yhdE-R2 | ACTGGAACTAGAGCTTGAAG | *nsrR* mutation |

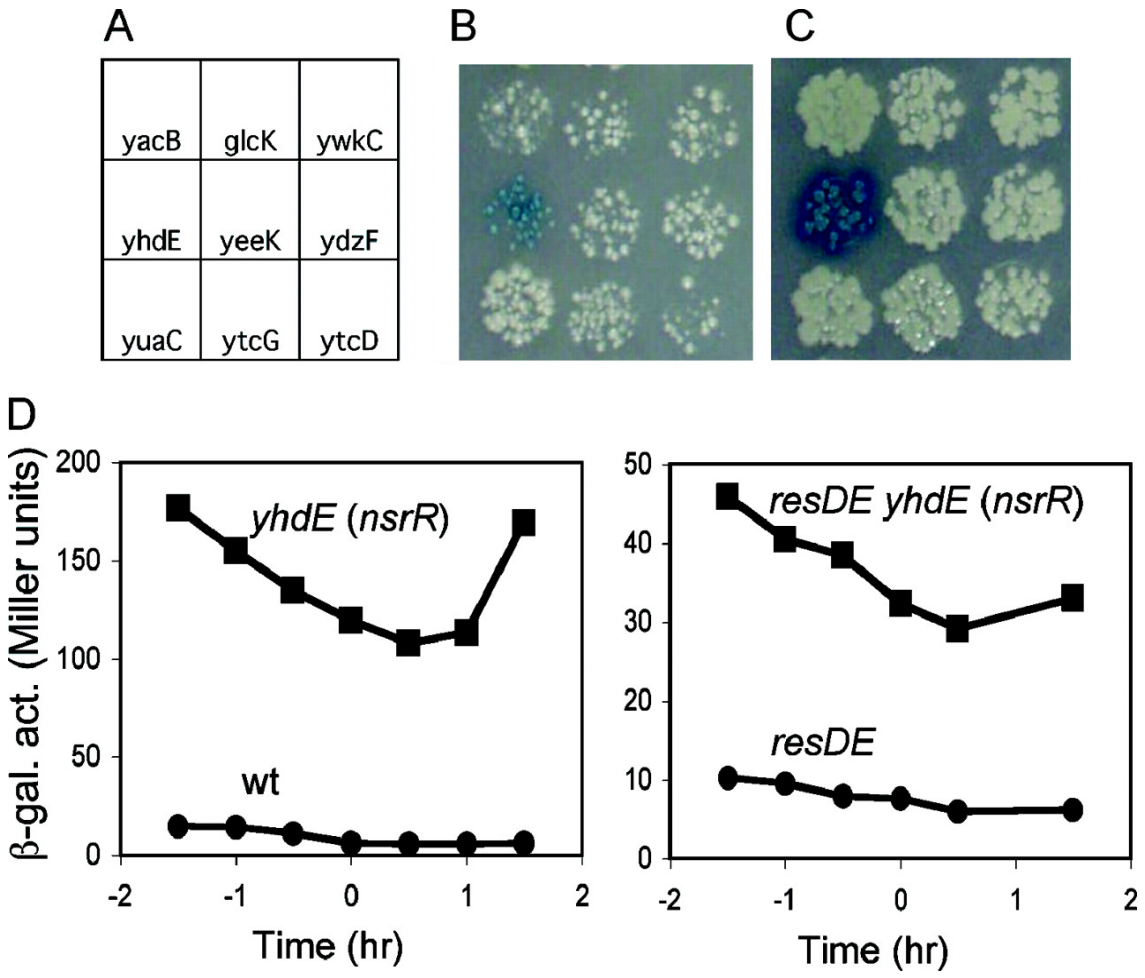



FIG. 5.1. TF array experiment to identify a transcriptional regulator of *hmp*. The figure shows only 9 of 285 spots. (A) Assignment of a mutation in each array is indicated. *yhdE* is now named *nsrR*. (B) ORB6126 (*hmp-lacZ resDE*) was used as the recipient of transformation. (C) ORB6120 (*hmp-lacZ*) was used for transformation. The *resDE nsrR* and *nsrR* mutants expressed *hmp-lacZ* under aerobic conditions, as indicated by the blue area, on LB agar supplemented with X-Gal. Details are described in Materials and Methods. (D) Effect of the *nsrR* mutation on *hmp-lacZ* expression is shown in the wild-type (left) and *resDE* mutant (right) strains grown aerobically in 2xYT supplemented with 1% glucose and 0.2% $KNO_3$.

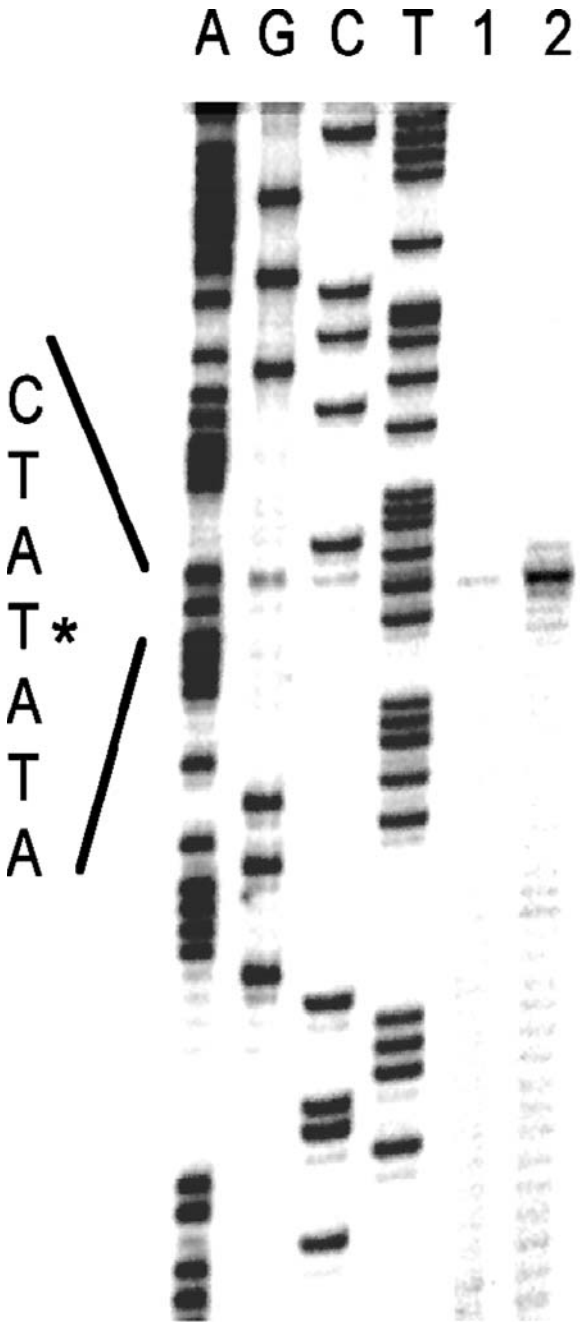



FIG. 5.2. Identification of the transcription start site of *hmp*. Primer extension analysis was carried out using RNA isolated from ORB6199 (*resDE nsrR*) grown under aerobic conditions (lane 1) and JH642 (wild type) grown under anaerobic conditions (lane 2). Nucleotide sequence ladders with the same primer as that used for the primer extension experiment are shown on the left. The transcription start site is marked with an asterisk.

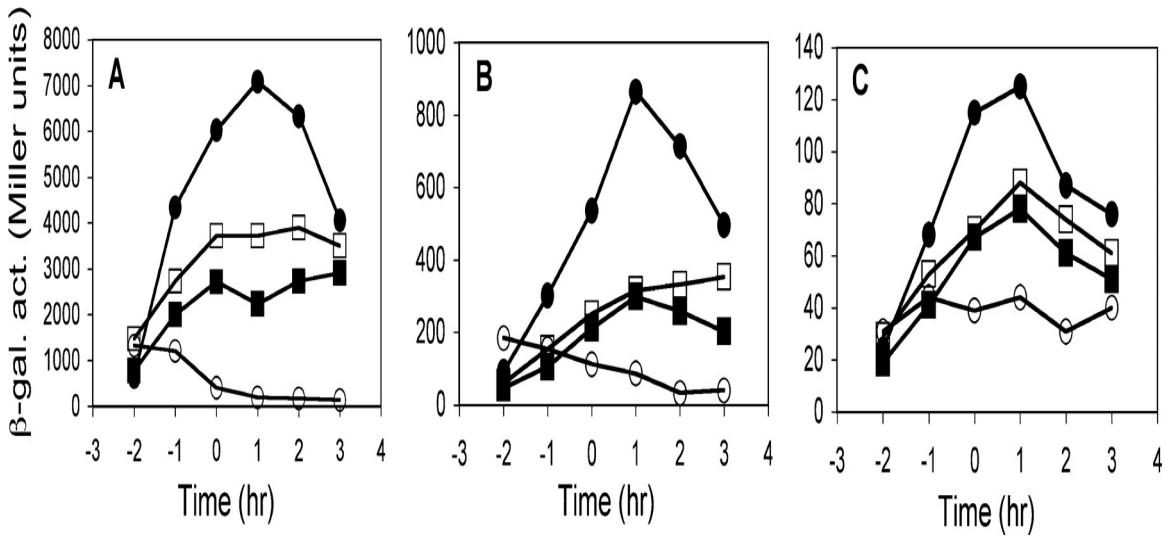



FIG. 5.3. Effect of the *nsrR* mutation on anaerobic expression of *hmp-lacZ* (A), *nasD-lacZ* (B), and *fnr-lacZ* (C). *B. subtilis* strains were grown anaerobically in 2xYT supplemented with 1% glucose and 0.2% $KNO_3$ (closed symbols) or with 0.5% glucose and 0.5% pyruvate (open symbols). (A) Circle, LAB2518 (wild type); square, ORB6189 (*nsrR*). (B) Circle, LAB2854 (wild type); square, ORB6188 (*nsrR*). (C) Circle, LAB2252 (wild type); square, ORB6187 (*nsrR*). Time zero is the end of the exponential growth phase.

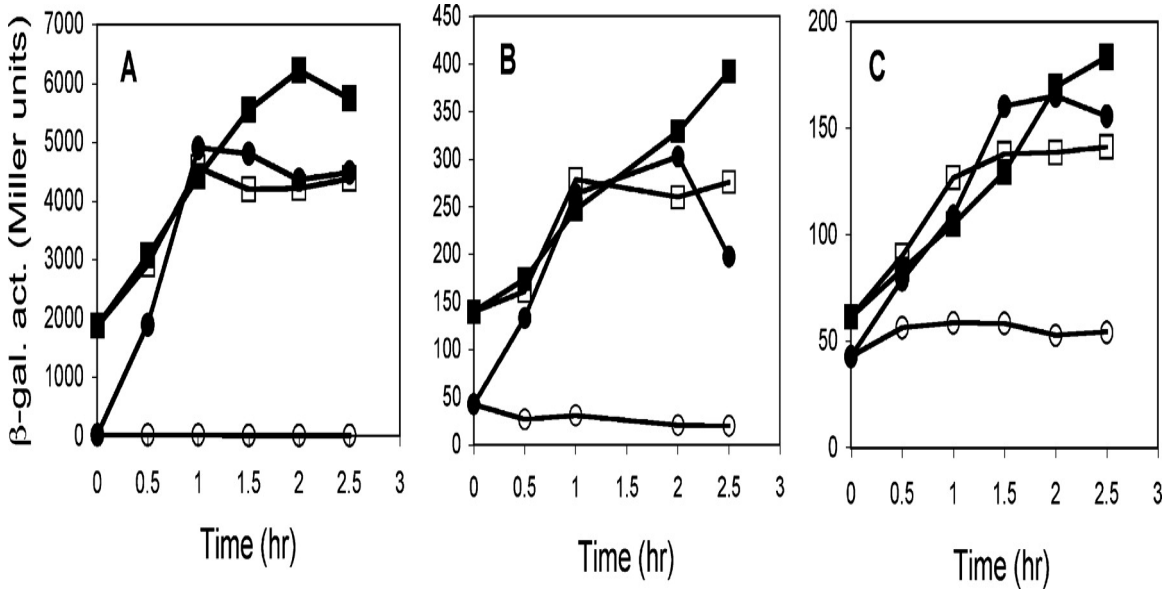



FIG. 5.4. Effect of spermine NONOate on anaerobic expression of *hmp-lacZ* (A), *nasD-lacZ* (B), and *fnr-lacZ* (C). *B. subtilis* strains were grown anaerobically in 2xYT supplemented with 0.5% glucose and 0.5% pyruvate. At time zero, samples were withdrawn to measure β-galactosidase activity, and spermine NONOate was added to the rest of the tubes at a final concentration of 100 μM. β-Galactosidase activities were measured in cells harvested at indicated times. Open symbols and closed symbols indicate cultures without and with spermine NONOate, respectively. (A) Circle, ORB3782 (*narG*); square, ORB6441 (*narG nsrR*). (B) Circle, LAB2966 (*narG*); square, ORB6440 (*narG nsrR*). (C) Circle, ORB6437 (*narG*); square, ORB6439 (*narG nsrR*).

