Dissecting Functional Domains of the ResD Transcription Factor by Mutational Analysis

By

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A THESIS

presented to the Institute of Environmental Health

at Oregon Health & Science University

School of Medicine

in partial fulfillment of the

requirements for the degree of

Master of Science

In Biochemistry and Molecular Biology

June 2017

School of Medicine

Oregon Health & Science University

Certificate of Approval

This is to certify that the Master's Thesis of

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ACKNOWLEDGMENTS

My experience at OHSU thus far has left me grateful for the opportunity to learn in such a supportive, collaborative, and innovative environment. I am sincerely thankful to my advisor, Dr. Michiko M. Nakano, for cultivating such an environment in her laboratory. I am in awe of, and desire to emulate her remarkable work ethic and ability to think critically. Her kindness, patient guidance, and continual encouragement have provided a strong foundation for my graduate studies, and will not be forgotten.

I owe a special thanks to my thesis advisory committee members, Drs. Michiko Nakano, Peter Zuber, and Pierre Moënne Loccoz for their guidance, technical advice, and valuable suggestions throughout my studies. I am also truly thankful to Drs. Michiko Nakano, Peter Zuber, and Dr. Michael Bartlett for giving their time and effort in carefully reviewing my thesis. Moreover, I appreciate the kindness and wonderful support I have received from members of the Nakano and Zuber lab, including Cierra Birch, Lea Mbengi, Nathan Halow, and Victoria Meadows.

Finally, I owe my deepest gratitude to my family for their constant love and prayers.

ABSTRACT

Dissecting Functional Domains of the ResD Transcription Factor by Mutational Analysis

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June, 2017

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Regardless of oxygen availability, *Bacillus subtilis* is able to survive by maintaining highly regulated and coordinated metabolic pathways, with activation or repression of target genes and/or modulation of protein activity. The ResDE two-component regulatory system of *B. subtilis* controls gene expression globally at the transcriptional level in order to allow for either aerobic respiration or anaerobic nitrate respiration. Our long-term research goal is to understand how ResD is able to respond to various environmental signals including oxygen and NO and display altered transcriptional regulation depending on the presence of and interactions with RNA polymerase (RNAP) and other transcription factors (TFs). A major aim of this work is to further current understanding of the mechanistic and functional interactions between ResD and another RNAP-associated TF, Spx, under nitrate respiration conditions. *B. subtilis* relies on the

global transcriptional regulator, Spx, to exert both positive and negative control over transcription initiation in response to oxidative stress. ResD and Spx likely coordinate interactions with RNAP in a manner that supports growth under aerobic or anaerobic conditions as the two TFs function under opposite physiological conditions in terms of oxygen levels.

The overall aim of my research is to investigate the interaction surface of RNAP with ResD in order to uncover the physiological relevance of a transcriptional profile governed by ResD and Spx. Previous genetic and crystal structure studies had identified that residues 47-54 of Spx constitute the interaction surface with αCTD of RNAP. Evidence gathered from previous work and subsequent recognition of sequence similarity between residues 153-160 of ResD and residues 47-54 of Spx suggested that the two RNAP-associated TFs may use similar residues to interact with RNAP. In accordance with the assumption, previous work also showed that alanine substitution mutations in the α-loop (the transactivation loop identified with OmpR) at positions G198 and R201 affected DNA-binding ability of ResD.

Amino acid substitution mutations of ResD were generated in the present study in order to elucidate the interaction surface of ResD with RNAP. The proposed αCTD interaction surface of ResD is located in the region corresponding to the N-terminal four-stranded antiparallel β sheet in the C-terminal domain of OmpR subfamily of response regulators. Dissecting the putative αCTD interaction surface of ResD led to the new finding of two regions A and B (corresponding to residues 142-148 and 153-160, respectively) of ResD that may play distinctive roles in transcriptional control. G52 of Spx is the site of interaction with Y263 of αCTD, and the same amino acid substitution at the corresponding ResD residue, G158, suggests by genetic analysis that G158 also directly, but weakly, interacts with Y263. Together, the results of this study have shown that the two conserved regions in Spx and ResD may differ in their interaction

with RNAP, providing insight into mechanistic and functional interactions between ResD and Spx.