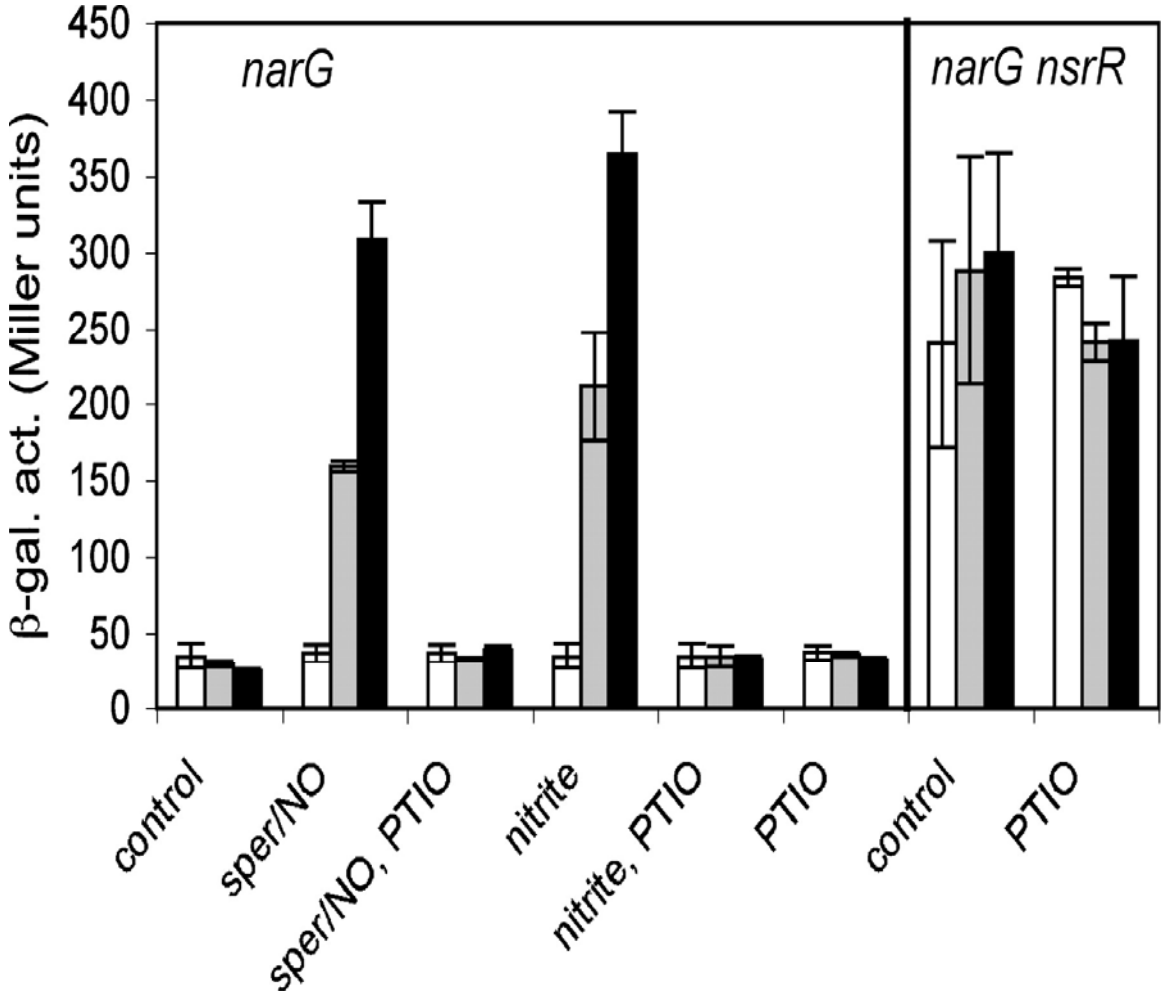



FIG. 5.5. Effect of carboxy-PTIO on NO- or nitrite-dependent induction of *nasD-lacZ* expression. Spermine NONOate (sper/NO; 100 μM) or nitrite (5 mM) was added at time zero to the *narG* (LAB2966) or *narG nsrR* (ORB6440) strain grown anaerobically in 2xYT supplemented with 0.5% glucose and 0.5% pyruvate. β-Galactosidase activity was measured at 0 h (before the addition of spermine NONOate or nitrite; white column) and at 0.5 h (gray column) and 1 h (black column) after the addition of spermine NONOate or nitrite. Carboxy-PTIO (1 mM) was added at time zero where indicated. The experiments were repeated two to three times, and average results are shown with standard deviations.

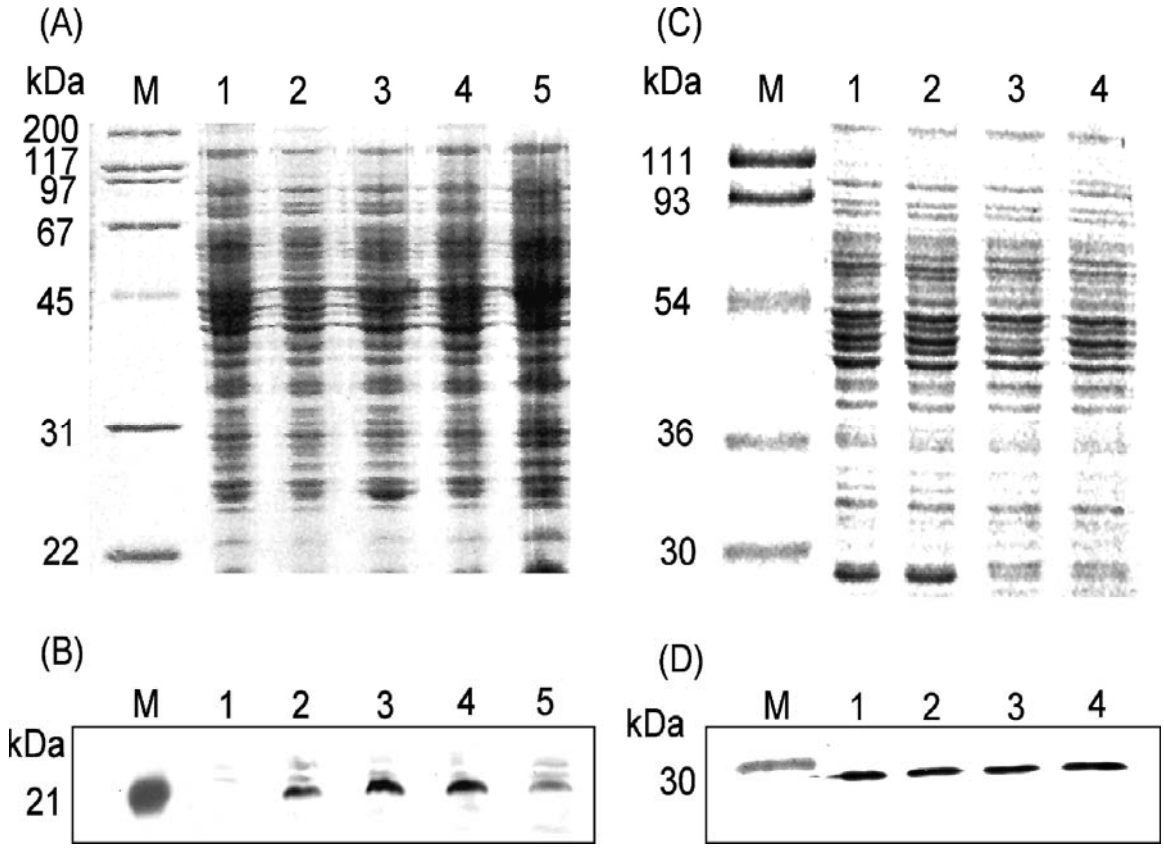



FIG. 5.6. Western blot analysis of NsrR (A and B) and ResD (C and D) in cells grown under different culture conditions. (A) Coomassie-stained gel. (B) Immunoblot using anti-NsrR antibody. Cleared lysates were prepared from the following: lane 1, ORB6179 (*nsrR*) cultured anaerobically in 2xYT supplemented with 0.5% glucose and 0.5% pyruvate; lane 2, JH642 (wild type) cultured anaerobically in 2xYT supplemented with 0.5% glucose and 0.5% pyruvate; lane 3, JH642 cultured anaerobically in 2xYT supplemented with 1% glucose and 0.2% $KNO_3$; lane 4, JH642 cultured anaerobically in 2xYT supplemented with 0.5% glucose and 0.5% pyruvate, followed by exposure to 100 μM spermine NONOate for 1 h; lane 5, JH642 cultured aerobically in 2xYT supplemented with 1% glucose and 0.2% $KNO_3$. Molecular mass markers are shown on the lane marked with M. (C and D) Western blot analysis of ResD protein in cells grown under anaerobic conditions. (C) Coomassie-stained gel. (D) Immunoblot using anti-ResD antibody. Cleared lysates were prepared from JH642 (lane 1) and ORB6179 (lane 2) cultured in 2xYT supplemented with 1% glucose and 0.2% $KNO_3$, and JH642 (lane 3) and ORB6179 (lane 4) cultured in 2xYT supplemented with 0.5% glucose and 0.5% pyruvate.

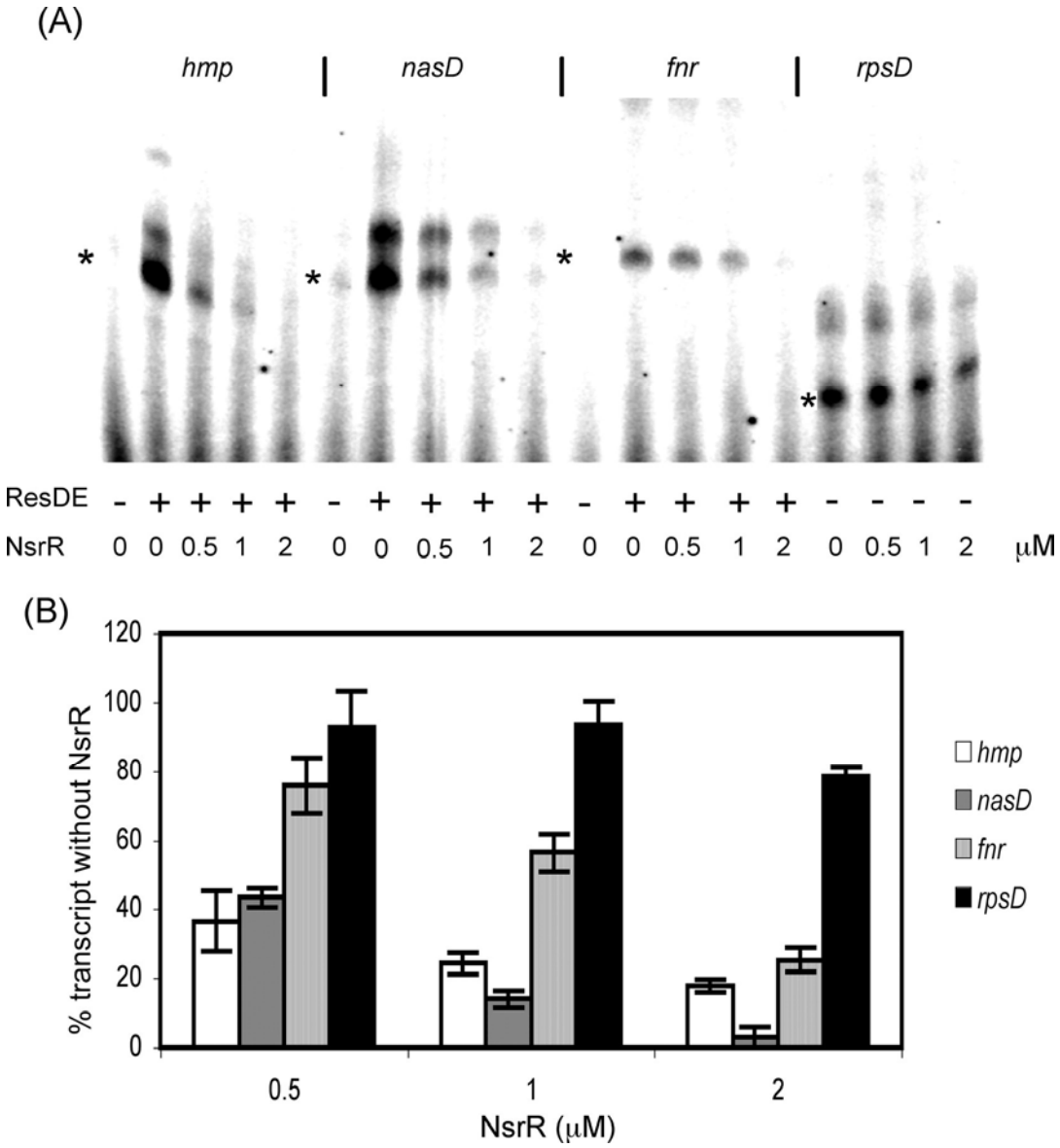



FIG. 5.7. Effect of NsrR on ResDE-dependent transcription in vitro. (A) The indicated amount of NsrR protein was incubated with ResD (1 μM), ResE (1 μM), and RNAP (50 nM). A 50 nM concentration of $\sigma^A$ was also included. *rpsD* was used as a negative control. The asterisks indicate the positions of each transcript, *hmp* (86 bases), *nasD* (96 bases), *fnr* (101 bases), and *rpsD* (71 bases). (B) The intensity of each band was quantified with ImageQuant and is shown as percent transcript detected in the absence of NsrR. The data shown are the average of two independent experiments, and the standard deviations are indicated.