CHAPTER 1

INTRODUCTION

Apart from its ability to secrete industrial enzymes, such as amylases, and produce heterologous proteins, *Bacillus subtilis* is also valued in the production of Natto, a traditional Japanese dish of fermented soybeans. With a well-developed natural transformation system invaluable for genetic manipulation and analysis, this Gram-positive, rod-shaped, endospore-forming bacterium rivals *Escherichia coli* in contributions to the study of bacterial biochemistry, physiology, and genetics.

1.1 THE AEROBIC/ANAEROBIC INTERFACE IN *BACILLUS SUBTILIS*

In nature, *B. subtilis* is commonly found in soil, water sources, and in association with plants (Harwood, 1992). A low level of reported incidence of pathogenicity amongst strains of *B. subtilis* has allowed for the use of *B. subtilis* spores as probiotics and competitive exclusion (CE) agents for both human and animal consumption. Probiotics and CE agents are thought to enhance the gut microflora by preventing the colonization of the gastrointestinal (GI) tract by pathogenic bacteria (Casula *et al.*, 2002).

In environments such as soil and the GI tract, oxygen availability for bacteria is not constant. For example, a rain shower drastically reduces the accessibility of oxygen in soil since the diffusion rate of oxygen in water is approximately 10,000 times lower compared to the diffusion in the gas phase (Hoffmann *et al.*, 1995). Similarly, oxygen concentrations decrease to near anoxia at the midpoint of the human GI tract lumen (Espey, 2013). Under low-oxygen or anoxic conditions, some bacteria can still generate ATP by induction of high-affinity terminal

oxidases to respire traces of oxygen, or by utilizing other substrates as final electron acceptors, such as nitrate (Bueno *et al.*, 2012). As a facultative anaerobe (Nakano *et al.*, 1998b), *B. subtilis* maintains highly regulated and coordinated metabolic pathways, with activation or repression of target genes and/or modulation of protein activity, in order to survive regardless of oxygen availability.

Global systems, such as the two-component regulatory pair of *B. subtilis*, ResD and ResE, control gene expression at the transcriptional level in order to allow for either aerobic respiration or anaerobic nitrate respiration (Sun *et al.*, 1996b). These pathways however promote the internal production of reactive oxygen species (ROS) and nitric oxide (NO), which can interact with each other and oxygen to yield reactive nitrogen species (RNS) (Zuber, 2009). *B. subtilis* relies on the global transcriptional regulator, Spx, to exert both positive and negative control over transcription initiation in response to oxidative stress (Zuber, 2004). As ResD and Spx were identified as RNA polymerase (RNAP)-binding transcription factors (TFs) (Delumeau *et al.*, 2011) and the two TFs function under opposite physiological conditions in terms of oxygen levels, ResD and Spx likely coordinate interactions with RNAP in a manner that supports growth under aerobic or anaerobic conditions. The aim of this study is to characterize the interaction surface of RNAP with ResD in order to uncover the physiological relevance of a transcriptional profile governed by ResD and Spx.

1.1.1 The aerobic/anaerobic interface in bacteria

Bacteria generally have three modes to sense oxygen levels: by direct interaction of O_2 with a membrane protein receptor, interaction with a regulatory protein, or through the monitoring of environmental oxygen concentration by redox-responsive regulatory systems