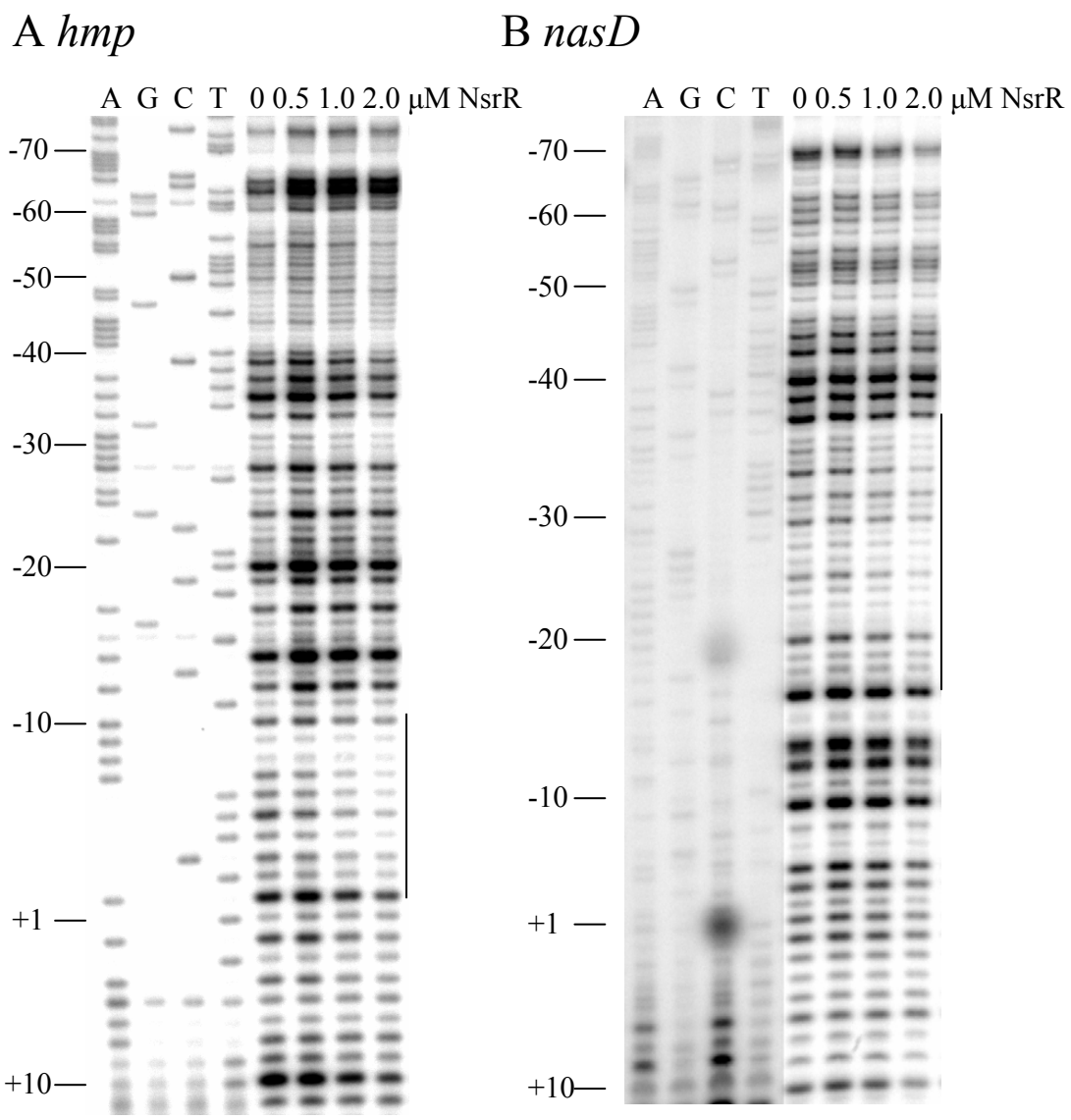



FIG. 5.8. DNase I footprinting analysis of *hmp* (A) and *nasD* (B) promoters. The labeled noncoding strands of each promoter fragment were used for binding reactions with NsrR. Regions strongly protected by NsrR from DNase I digestion are marked by solid lines. Dideoxynucleotide sequencing reactions using the same primer are also shown and nucleotide positions are marked relative to the transcription start site.

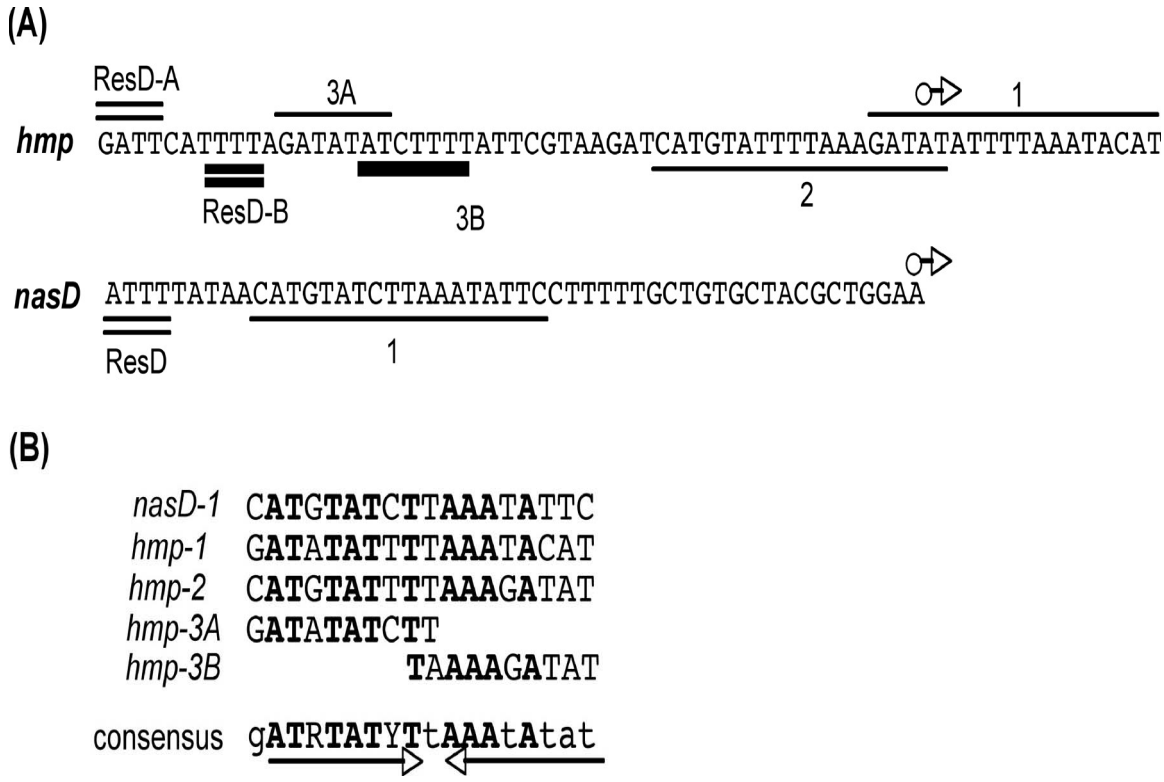



FIG. 5.9. Putative NsrR-binding sites in the *hmp* and *nasD* promoters. (A) Sequences similar to the proposed NsrR-binding site for gram-positive bacteria (Rodionov *et al.*, 2005) are shown by numbered lines. The 3A and 3B sites are half-sites, and the 3B site is on the noncoding strand. The ResD-binding sites proximal to the promoter identified in a previous work (Geng *et al.*, 2004) are marked by double lines labeled ResD. The most proximal ResD-binding site (ResD-B) is present on the opposite strand from the other ResD boxes. An arrow with a circle shows the start site and direction of transcription (LaCelle *et al.*, 1996; Nakano *et al.*, 1998). (B) Comparison of the putative NsrR-binding sites. Highly conserved and weakly conserved nucleotides in the putative consensus sequence are shown in uppercase bold and lowercase letters, respectively. An imperfect dyad symmetry sequence is shown by arrows. Abbreviations: R, A or G; Y, C or T.

# CHAPTER 6
# *BACILLUS SUBTILIS* RESD INDUCES EXPRESSION OF THE PUTATIVE REGULATORY GENES *YCLJK* UPON OXYGEN LIMITATION[4]

## 6.1 INTRODUCTION

Current knowledge about the adaptation of *Bacillus subtilis* to oxygen limitation in the environment has revealed a redox-dependent regulation of gene expression at the transcriptional level (Marino *et al.*, 2000; Nakano and Zuber, 1998, 2002; Ye *et al.*, 2000). A two-component regulatory system, composed of a histidine sensor kinase (ResE) and a response regulator (ResD), has a pivotal role in the metabolic adjustment required for anaerobic growth, with nitrate as a terminal electron acceptor(Nakano *et al.*, 1996; Sun *et al.*, 1996b). ResD and ResE are required for the transcription of genes involved in anaerobic nitrate respiration, including *fnr* (anaerobic gene regulator; Fnr), *nasDEF* (nitrite reductase operon), and *hmp* (flavohemoglobin) (Hoffmann *et al.*, 1998; LaCelle *et al.*, 1996; Nakano *et al.*, 1996; Nakano *et al.*, 1998). Furthermore, ResD and ResE also play an important role in another mode of anaerobic growth, i.e., fermentation (Cruz Ramos *et al.*, 2000; Nakano *et al.*, 1997), in which they are required for the full induction of *ldh* (lactate dehydrogenase) and *lctP* (lactate permease) expression (Cruz Ramos *et al.*, 2000). The genes encoding ResA, ResB, and ResC, which constitute an operon with the genes for ResD and ResE, are thought to be involved in cytochrome *c* biosynthesis (Sun *et al.*, 1996b). A recent study showed that ResA is required for the reduction of cysteinyl residues during heme binding to apocytochrome *c* (Erlendsson *et al.*, 2003). ResD and ResE are also required for the transcription of their own genes, which is initiated at the

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Härting, E., Geng, H., Hartmann, A., Hubacek, A., Münch, R., Ye, R.W., Jahn, D., and Nakano, M.M. (2004) *Bacillus subtilis* ResD induces expression of potential regulatory genes *yclJK* upon oxygen limitation. *J. Bacteriol. 186*: 6477-6484.

*resA* operon promoter (Sun *et al.*, 1996b). Previous studies demonstrated the physical interaction of ResD with the regulatory regions of *ctaA* (Zhang and Hulett, 2000), *resA* (Zhang and Hulett, 2000), *hmp* (Nakano *et al.*, 2000b), *nasD* (Nakano *et al.*, 2000b), and *fnr* (Nakano *et al.*, 2000b). ResD activates the transcription of *ctaA* (Paul *et al.*, 2001), *hmp* (Geng *et al.*, 2004; Nakano *et al.*, 2003b), *nasD* (Geng *et al.*, 2004), and *fnr* (Geng *et al.*, 2004) in vitro.

The ResD-ResE signal transduction system functions early in the anaerobic gene regulatory cascade. It activates the expression of other regulatory genes that also play important roles in anaerobic gene expression. One such gene encodes Fnr, which is needed for activation of the respiratory nitrate reductase operon *narGHJI* (Cruz Ramos *et al.*, 1995). Fnr also activates the transcription of another regulator gene, *arfM*. Anaerobic expression of the fermentative operons *ldh-lctP* (lactate fermentation) and *alsSD* (acetoin formation)(Marino *et al.*, 2001) and of the heme biosynthesis genes *hemN* and *hemZ* is partly dependent on ArfM (Homuth *et al.*, 1999).

A previous DNA microarray analysis showed that the two-component regulatory genes *yclJ* (encoding a potential response regulator) and *yclK* (encoding a potential sensor kinase) are induced by oxygen limitation (Ye *et al.*, 2000). YclK is likely one of the class IIIA family of kinases according to a classification based on sequence similarities in the vicinity of the phosphorylated histidine. The potential response regulator YclJ belongs to the OmpR family of transcriptional regulators according to alignments of sequences within the C-terminal domains of response regulators (Fabret *et al.*, 1999). A comprehensive DNA microarray analysis was undertaken to identify target genes of uncharacterized *B. subtilis* two-component regulatory systems (Kobayashi *et al.*, 2001). Seventeen genes were designated as being positively controlled by YclJK, while 11 appeared to be negatively controlled. For this paper, we examined how *yclJK* transcription is activated in response to oxygen limitation and determined whether the candidate target genes are regulated by YclJK.

## 6.2 RESULTS

### 6.2.1 Analysis of *yclJK* operon structure

In a microarray analysis, the *yclJ* and *yclK* genes for a potential two-component system were found to be induced under anaerobic conditions (Ye *et al.*, 2000). In order to analyze the expression and organization of these genes in detail, we performed a Northern blot analysis. A sequence analysis of the *yclJK* region showed that the *yclK* start codon resides eight nucleotides upstream of the *yclJ* stop codon (http://genolist.pasteur.fr/SubtiList/). The partial overlap between the *yclJ* and *yclK* open reading frames suggested that these genes constitute an operon. Northern analysis results listed in the BSORF *Bacillus subtilis* Genome Database (http://bacillus.genome.ad.jp/) indicated that *yclJ* and *yclK* are cotranscribed. We examined whether the two genes are coinduced by anaerobiosis by examining *yclJK* transcript levels on Northern blot membranes. Equal amounts of total RNAs isolated from wild-type cells cultured under aerobic and three different anaerobic conditions (fermentative, anaerobic with nitrate, and anaerobic with nitrite) were analyzed with a *yclJ*-specific RNA probe (Fig. 6.1). A single transcript of 2.1 kb, which corresponds to the size of the *yclJK* operon, was detected for RNAs isolated from anaerobically grown cells, whereas the transcript was barely detected in RNAs from aerobic cultures. A transcript of the same size was detected with a *yclK*-specific probe (data not shown). The *yclJK* transcript was not present in RNA prepared from a *resDE* mutant strain that was grown anaerobically. This result clearly demonstrated that the anaerobic induction of *yclJK* is dependent on *resDE*. The Northern blot analysis showed that *yclJ* and *yclK* are likely cotranscribed from a promoter residing upstream of the *yclJ* gene. The transcription start site was identified by primer extension analysis (Fig. 6.2). Upstream of the transcription start site a potential $\sigma^A$-type –10 (TATTAT) sequence was detected, but no sequence resembling a –35 region was present, suggesting the involvement of an additional activator for efficient transcription of *yclJK*.

### 6.2.2 Examination of *yclJ-lacZ* expression in various regulatory mutant strains

To gain further insights into the regulation of *yclJK* transcription, we fused the promoter region of *yclJ* to a promoterless *E. coli lacZ* gene. The expression of *yclJ-lacZ*

in wild-type cells was induced 8- to 10-fold under all employed anaerobic conditions compared to aerobic conditions (Fig. 6.3). In accordance with the results of the Northern blot analysis, the introduction of the *resDE* mutation completely abolished the anaerobic induction of *yclJK*. Only a residual expression of *yclJ-lacZ* was found in a *resE* mutant strain, indicating that the phosphorylation of ResD by ResE is required for the anaerobic induction of *yclJK* (Fig. 6.3). In contrast to the effect observed for the *resDE* mutant, mutations in *fnr* had no significant effect on *yclJ-lacZ* expression. In agreement with this finding, no obvious Fnr binding site was detected in the *yclJK* promoter region. The expression level of *yclJ-lacZ* in an *arfM* mutant strain was similar to the wild-type level as well (data not shown). These results indicate that ResDE does not exert its positive role through Fnr or ArfM and that it may play a more direct role in *yclJK* activation. The β-galactosidase activity in a *yclJ* mutant was reduced by half compared to that in the wild type, suggesting that *yclJK* expression is under moderate autoregulatory control. Nevertheless, ResD is the essential regulator of *yclJK* expression. YclJK is able to increase *yclJK* expression to some extent, but only in cooperation with ResD. Therefore, a *resD* mutation abolishes the expression of *yclJK*.

### 6.2.3 ResD binds to the *yclJ* promoter

We examined by eletrophoretic mobility shift assay (EMSA) the possibility that ResD directly binds to the *yclJ* promoter. A 250-bp DNA fragment carrying the *yclJ* promoter (positions –220 to +30) was end labeled with [$\gamma$-$^{32}$P]ATP and incubated with increasing amounts of purified recombinant ResD in the presence or absence of ResE, followed by electrophoresis in order to resolve DNA-protein complexes (Fig. 6.4A). The results indicated that ResD forms a stable complex with the *yclJ* promoter. However, the phosphorylation of ResD via ResE only slightly stimulated DNA binding.

To localize the ResD binding site in the *yclJ* promoter, we performed DNase I footprinting analysis (Fig. 6.5). A strongly protected area between positions –92 and –73 relative to the transcription start site was observed on both strands, and ResD-P protected the same regions with a similar affinity as unphosphorylated ResD.

### 6.2.4 Phosphorylation of ResD by ResE is needed for maximal transcriptional activation of *yclJK*

Since the binding of ResD to the *yclJ* promoter was not stimulated by phosphorylation, we investigated whether phosphorylation is mainly required for transcriptional activation by using an in vitro transcription assay. In the absence of ResD and ResE, the transcript of *yclJ* was barely detected (Fig. 6.6, lane 1). Increasing amounts of unphosphorylated ResD only slightly stimulated in vitro transcription (Fig. 6.6, lanes 2 to 4). However, in vitro transcription was significantly stimulated when both ResD and ResE were present (Fig. 6.6, lanes 5 to 7). This indicated that the phosphorylation of ResD is required for full transcriptional activation. It was shown previously that the aspartate residue at position 57 of ResD is the phosphorylation site of the response regulator. A mutant ResD protein carrying alanine at position 57 (D57A) can no longer be phosphorylated by ResE (Geng *et al.*, 2004). To further determine the effect of ResD phosphorylation on *yclJK* transcription in vitro, we tested the mutant ResD D57A protein with the in vitro transcription assay. Transcription was stimulated by the mutant ResD D57A protein to a small extent, similar to that by unphosphorylated wild-type ResD (Fig. 6.6, compare lanes 2 to 4 with lanes 8 to 10). Moreover, the level of the *yclJK* transcripts was not further increased by ResD D57A in the presence of ResE (Fig. 6.6, lanes 11 to 13). These results clearly demonstrated that unphosphorylated ResD, it binds to the promoter region, but is not sufficient to activate the maximal transcription of *yclJK*. The low level of transcription that was activated by unphosphorylated ResD and the D57A mutant was likely due to the phosphorylation-independent activation of ResD as previously described (Geng *et al.*, 2004). These results, together with the results of the *yclJ-lacZ* expression analysis with the *resE* mutant strain, showed that the phosphorylation of ResD by ResE is required for full anaerobic induction of *yclJK*.

### 6.2.5 New definition of ResD binding sites by a bioinformatic approach

We used an extended information weight matrix model to find a better definition of ResD binding sites. The DNA binding regions of ResD defined by footprinting analyses are part of the Prodoric database (Münch *et al.*, 2003). They were used to create a weight matrix model, which is presented as a sequence logo (Fig. 6.7A) (Schneider and

Stephens, 1990). The newly defined sequence is 21 bp long, with two stretches of conserved residues, specifically TTGT (positions 4 to 7) and TTTT (positions 13 to 16). The information content of these sequences is higher than 1, indicating the occurrence of major groove contacts with the DNA helix (Schneider and Stephens, 1990). The spacing of 10 between the conserved regions corresponds to one turn of the DNA helix and is typical for DNA binding motifs. The formerly proposed consensus recognition sequence for ResD, TTTGTGAAT (Nakano *et al.*, 2000b; Zhang and Hulett, 2000), corresponds to the first part of the newly defined binding sequence. In the *yclJ* promoter, the binding motif was detected on the opposite strand at positions –74 to –94 (Fig. 6.7B) and corresponds to the protected region from the footprinting analysis (Fig. 6.5).

### 6.2.6 Mutagenesis studies of *yclJ* promoter

Although ResD binds directly to several promoters, no detailed mutagenesis study of the ResD binding sequence is available. Based on the new computer-aided model of the ResD binding site, we exchanged three highly conserved T and two A residues in each binding part. The two mutated promoters were fused to *lacZ* and tested for β-galactosidase activity as described above. Both series of mutations almost completely abolished the anaerobic induction of *yclJ-lacZ* (Fig. 6.8), indicating that the mutated regions bear one or more nucleotide substitutions that impair ResD-dependent activation. These results suggest that the mutationally altered sequence contains the site of the ResD-DNA interaction. Confirmation of the new consensus awaits more detailed mutational analysis. To examine whether ResD binds to the mutant promoters, we performed EMSAs with the *yclJ* promoter containing the mutated a and b ResD binding sites. A concentration of 0.5 μM ResD was sufficient for almost complete binding to the wild-type *yclJ* promoter fragment, but this concentration of ResD resulted in little, if any, binding to the mutant *yclJ* promoters (Fig. 6.4B). A competition experiment using excess cold DNA showed that the wild-type DNA fragment, at concentrations as low as 2.5 nM, showed significant competition with the labeled probe. In contrast, a 10 nM concentration of the mutant *yclJ* promoters failed to compete for ResD binding to the *yclJ* promoter (Fig.6.4C).

### 6.2.7 The *YclJK* regulon

To investigate the potential participation of YclJK in anaerobic growth processes, we analyzed the phenotype caused by an *yclJ* mutation. The deletion of *yclJ* had no obvious influence on aerobic or anaerobic growth. Moreover, the processes of sporulation and competence were not influenced by the *yclJ* mutation (data not shown). Subsequently, we analyzed the expression of several genes which are known to play an important role in the anaerobic metabolism of *B. subtilis*. The expression of *narG* (nitrate reductase), *nasD* (nitrite reductase), *arfM* (modulator of anaerobic respiration and fermentation), *hmp* (flavohemoglobin), *ldh* (L-lactate dehydrogenase), and *alsS* (alpha-acetolactate synthase) was studied by using reporter gene fusions in the *yclJ* mutant strain during aerobic and anaerobic growth. However, the *yclJ* mutation had no significant effect on the expression of any of these genes (data not shown).

A previous DNA microarray study identified genes that are possibly regulated by YclJK (Kobayashi *et al.*, 2001). Candidate genes activated by YclJK were *gerKB*, *pyrR*, *dhbA*, *dhbB*, *fhuD*, and several genes of unknown function. Those repressed by YclJK included *acoA*, *acoB*, *acoC*, *acoL*, *atpA*, *atpD*, *atpE*, *qoxB*, and *qoxC*. Since *dhbA* and *dhbB*, which are involved in siderophore 2,3-dihydroxybenzoate biosynthesis(Rowland *et al.*, 1996), were shown to be induced by oxygen limitation(Ye *et al.*, 2000), we tested whether these genes are regulated by YclJK. The expression of a *dhbA-lacZ* reporter gene fusion (Rowland and Taber, 1996) was examined in the wild type and the *yclJ* mutant strain during aerobic and anaerobic growth. As expected, *dhbA-lacZ* expression was higher upon anaerobic cultivation. However, the higher level of anaerobic expression was not dependent on YclJ (data not shown). The *yclI* gene was also reported to be activated by YclJK(Kobayashi *et al.*, 2001). Since *yclI* is transcribed divergently from the *yclJK* operon, one might expect that its expression would be affected by YclJK. We constructed an *yclI-lacZ* reporter gene fusion and compared the β-galactosidase activities of wild-type cells and the *yclJ* mutant strain cultivated under aerobic and anaerobic conditions. The expression of *yclI-lacZ* was two- to threefold higher under anaerobic conditions than under aerobic conditions. However, there was no significant difference in observed expression between the wild-type and *yclJ* mutant strains.

The *qoxB* and *qoxC* genes, which were identified as being negatively affected by YclJK, encode subunits of cytochrome *aa3* quinol oxidase. They are transcribed from the *qoxA* promoter (Santana *et al.*, 1992). An examination of a *qoxA-lacZ* reporter gene fusion revealed that YclJK does not affect expression of the *qox* operon.

The discrepancy between the results of our *lacZ* reporter gene experiments and the DNA microarray analysis described by Kobayashi and coworkers was most likely caused by the different experimental conditions employed. For their DNA microarray analysis, Kobayashi et al. overproduced the response regulator YclJ in the absence of the sensor kinase YclK under aerobic conditions. This strategy is applicable to certain classes of two-component regulatory systems, such as CitST and DesKR. However, it is not applicable to others. For example, the overproduction of ResD in the absence of ResE does not result in activation of the ResDE regulon under anaerobic conditions (M. M. Nakano, unpublished result).

In order to identify genes of the YclJK regulon, we isolated RNAs from the wild type and a *yclJK* mutant grown under a variety of anaerobic conditions and then performed various DNA microarray analyses. Since *yclJK* is almost exclusively expressed under anaerobic conditions, the stimulus for the YclJK system might be present only under anaerobic conditions. As expected from the initial *lacZ* fusion analysis described above, the expression of the genes identified as YclJK targets in the previous microarray experiment (Kobayashi *et al.*, 2001) was not significantly affected by the *yclJ* mutation in our microarray experiment (data not shown). Moreover, in six independent microarray tests, we reproducibly detected only minor changes of less than twofold in the transcriptional profiles when the wild type and the *yclJ* mutant were compared. The only exception was the expression of the *yclJK* operon itself, which was found to be repressed by a factor of about 30 in the *yclJ* mutant, which is in agreement with the results for *yclJ-lacZ* reporter gene expression. Besides *yclJK* itself, no target genes of YclJK have been identified. YclJK-denpedent genes can not be identified by the approach of microarray analysis using the *yclJK* strain probably because an additional signal is required for activation of YclJK-dependent genes. To identify YclJ binding regions, chromatin immunoprecipitation in conjunction with gene microarray analysis (ChIP-on-chip) might be useful (Ren *et al.*, 2000).

## 6.3 DISCUSSION

The ResD-ResE signal transduction system plays an important role in the anaerobic metabolism of *B. subtilis* and probably other low-GC-content gram-positive bacteria (Nakano and Zuber, 2002). A recent genome sequence analysis has shown that ResD orthologs exist in other gram-positive bacteria, such as *Bacillus anthracis*, *Bacillus stearothermophilus*, *Bacillus halodurans*, *Listeria monocytogenes*, and *Staphylococcus aureus* (NCBI Microbial Genome BLAST databases). The *resD* and *resE* orthologs of *S. aureus*, *srrAB* (or *srhSR*), participate in the regulation of energy metabolism in response to variations in oxygen tension (Throup *et al.*, 2001) and in the control of virulence factor expression (Yarwood *et al.*, 2001). An *srhSR* mutant exhibited reduced survival in animal hosts (Yarwood *et al.*, 2001). In this paper, we have shown that ResDE regulates the anaerobic induction of the potential two-component *yclJK* system. Northern blot, reporter gene fusion, and in vitro transcription experiments showed that ResD upregulates *yclJK* transcription upon oxygen limitation. EMSA and DNase I footprinting experiments demonstrated a ResD interaction with the *yclJ* promoter region. Although the phosphorylation of ResD only slightly enhanced binding to the *yclJ* promoter, in vitro transcription experiments showed that the phosphorylation of ResD greatly stimulates *yclJ* transcription. It is likely that the phosphorylation of ResD affects the interaction of ResD with RNA polymerase at the *yclJ* promoter.

We proposed a putative ResD binding site by using a bioinformatic approach. ResD likely binds as a dimer to the 21-bp sequence of the ResD box consisting of two tandemly arranged 10-bp half-sites (Fig. 6.7A). In the 21-bp sequence, positions 4, 5, 7, 14, and 15 were the most conserved. Position 6 is usually a G (but is a C in one of the ResD binding sites in *ctaA*), and positions 16 and 18 are occupied by T, except for an A in *nasD*. Stretches of sequences from positions 1 to 3 (ATT) and positions 9 to 11 (ACA) are identical only in *hmp* and *nasD*. Among known members of the ResDE regulon, the oxygen-dependent induction of *hmp* and *nasD* is the highest. It remains to be examined whether these sequences are important for the high-affinity binding of ResD to these promoters.