(Bueno *et al.*, 2012). In the photosynthetic *Rhodobacter* bacterial species, the RegBA/PrrBA two-component systems function in low oxygen tension. The membrane-associated RegB/PrrB histidine protein kinases sense changes in redox state and activate the cognate RegA/PrrA response regulators (Elsen *et al.*, 2004; Eraso *et al.*, 2008). Four global systems regulate the aerobic/anaerobic interface in *E. coli*. O_2 is the preferred electron acceptor and represses the terminal reductases of *E. coli* anaerobic respiration. In anaerobic respiration, nitrate represses other terminal reductases, such as fumarate or DMSO reductases. Energy conservation is maximal with O_2 and lowest with fumarate (Unden *et al.*, 1997). The Fnr TF acts as a direct oxygen sensor and is the primary transcriptional regulator of the switch between aerobic and anaerobic growth by coordinating with the aerobic respiration control of the ArcB/ArcA two component system (Cotter *et al.*, 1997; Shalel-Levanon *et al.*, 2005). The NarXL and NarQP two-component regulatory systems are involved in nitrate regulation of anaerobic respiratory gene expression. The two homologous sensor proteins, NarX and NarQ, mediate both nitrate and nitrite signaling. Of the two response regulators, NarL controls nitrate induction of nitrate respiration genes and repression of genes encoding alternate anaerobic respiratory proteins, while NarP controls nitrite induction of several operons (Stewart, 1993). In the opportunistic pathogen *Pseudomonas aeruginosa*, the Anr global transcriptional regulator plays a pivotal role in adaptation to microoxic or anoxic conditions. Anr is a homolog of *E. coli* Fnr and regulates expression of the enzymes required for nitrate respiration (Zimmermann *et al.*, 1991).

1.1.2 Aerobic respiration in *Bacillus subtilis*

Under oxic conditions, *B. subtilis* utilizes a branched electron transport chain, with electrons transferred either sequentially through the cytochrome *c* and cytochrome oxidase, or to

a quinol oxidase. Terminal oxidases, complexed with different hemes, at the end of each branch reduce molecular oxygen to water. Currently, terminal oxidases that have been identified include one cytochrome *c* oxidase, cytochrome caa_3, and quinol oxidases, cytochrome aa_3, *bd*, and a *bb'*-type oxidase (Nakamura *et al.*, 2011) (Fig. 1.1). caa_3 and aa_3 are heme-A copper oxidases, with the subunits of caa_3 encoded by *ctaCDEF* (Saraste *et al.*, 1991) and aa_3 encoded by the *qoxABCD* operon (Santana *et al.*, 1992). The whole genome sequence of *B. subtilis* suggests two potential cytochrome *bd*-type menaquinol oxygen reductases, *cydABCD* and *ythABC* (Winstedt *et al.*, 1998), and the novel *bb'* oxidase encoded by genes of unknown function (Azarkina *et al.*, 1999).

The presence of one quinol oxidase, either cytochrome aa_3 or cytochrome *bd*, is essential for vegetative aerobic growth, whereas the mutant lacking both quinol oxidases is able to grow anaerobically through nitrate respiration (Winstedt *et al.*, 2000).

1.1.3 Growth of *B. subtilis* during aerobic to anaerobic shift

Two-dimensional gel electrophoresis analysis of *B. subtilis cells* has identified proteins exclusively produced under aerobic growth conditions, including glycerol kinase (GlpK) and a protein with similarity to NADH dehydrogenase (YjlD, Ndh) (Marino *et al.*, 2000). *ndh* transcription is negatively regulated by Rex, a global redox-sensing protein, that responds to the cellular NADH/NAD^+ ratio (Gyan *et al.*, 2006). Microarray analysis has suggested that during the transition from aerobic to microaerophilic and finally to anaerobic growth, the coordination of certain respiratory genes, including *cyd*, as well as *ldh* (lactate dehydrogenase) and *lctP* (lactate permease), is also negatively regulated by Rex (Larsson *et al.*, 2005).