The physiological role of YclJK remains unsolved. Which signal is transmitted by the YclJK regulatory system and whether its involvement in gene regulation is related to anaerobiosis are still unknown. The *yclJ* mutant showed no obvious growth defect when it was tested under various aerobic and anaerobic conditions. The *yclJ* mutant was able to grow well under anaerobic conditions that facilitated either nitrate respiration or fermentation (data not shown). Mutant phenotypes of *yclJ* are not observed in studies listed in the BSORF *Bacillus subtilis* Genome Database. We also examined the effect of the *yclJ* mutation on the expression of genes that were predicted to be regulated by YclJK according to a previously reported DNA microarray analysis (Kobayashi *et al.*, 2001), and we examined DNA arrays ourselves. Our results with promoter-*lacZ* fusions showed that none of the genes tested was significantly regulated by *yclJK* under the growth conditions used. It is possible that oxygen limitation, which activates *yclJK* transcription, is not sufficient to activate the YclJK signal transduction system. Further studies are needed to identify the signal that is transmitted by YclJK, the genes that are regulated by YclJK, and the physiological role of YclJK in *B. subtilis*.

## 6.4 MATERIALS AND METHODS

### 6.4.1 Bacterial strains and growth conditions

All *B. subtilis* strains used for this work are listed in Table 6.1. Luria-Bertani medium was used for standard cultures of *B. subtilis* and *Escherichia coli* unless otherwise indicated. For investigations of the expression of various *lacZ* fusions and for preparations of RNA, the strains were grown at 37°C in minimal medium (80 mM $K_2HPO_4$, 44 mM $KH_2PO_4$, 0.8 mM $MgSO_4 \cdot 7H_2O$, 1.5 mM thiamine, 40 μM $CaCl_2 \cdot 2H_2O$, 68 μM $FeCl_2 \cdot 4H_2O$, 5 μM $MnCl_2 \cdot 4H_2O$, 12.5 μM $ZnCl_2$, 24 μM $CuCl_2 \cdot 2H_2O$, 2.5 μM $CoCl_2 \cdot 6H_2O$, 2.5 μM $Na_2MoO_4 \cdot 2H_2O$, 50 mM glucose, 50 mM pyruvate, 1 mM L-tryptophan, 0.8 mM L-phenylalanine), and where indicated, 10 mM nitrate or 10 mM nitrite was added. For aerobic growth, 100 ml of medium was inoculated at an optical density at 578 nm ($OD_{578}$) of 0.05 with an aerobically grown overnight culture and then incubated in a 500-ml baffled flask with shaking at 250 rpm. For anaerobic fermentative growth, the bacteria were incubated in completely filled flasks

with rubber stoppers and with shaking at 100 rpm in an incubation shaker to minimize aggregation of the bacteria. Inoculation was performed aerobically with an aerobically grown overnight culture with an $OD_{578}$ of 0.3. Anaerobic conditions were achieved after a short time through the consumption of residual oxygen by the inoculated bacteria. After 3 h in the midst of the exponential growth phase and after 6 h at the beginning of the stationary growth phase, samples for β-galactosidase assays were taken. The cells for preparations of RNA were harvested after 3 h in the midst of the exponential growth phase.

### 6.4.2 Construction of B. subtilis *yclJK* mutant strain

A 1,197-bp PCR fragment containing parts of the coding region of *yclJK* was amplified by PCR with primers EH100 5'-GGTTTTGAAGCCGAATTCGTTCATGAC-3' (the 5' end corresponds to position 69 of the *yclJ* coding sequence) and EH101 5'-CGCTTCTCTCAGCTCTAGAATCCGCTTGAC-3' (the 5' end corresponds to position 593 of the *yclK* coding sequence, with two base changes to create an internal XbaI restriction site [underlined]). This fragment was cleaved at an internal KpnI site (positions 106 to 112 of the *yclJ* coding sequence) and at the XbaI site and ligated into vector pBluescript SK(+) II (Stratagene) digested with the same restriction enzymes. The plasmid was then cut at an internal HindIII restriction site in the *yclJK* fragment, and an erythromycin resistance gene cassette liberated from the vector pDG646 (Guérout-Fleury *et al.*, 1995) by HindIII digestion was inserted. The plasmid was transformed into *B. subtilis* strain JH642 and screened for erythromycin-resistant clones. The desired double-crossover event was confirmed by PCR and Southern blot analysis. The resulting mutant strain, BEH1, carries an inactivated *yclJ* gene.

### 6.4.3 Preparation of RNA and Northern blot analysis

For preparations of RNA, 25 ml of cell culture was added to 25 ml of an ice-cold solution containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 20 mM $NaN_3$. The cells were harvested by centrifugation (5 min at 7,155 x *g* and 4°C). The cell pellets were resuspended in 200 µl of supernatant, immediately dropped into a Teflon disruption vessel, filled, and precooled with liquid $N_2$. The cells were disrupted with a Mikro-

Dismembrator S instrument (B. Braun Biotech International, Melsungen, Germany) for 2 min at 2,600 rpm. The resulting frozen powder was resuspended in 1 ml of prewarmed (50°C) cell lysis solution consisting of 4 M guanidine thiocyanate, 25 mM sodium acetate (pH 5.2), and 0.5% (wt/vol) *N*-laurylsarcosine. After complete cell lysis, the solution was immediately placed on ice. The RNA was extracted twice with acidic phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol/vol]) and once with chloroform-isoamyl alcohol (24:1 [vol/vol]). After ethanol precipitation, the RNA pellet was resuspended in 180 μl of 20 mM phosphate buffer (pH 6.5)-1 mM EDTA and 20 μl of a solution containing 200 mM sodium acetate (pH 4.5), 180 mM $MgCl_2$, 100 mM NaCl, and 15 U of DNase I and then was incubated for 30 min at room temperature. Twenty microliters of 250 mM EDTA, pH 7.0, was added, followed by phenol-chloroform extraction and ethanol precipitation. The resulting RNA pellet was dissolved in 50 μl of $H_2O$. For Northern blot analysis, 10 μg of RNA was separated under denaturing conditions in a 1% agarose-670 mM formaldehyde-morpholinepropanesulfonic acid gel, stained with ethidium bromide, and transferred to a positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) by vacuum blotting. The approximate sizes of the mRNAs were estimated by the use of RNA standards (Bethesda Research Laboratories, Inc.) labeled with digoxigenin. Hybridization and detection was performed as described elsewhere (Engler-Blum *et al.*, 1993). A digoxigenin-labeled RNA probe was synthesized in vitro with T7 RNA polymerase and a 556-bp *yclJ*-specific PCR fragment as a template, previously amplified with the following primers: EH34, 5'-TATGTACGATGACGGAGATG-3'; and EH35, 5'-CTAATACGACTCACTATAGGGAGATAAAATTGATAGCCCCATAC-3'.

### 6.4.4 Construction of reporter gene fusion and site-directed mutagenesis of *yclJ* regulatory region

A transcriptional fusion between the *E. coli lacZ* gene and the *yclJ* upstream region was constructed. A 545-bp PCR fragment spanning the region from positions –495 to +50 relative to the translational start of *yclJK* was amplified with the primers EH29 (5'-CGAGGAATTCGCATCAGACACTTT-3') and EH28 (5'-TAATGGATCCGTCATCGTACATACA-3'). Using the restriction sites for EcoRI and BamHI created by the primers (underlined), we cloned the promoter region of *yclJK* into

the plasmid pDIA5322(Martin-Verstraete *et al.*, 1992), resulting in plasmid P*yclJ-lacZ*. This plasmid was transformed into *B. subtilis* strains JH642, LAB2135 (Δ*resDE*) (Nakano *et al.*, 1996), LAB2234 (Δ*resE*) (Nakano *et al.*, 1997), THB2 (Δ*fnr*) (Hoffmann *et al.*, 1998), and BEH1 (Δ*yclJ*), and transformants were screened for double-crossover integration at the *amyE* locus.

The potential ResD binding sites were changed from TATTTTTTTCATAC to TcTTggTcTCATgC (part a) and from TAGATTGTTCATAT to TcGAggGcTCATgT (part b), respectively (exchanged bases are shown in lowercase). Crossover PCRs were performed with the following two primers containing the desired base exchanges (in bold letters): EH88 (5'-GAAAAAAA**C**ATGA**G**C**C**TC**G**ATTTCTAG-3') and EH89 (5'-CTAGAAAT**C**GA**GG**G**C**TCAT**G**TTTTTTTC-3') for part a and EH86 (5'-TCCAAAGG**C**ATGA**G**A**C**CAA**G**ATGAAC-3') and EH87 (5'-GTTCAT**C**TT**GG**T**C**TCAT**G**CCTTTGGA-3') for part b. Two PCR products were generated with primer pairs EH28-EH88 (180 bp) and EH29-EH89 (393 bp) for part a and with primer pairs EH28-EH86 (191 bp) and EH29-EH87 (380 bp) for part b. In a second PCR, we used the first two PCR products as templates and amplified the whole promoter with the primer pair EH28-EH29. The complete promoter fragments were cloned into the plasmid pDIA5322, as described above for the wild-type sequence, resulting in the plasmids pmuta *yclJ-lacZ* and pmutb *yclJ-lacZ*. After transformation into *B. subtilis* strain JH642, strains BEH6 and BEH7 were obtained.

### 6.4.5 Identification of *yclJK* transcription start site

Fifty micrograms of RNA was used for a primer extension analysis of the *yclJ* transcript. Reverse transcription was initiated from the γ-$^{32}$P-end-labeled primer EH36 (5'-CGTCATCGTACATACACTAACATTATC-3') by a standard procedure (Ausubel *et al.*, 1995). The sequencing reaction was performed with the same primer. The primer extension products and the sequencing reactions were analyzed in a 6% denaturing polyacrylamide gel in Tris-borate buffer. The dried gel was analyzed by use of a phosphorimager.

### 6.4.6 Measurement of *yclJ-lacZ* expression

For β-galactosidase assays, cells were harvested by centrifugation 3 h after inoculation. β-Galactosidase activities were determined by a standard method and are given in Miller units (Miller, 1972).

### 6.4.7 Prediction of ResD binding sites

A model of the ResD binding site was created by computing an information weight matrix with the following equation: $R_{iw} = 2 + \log_2 f(b,l) - e(n)$ (bits per base), where $f(b,l)$ is the frequency of each base ($b$) at position $l$ in the aligned binding sites and $e(n)$ is a small sample size correction factor(Schneider, 1997). By adding the weights together for various positions in a particular binding site, we could measure the total individual information ($R_i$) in bits. We employed Virtual Footprint software for pattern searches and the prediction of potential binding sites (Münch *et al.*, 2003). This program is able to use the information weight matrix model and is connected interactively to the Prodoric database (http://prodoric.tu-bs.de). The sequence logo was created with Web-Logo software (http://weblogo.berkeley.edu).

### 6.4.8 Electrophoretic mobility shift assays (EMSAs)

The ResD and ResE proteins were overproduced in *E. coli* carrying pXH22 or pMMN424, respectively, and were purified by chromatography as described previously (Nakano *et al.*, 2000b). DNA fragments carrying the *yclJ* promoter region (–220 to +30 with respect to the *yclJ* transcription start site) were amplified by PCR with the primers oMN02-201 (5'-GCCTTCATATTCCAAAAG-3') and oMN02-202 (5'-TATATCCTCCGGTTGTTT-3') and with JH642 chromosomal DNA as a template. Mutant *yclJ* promoters were amplified from plasmids pmuta *yclJ-lacZ* and pmutb *yclJ-lacZ* with the same oligonucleotides. The primers were 5' end labeled on the coding and noncoding strands with T4 polynucleotide kinase and [$\gamma$-$^{32}$P]ATP. DNA fragments were produced by PCR as described above, but with one labeled and one unlabeled primer. The radiolabeled promoter fragments were separated in 6% nondenaturing polyacrylamide gels and then purified in Elutip-d columns (Schleicher & Schuell, Dassel, Germany). Unlabeled probes employed in competition experiments were produced by PCR as well

and were purified in 1.2% agarose gels, followed by fragment recovery by use of a gel extraction kit (Qiagen, Hilden, Germany).

EMSAs were performed by incubating the labeled fragments (0.18 pmol or 1,000 cpm per reaction) with the indicated amounts of the ResD protein, with or without ResE, in 20 μl of reaction buffer [50 mM Tris-HCl (pH 7.5), 100 mM KCl, 0.1 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol, 5 mM $MgCl_2$, 50 μg of poly(dI-dC)/ml, 50 μg of bovine serum albumin (BSA)/ml, 0.25 mM ATP]. After incubation for 15 min at room temperature, the reaction mixtures were separated in 5% nondenaturing polyacrylamide gels in Tris-acetate buffer. The gels were dried and analyzed by use of a phosphorimager (Molecular Dynamics, Sunnyvale, Calif.). In vitro phosphorylation assays using [$\gamma$-$^{32}$P] ATP showed that ResD was phosphorylated under these conditions.

### 6.4.9 DNase I footprinting analysis

The *yclJ* fragment, labeled as described above (50,000 cpm per reaction), was incubated with 2 to 6 μM ResD and/or ResE protein in the same buffer as that used for EMSAs, except that glycerol, poly (dI-dC), and BSA were omitted. The reaction was treated with 60 ng of DNase I at room temperature for 20 s for free probes and 40 s for reactions containing the protein(s). The same primers used for the labeling of DNA fragments were used for sequencing of the template DNAs with a Thermo Sequenase cycle sequencing kit (USB, Cleveland, Ohio). The sequencing reactions were run together with the footprinting reactions in 8% polyacrylamide-urea gels in Tris-borate buffer. The dried gels were analyzed by phosphorimaging.

### 6.4.10 In vitro transcription assay

A mutant ResD protein (D57A) was overproduced in *E. coli* pMMN539. ResE and wild-type and mutant ResD proteins were chromatographically purified as described previously (Geng *et al.*, 2004). The linear template of *yclJ* (–129 to +116) used for in vitro transcription assays was PCR amplified with the primers oHG-11 (5'-CCCCTTGCTGATAAATTAATA-3') and oHG-12 (5'-AATTCGGCTTCAAAACCTTCT-3'). The PCR products were purified with a PCR purification kit (Qiagen). In vitro transcription buffer contained 25 mM Tris-HCl (pH 7.5),

100 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 5 mM $MgCl_2$, 0.25 mM ATP, 50 μg of BSA/ml, 10% glycerol, and 0.4 U of RNasin RNase inhibitor (Promega)/μl. ResD (0.35, 0.7, and 1.4 μM), ResE (1 μM), or both were incubated in 20 μl of transcription buffer at room temperature for 10 min. RNA polymerase and templates were added at final concentrations of 25 and 5 nM, respectively, and the reaction mixtures were incubated for 10 min at room temperature. ATP, GTP, CTP (each at 100 μM), UTP (25 μM), and 5 μCi of [$\alpha$-$^{32}$P] UTP (800 Ci/mmol) were added to start transcription. After incubation at 37°C for 20 min, 10 μl of stop solution (1 M ammonium acetate, 100 μg of yeast RNA/ml, 30 mM EDTA) was added. The nucleic acids in the reaction mixture were precipitated with ethanol, and the pellet was dissolved in 3.5 μl of loading dye solution (7 M urea, 100 mM EDTA, 5% glycerol, 0.05% bromophenol blue). The transcripts were analyzed in a urea-8% polyacrylamide gel. RNA markers were prepared according to the Decade marker system protocol (Ambion Inc., Austin, Tex.).

**TABLE 6.2 Bacterial strains used for this study**

| *B. subtilis* strain | Genotype | Source or reference |
|---|---|---|
| JH642 | *trpC2 pheA1* | BGSC[a] |
| LAB2135 | *trpC2 pheA1* Δ*resDE*::*tet* | (Nakano *et al.*, 1996) |
| LAB2234 | *trpC2 pheA1* Δ*resE*::*spec* | (Nakano *et al.*, 1997) |
| THB2 | *trpC2 pheA1 fnr*::*spec* | (Hoffmann *et al.*, 1998) |
| BEH1 | *trpC2 pheA1* Δ*yclJ*::*ery* | This study |
| BEH2 | *trpC2 pheA1 amyE*::*yclJ-lacZ cat* | This study |
| BEH3 | *trpC2 pheA1* Δ*yclJ*::*ery amyE*::*yclJ-lacZ cat* | This study |
| BEH4 | *trpC2 pheA1 fnr*::*spec amyE*::*yclJ-lacZ cat* | This study |
| BEH5 | *trpC2 pheA1* Δ*resDE*::*tet amyE*::*yclJ-lacZ cat* | This study |
| BEH6 | *trpC2 pheA1 amyE*::*muta yclJ-lacZ cat* | This study |
| BEH7 | *trpC2 pheA1 amyE*::*mutb yclJ-lacZ cat* | This study |

[a] BGSC, *Bacillus* Genetic Stock Center.

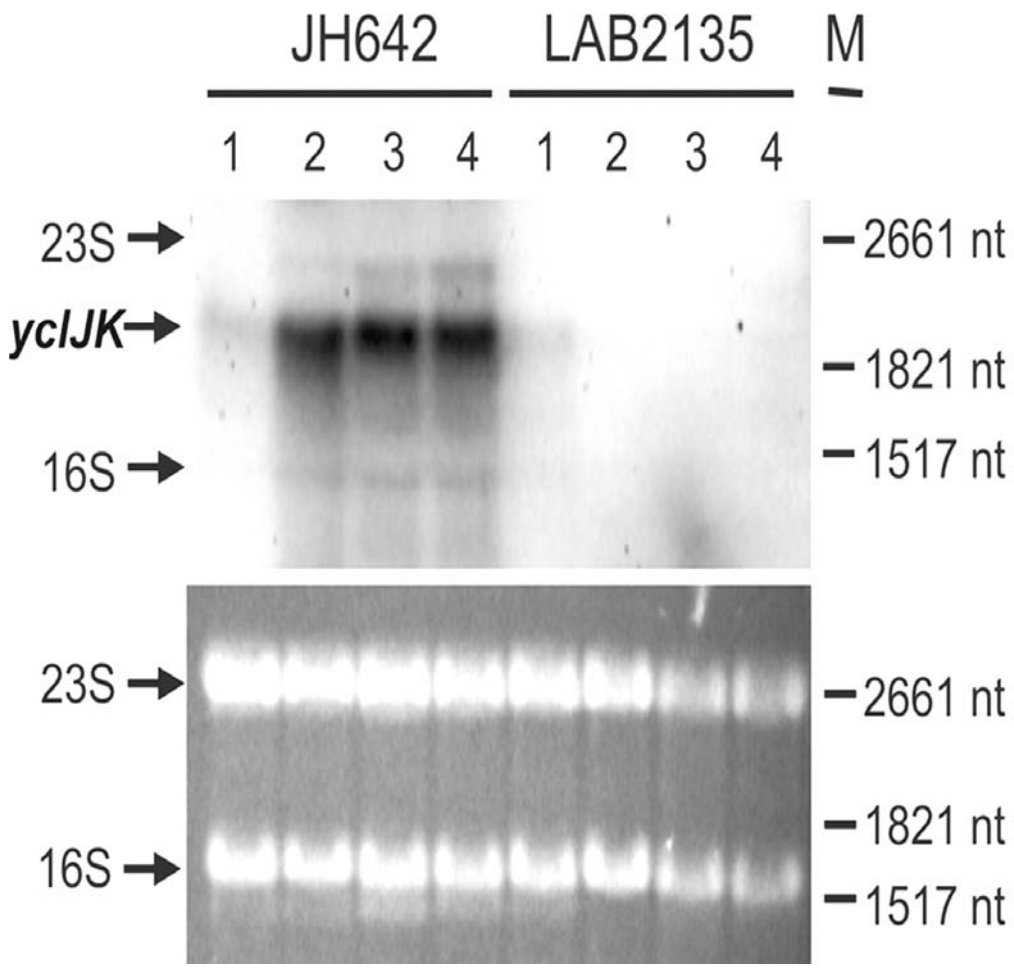


FIG. 6.1. Anaerobic expression of *yclJK* operon is ResDE dependent. Total RNAs were extracted from wild-type strain JH642 and the *resDE* mutant strain LAB2135 grown under the following growth conditions: aerobic (1), fermentative (2), anaerobic plus nitrate (3), and anaerobic plus nitrite (4). RNAs (10 μg) were separated in a 1% denaturing agarose gel and analyzed by Northern blotting. A *yclJ*-specific RNA probe was used for hybridization. A single transcript of 2.1 kb was detected, which corresponds to the size of a *yclJK* transcript. Ethidium bromide staining of the gel showed that equal amounts of RNA were analyzed. The size standards are RNA molecular weight marker no. 1 (Roche Diagnostics GmbH) and the 16S and 23S rRNA species.

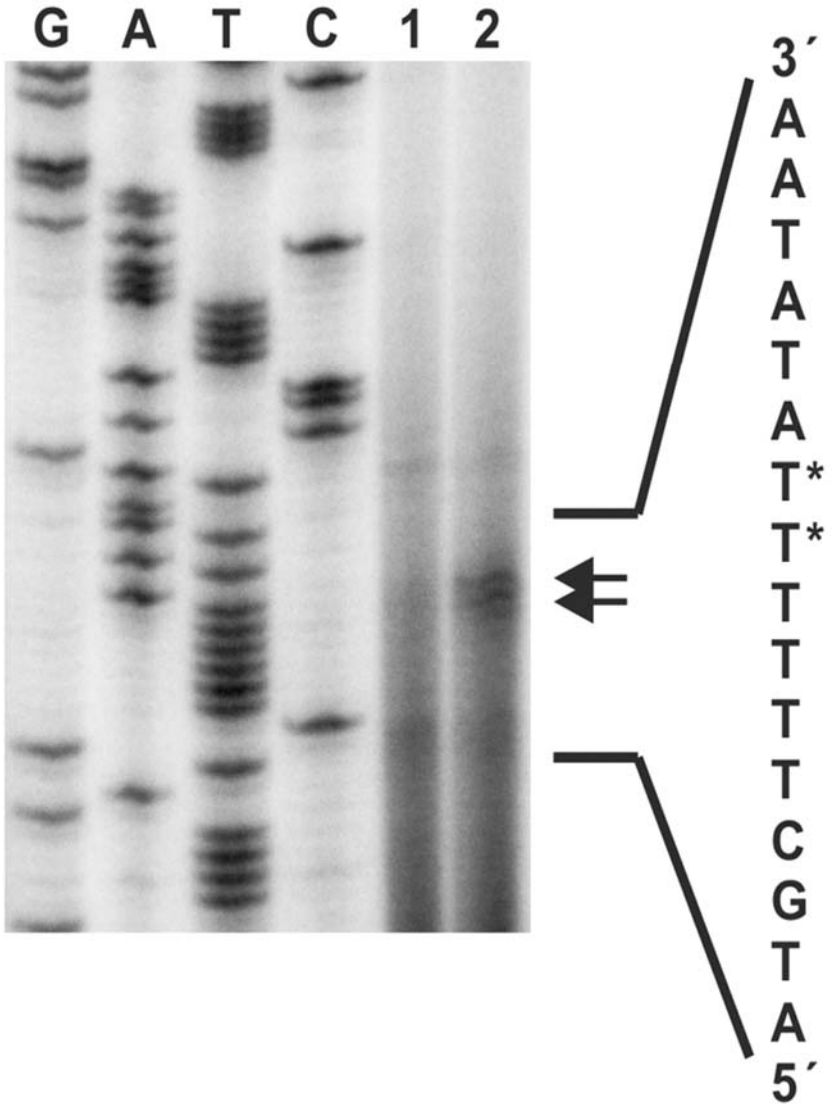


FIG. 6.2. Determination of transcription start site of *yclJK* by primer extension analysis. The total RNA was isolated and analyzed from JH642 cells grown aerobically (1) and under fermentative conditions (2). The same primer used for the primer extension analysis was used for sequencing reactions (lanes G, A, T, and C). Arrows indicate the primer extension products and asterisks mark the 5' end of the *yclJK* mRNA in the sequence.

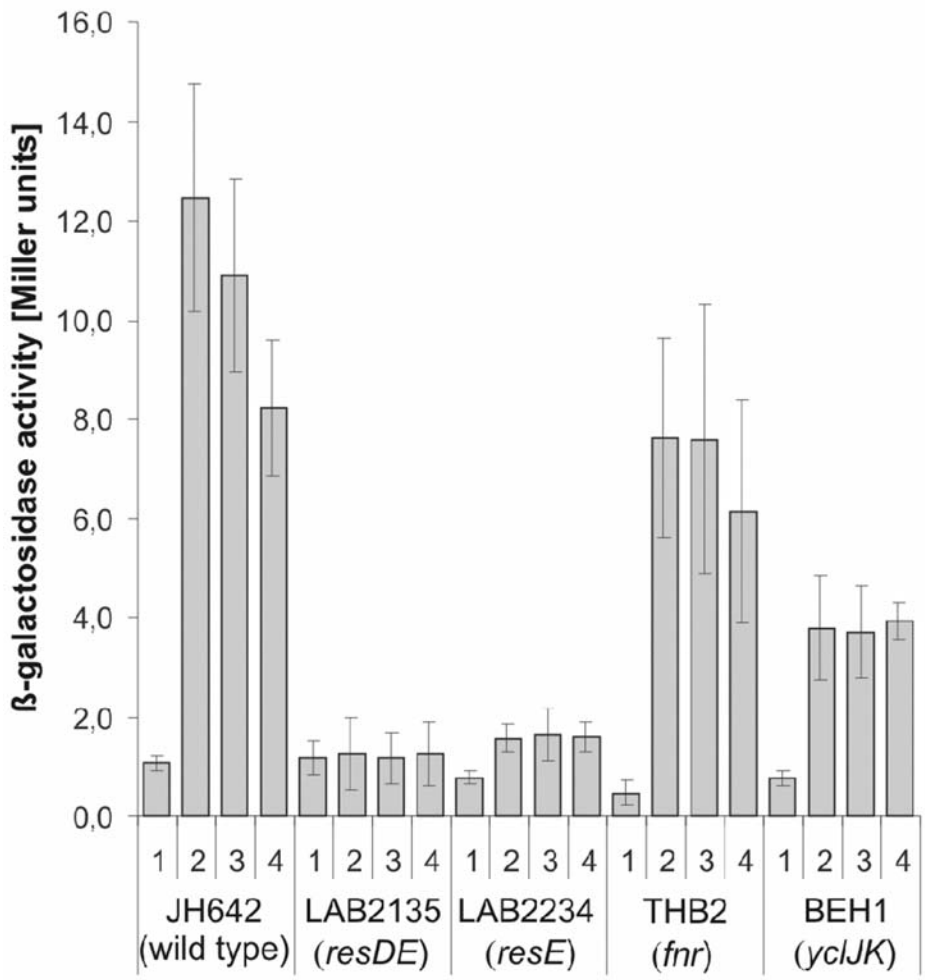


FIG. 6.3. Expression of *yclJ-lacZ* in various regulatory mutant strains. β-Galactosidase activities were measured in the JH642 wild-type strain and *resDE*, *resE*, *fnr*, and *yclJ* mutant strains after 3 h of culture under the following growth conditions: aerobic (1), fermentative (2), anaerobic plus nitrate (3), and anaerobic plus nitrite (4). Error bars indicate standard deviations ($n \geq 3$).

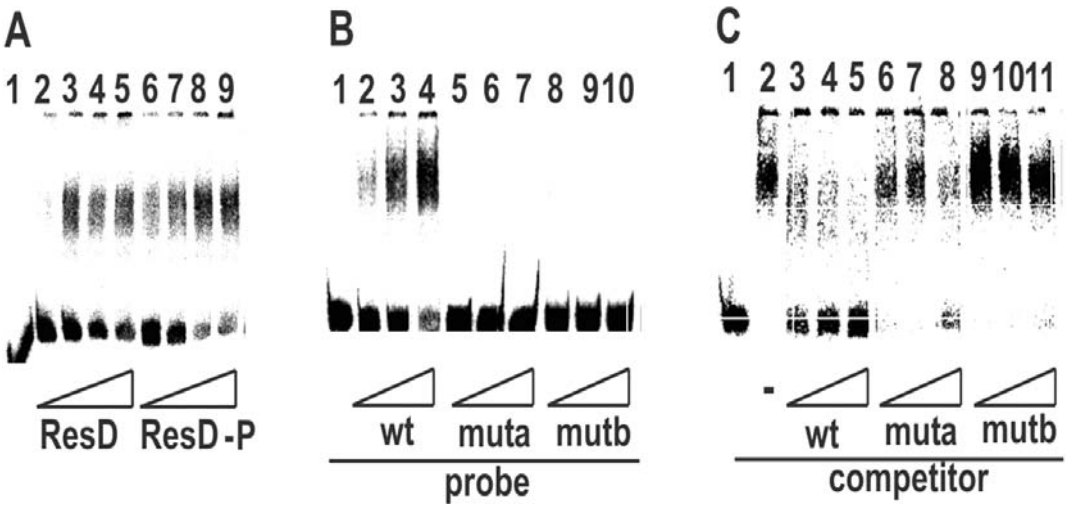


FIG. 6.4. EMSAs to detect binding of ResD to *yclJ* promoter. (A) An end-labeled DNA fragment containing the *yclJ* promoter was incubated with ResD in the presence (ResD-P) or absence (ResD) of 0.5 μM ResE. The amounts of ResD used were 0.06 μM (lanes 2 and 6), 0.13 μM (lanes 3 and 7), 0.25 μM (lanes 4 and 8), and 0.5 μM (lanes 5 and 9). Lane 1, probe only. (B) ResD does not bind to *yclJ* promoters carrying mutations in the putative ResD box. The same DNA fragments containing the wild-type and mutant promoters were used to examine interactions with ResD phosphorylated with 0.5 μM ResE. The ResD concentrations used were 0.13 μM (lanes 2, 5, and 8), 0.25 μM (lanes 3, 6, and 9), and 0.5 μM (lanes 4, 7, and 10). Lane 1, probe only. (C) Competition experiment with cold DNA fragment containing the wild-type and mutant promoters. The labeled *yclJ* promoter fragment was incubated with 0.5 μM ResD and ResE (lane 2). Increasing amounts of cold DNA, i.e., 2.5 nM (lanes 3, 6, and 9), 5 nM (lanes 4, 7, and 10), and 10 nM (lanes 5, 8, and 11), were included as competitor DNA in the reaction mixtures. Lane 1, probe only. The image shows the effect of the mutations in the putative ResD box on binding by ResD. Probes muta and mutb indicate the *yclJ* promoter carrying the binding site a and binding site b mutations, respectively.

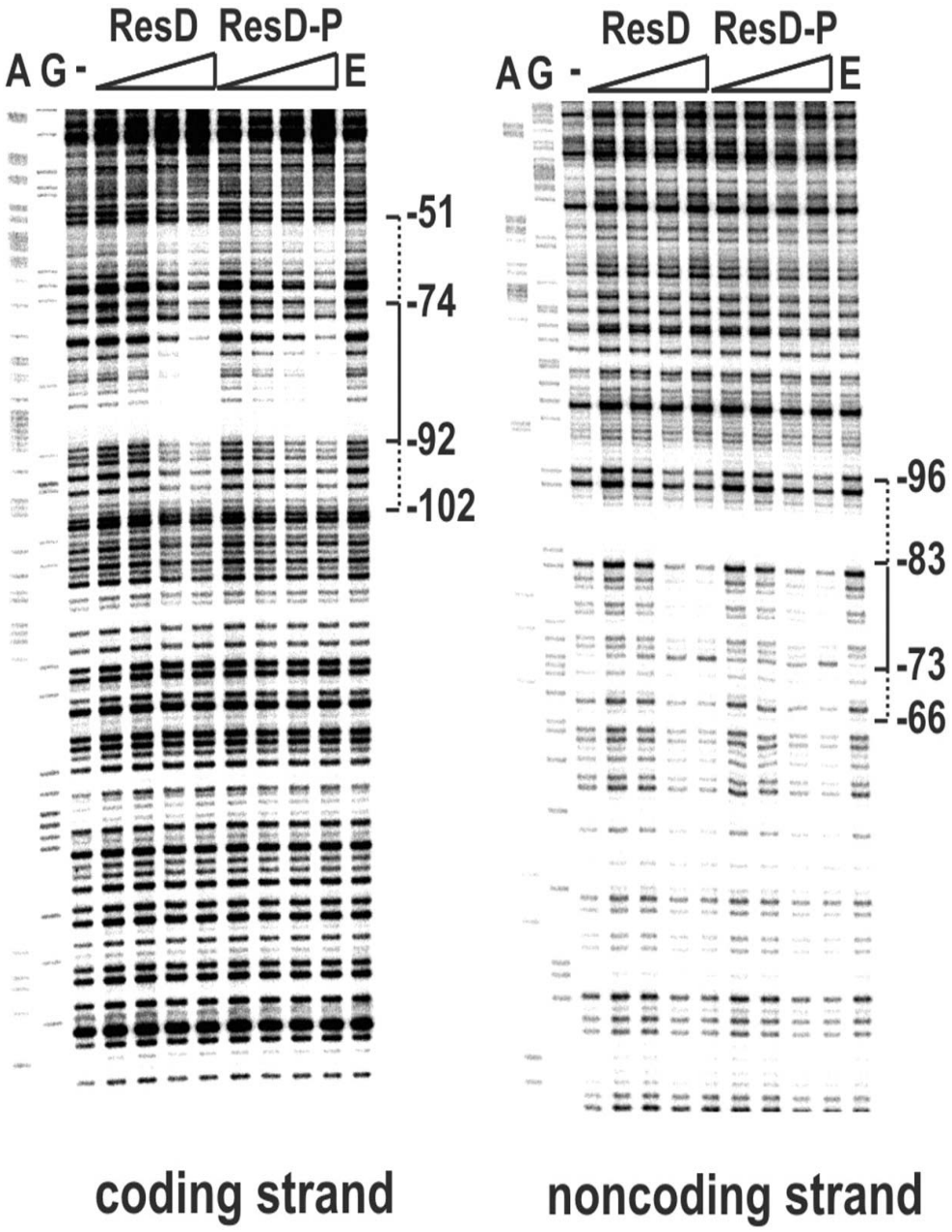


FIG. 6.5. DNase I footprinting experiment with ResD and the *yclJ* promoters. Increasing concentrations of ResD and/or ResE (2, 4, 8, and 16 μM) were incubated with 32P-end-labeled coding and noncoding strands of the *yclJ* promoter. G and A sequencing ladders are included to localize the binding sites. The vertical brackets indicate protected regions. Dotted brackets show weakly protected regions. Positions relative to the transcription start site are shown.

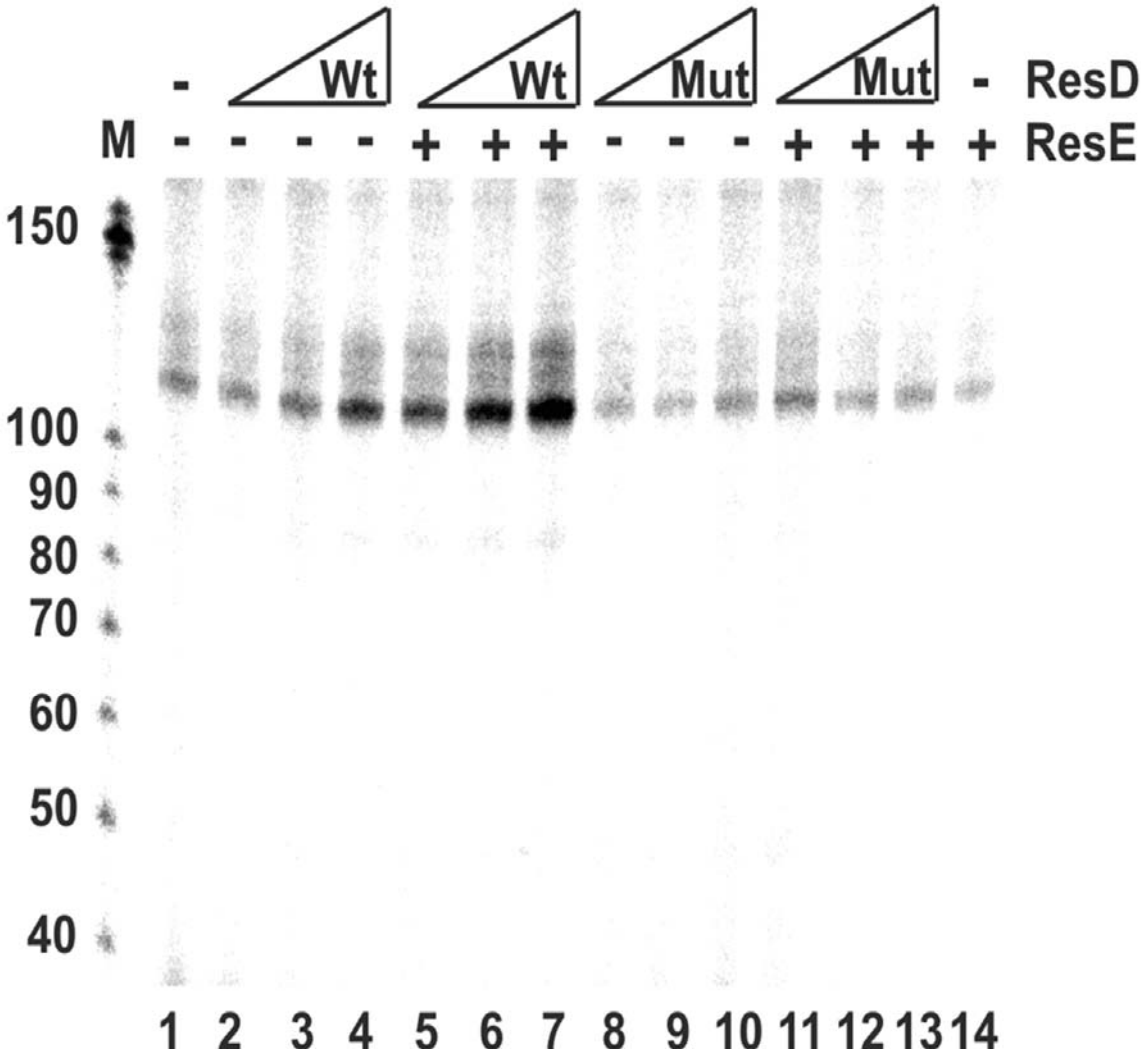


FIG. 6.6. In vitro transcription analysis of *yclJ* promoter. Transcription was carried out with 25 nM purified RNA polymerase and 5 nM templates without ResD and ResE (lane 1), with increasing amounts of wild-type ResD (lanes 2 to 4), with wild-type ResD and ResE (lanes 5 to 7), with the D57A mutant ResD (lanes 8 to 10), with the D57A mutant ResD and ResE (lanes 11 to 13), and with ResE only (lane 14). The amounts of ResD used were 0.35, 0.7, and 1.4 μM, and the amount of ResE used was 1 μM. An RNA size marker (in nucleotides) is shown (M).

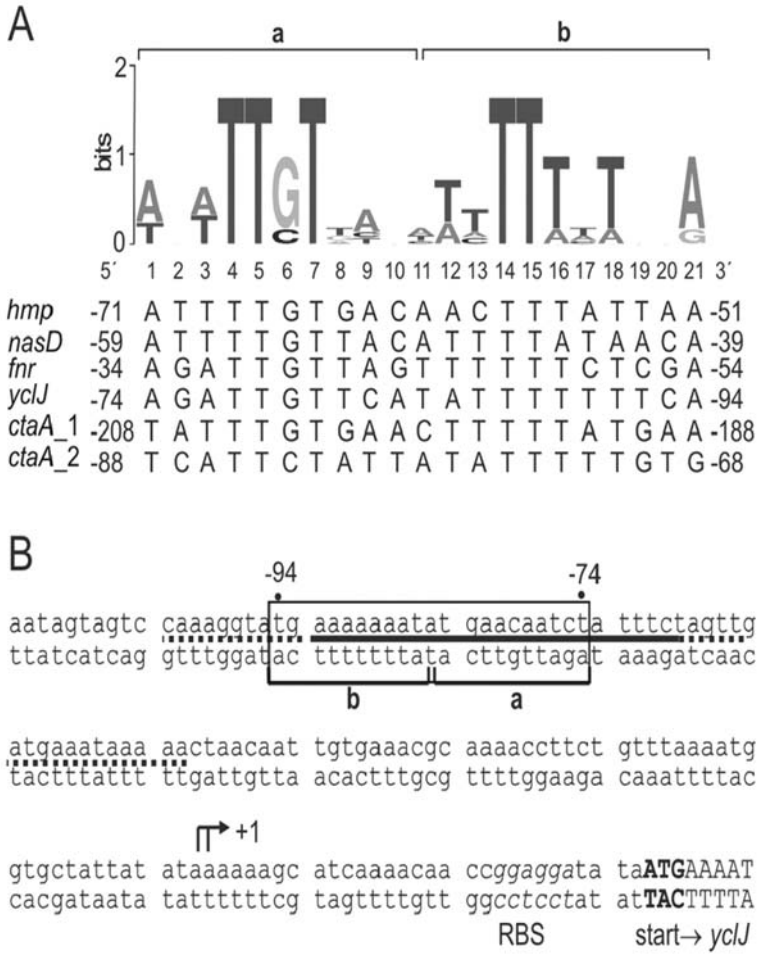


FIG. 6.7. New definition of ResD binding site and location in the *yclJ* promoter. (A) Sequence logo of the ResD binding site based on the information weight matrix model. The height of each stack of letters is the sequence conservation, measured in bits of information according to the equation given in Materials and Methods. The height of each letter within a stack is proportional to its frequency at that position in the binding site. The letters are sorted, with the most frequent on top. (B) ResD-dependent promoter of *yclJ*. The transcription start site obtained from primer extension analysis is marked "+1." Potential ResD binding sites, a and b, are boxed, and their positions with respect to the transcriptional start sites are given. The solid line marks the protected region from the footprinting experiments; the dashed lines mark weakly protected regions. RBS, ribosome-binding site.

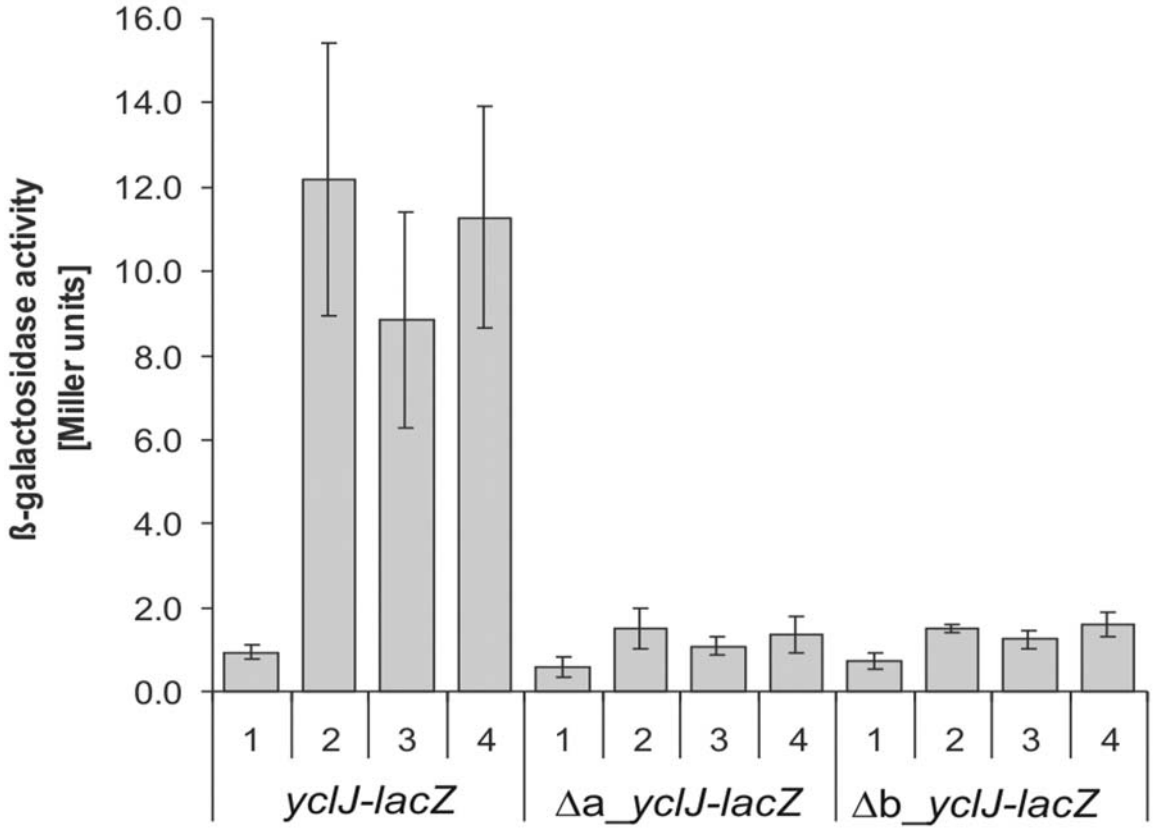


FIG. 6.8. Mutations in binding sites a and b prevent expression of *yclJ-lacZ*. ß-Galactosidase activities were measured from *yclJ-lacZ* wild-type and mutant promoter constructs in the JH642 wild-type strain after 3 h of culture under the following growth conditions: aerobic (1), fermentative (2), anaerobic plus nitrate (3), and anaerobic plus nitrite (4). Error bars indicate standard deviations ($n \geq 3$).

# CHAPTER 7
# CONCLUSIONS AND FUTURE DIRECTIONS

## 7.1 SUMMARY OF RESEARCH

### 7.1.1 Architecture of ResD binding to the *hmp*, *nasD* and *fnr* promoters

Hydroxyl radical footprinting analysis revealed that ResD tandemly binds to the promoter regions of *hmp* and *nasD*, and phosphorylation of ResD stimulates its binding activity. The ResD binding sites in *hmp* and *nasD* are located on the same face of the DNA helix, except that ResD binds to the opposite face of the helix at the most promoter-proximal binding site of the *hmp* promoter. ResD binds to *fnr* only in the presence of αCTD of RNAP. The bases in the *fnr* promoter critical for ResD activation were identified by mutational analysis. Based on these results, $TTGTAAN_3TTN_4A$ was proposed as a consensus ResD-binding sequence. The alignment of the consensus sequence in various ResD-controlled promoters led us to propose that ResD binds to the promoter DNA in more than one orientation.

### 7.1.2 ResD activation of *hmp*, *nasD* and *fnr* transcription in a phosphorylation-dependent and -independent manner

In vitro transcription experiments showed that ResD stimulates transcription and phosphorylation of ResD enhances transcriptional activation. Although phosphorylation stimulates transcription, unphosphorylated ResD and its phosphorylation site (Asp57) mutant are able to activate transcription at a low level. The D57A mutant activates transcription of the ResDE regulon in response to oxygen limitation, suggesting that either ResD itself, or together with an auxiliary factor, senses oxygen limitation via an unknown mechanism, leading to anaerobic gene activation.

### 7.1.3 Critical residues of ResD, αCTD and $\sigma^A$ for ResD-activated transcription of *fnr*, *nasD* and *hmp*

Alanine substitutions of amino acids in the ResD transactivation loop identified the critical residues required for transcription activation. Mutations F197A, G198A and D199A reduce *fnr* expression in vivo, while only G198A affects *nasD* expression. In contrast, none of the residues are important for *hmp* expression in vivo. DNase I footprinting assay showed that G198A abolishes the ResD DNA-binding ability. On the contrary, D199A moderately enhances ResD binding to the promoter DNA of *hmp* and greatly increased the binding affinity to the *nasD* promoter. These results strongly suggest that the mutations in the transactivation loop affect DNA binding and not RNAP interaction as previously proposed.

Alanine scanning mutagenesis of αCTD of RNAP identified E254, V260, Y263, K267, A269 and N290 as important residues for the activation of *fnr* and *nasD* transcription by ResD. E254, V260, Y263, K267, A269 constitute a surface-exposed patch likely to interact with ResD, while N290 may be involved in DNA binding. None of the single alanine substitutions of αCTD affect *hmp* expression in vivo, while *hmp* expression is highly reduced by single mutations in region 4.2 of $\sigma^A$ subunit of RNAP. The residues include K356, H359 and R362, which moderately reduce *fnr* and *nasD* expression. DNase I footprinting analysis indicated that ResD~P itself does not bind to *fnr*, but the interaction of ResD~P with αCTD facilitates cooperative binding of ResD~P and RNAP, thereby increasing *fnr* transcription initiation. The presence of α also enhances ResD binding to the *hmp* and *nasD* promoter regions. The K267A mutation of αCTD disrupts the ResD-αCTD interaction at the *fnr* promoter and the ResD-α interaction at the *nasD* promoter. These results strongly argue that ResD interacts with αCTD during *fnr* and *nasD* transcription initiation. During *hmp* transcription initiation, ResD may interact with $\sigma^A$, and the interaction of ResD with αCTD, if it exists, contributes less to ResD-controlled transcription activation.

### 7.1.4 NsrR-dependent regulation of *hmp*, *nasD* and *fnr* in response to NO

NsrR represses ResDE-dependent and -independent expression of *hmp* under aerobic growth conditions. Under anaerobic conditions, NsrR represses *hmp*, *nasD* and

*fnr* expression and the repression is alleviated by NO. NsrR likely has an iron-sulfur cluster that may sense NO to regulate NsrR activity. Apo-NsrR protein purified under aerobic conditions binds the *hmp* and *nasD* promoter regions and inhibits transcription in vitro. In addition, NsrR may act as a coactivator of ResD to activate expression of *hmp*, *nasD* and *fnr* in response to NO. Thus NsrR participates in upregulating genes involved in anaerobic respiration when nitrate is present. Unlike the Nar systems in *E. coli* that sense nitrate/nitrite, NsrR responds to NO, which is generated during nitrate respiration.

### 7.1.5 Activation of *yclJK* transcription by ResD

ResD directly activates the *yclJK* operon that encodes two-component regulatory proteins. The *yclJK* genes were originally identified as those induced by oxygen limitation; however the target genes and the physiological roles of the YclJK-signal transduction system are unknown.

## 7.2 FUTURE DIRECTIONS

To address how phosphorylation of ResD receiver domain (NTD) affects DNA-binding domain (CTD) activity, the transcriptional activation by ResD-CTD needs to be examined in vivo and in vitro. Although isolated ResD-CTD was shown to be unstable in *B. subtilis* (Karl Mullen and Michiko M. Nakano, unpublished results), I have successfully purified ResD-CTD protein that is overexpressed in *E. coli*. If ResD-NTD inhibits ResD-CTD DNA-binding ability, which can be released by the phosphorylation of Asp, ResD-CTD could activate transcription in vitro. If ResD-CTD is unable to activate transcription, the result suggests that either phosphorylation has a positive effect on CTD or ResD-NTD interacts with RNAP. If ResD-CTD does not activate transcription but still binds to DNA, NTD might be involved in the interaction with RNAP. Because the mutational analysis of the transactivation loop of ResD failed to identify residues that interact with RNAP, the information obtained from the experiments with ResD-CTD could provide an insight into an understanding of ResD-RNAP interaction.

ResD phosphorylation-independent regulation is unique among RRs, as it still responds to a signal derived from oxygen limitation. How ResD senses oxygen limitation remains to be elucidated. There is one cysteine (C69) on the helix α3 in the N-terminal receiver domain f ResD, although the corresponding residue in SrrA, the *S. aureus* ResD ortholog, is alanine. The substitution of the cysteine residue with either alanine or serine in both *B. subtilis* ResD and ResD (D57A) results in the loss of activity (M. M. Nakano, unpublished result). It is worth determining the role of the cysteine in the activity of ResD such as signal-sensing through the active thiol group in the cysteine. The role of cysteine in ResD activation needs to be examined in vivo and in vitro. I purified the mutant ResD proteins carrying C69A or C69S mutations, which can be used for in vitro studies. Another possibility of oxygen-sensing by unphosphorylated ResD is that an auxiliary protein that interacts with ResD senses oxygen limitation. The possibility could be investigated by searching for such a protein using the yeast two-hybrid system. We have obtained a *B. subtilis* genomic library fused to the GAL4 activation domain from Dr. Hirofumi Yoshikawa (Tokyo University of Agriculture, Japan), which was successfully used to identify the αCTD-Spx interaction.

The critical residues of ResD, αCTD and $\sigma^A$ for activation of *hmp*, *nasD*, and *fnr* identified in vivo need to be reassessed in the absence of NsrR because NsrR could be involved in ResD-dependent activation. I have constructed strains carrying desired mutations in the *nsrR* background. Another way to eliminate potential NsrR effect on activation is to examine mutational effects on ResDE-dependent gene transcription by in vitro analysis. In order to mimic in vivo conditions, the supercoiled plasmid carrying target DNA fragments, instead of linear PCR products, should be considered to use as templates for in vitro experiments. Since the single mutations of $\sigma^A$ also affect ResD activation of *hmp*, and less so *nasD* and *fnr*, the possible interaction between ResD and $\sigma^A$ needs to be checked by DNase I footprinting experiments. The possible roles of ResD rather than recruiting RNAP to promoters need to be considered. For this purpose, the potassium permanganate footprinting experiments can be used to examine whether ResD affects a formation of open-complex during transcriptional initiation. DNase I footprinting analysis identified regions protected in the presence of ResD and α; however, it did not reveal topology of the transcription initiation complex. In order to determine

the arrangement of protein-DNA complex during transcriptional activation by ResD, the use of cross-linking method is an option.

NsrR likely senses NO via the Fe-S center. The conserved cysteines that may coordinate iron will be substituted with alanine or serine to examine whether the Fe-S center is required for NsrR activity. The mutational analysis of putative NsrR binding sites on *hmp* and *nasD* promoter regions, especially the sites close to ResD binding sites, will be carried out to investigate whether NsrR is involved in ResD-controlled transcription activation. To further characterize the NO-dependent modification of the Fe-S center in vitro, we need to purify NsrR protein under anaerobic condition to isolate NsrR protein that contains the Fe-S cluster.

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# BIOGRAPHICAL SKETCH

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