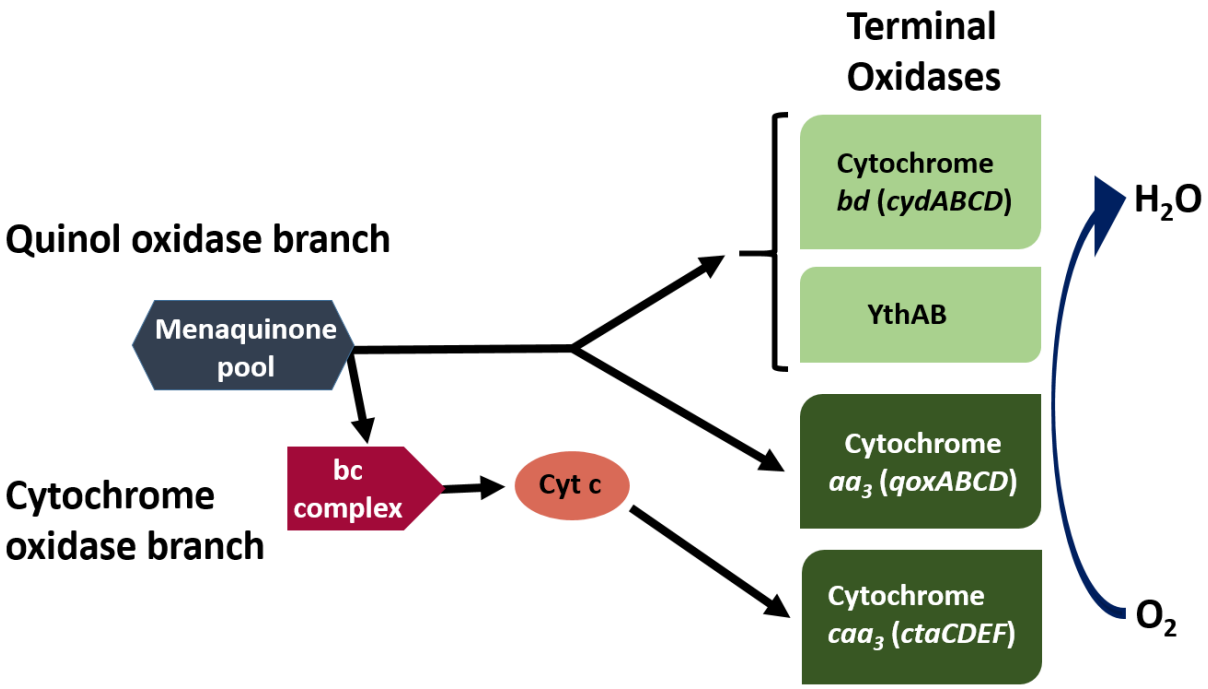

FIG. 1.1. Aerobic respiratory pathways in *B. subtilis*. Under oxic conditions, *B. subtilis* utilizes a branched electron transport chain. Terminal oxidases at the end of each branch reduce molecular oxygen to water.

In addition to Rex, the successful transition from an aerobic to anaerobic environment is dependent on other regulatory proteins, including the two-component ResD-ResE regulatory system (Fig. 1.2). The partially overlapping *resD* and *resE* genes are transcribed during vegetative growth from a weak promoter directly upstream of *resD*. *resD* and *resE* also belong to a larger operon that includes three upstream genes, *resABC* (Sun *et al.*, 1996b). ResA (extracytoplasmic membrane-anchored thiol-disulfide oxidoreductase) (Erlendsson *et al.*, 2003), ResB and ResC (integral membrane proteins that likely function in transmembrane heme transport), are essential for cytochrome *c* maturation (Le Brun *et al.*, 2000). Aerobic expression of the ResDE regulon is derepressed by a mutation in the *qox* operon (Nakano *et al.*, 1998b). The *qox* operon codes for the aa_3 oxidase necessary for regenerating the major electron carrier in *B. subtilis*, menaquinone (Santana *et al.*, 1992). The redox state of intracellular menaquinone has been postulated to be a signal for the autophosphorylation of the membrane-bound ResE sensor kinase. ResE subsequently donates a phosphate to its cognate response regulator, ResD. ResE dually functions as a kinase and a phosphatase for ResD, with phosphatase activity dominant under aerobic conditions, and kinase activity more dominant when oxygen becomes limiting (Nakano *et al.*, 2001b). Transcription of *resDE* from the *resA* promoter requires phosphorylated ResD (ResD~P) during anaerobic growth or at the end of aerobic exponential growth, while transcription from the promoter upstream of *resD* is ResD-independent. However, the requirement of ResD for activation of *resA* is likely indirect because a number of spontaneous compensatory mutations unlinked to the *res* locus in a *resDE* mutant have been shown to restore transcription to the *resA* operon (Hulett, 1996). In contrast, ResD directly binds to the promoter regions of *ctaA* (encoding a membrane-bound protein that catalyzes heme O to heme A conversion) (Sun *et al.*, 1996b; Zhang *et al.*, 2000). The ResDE system has been suggested to

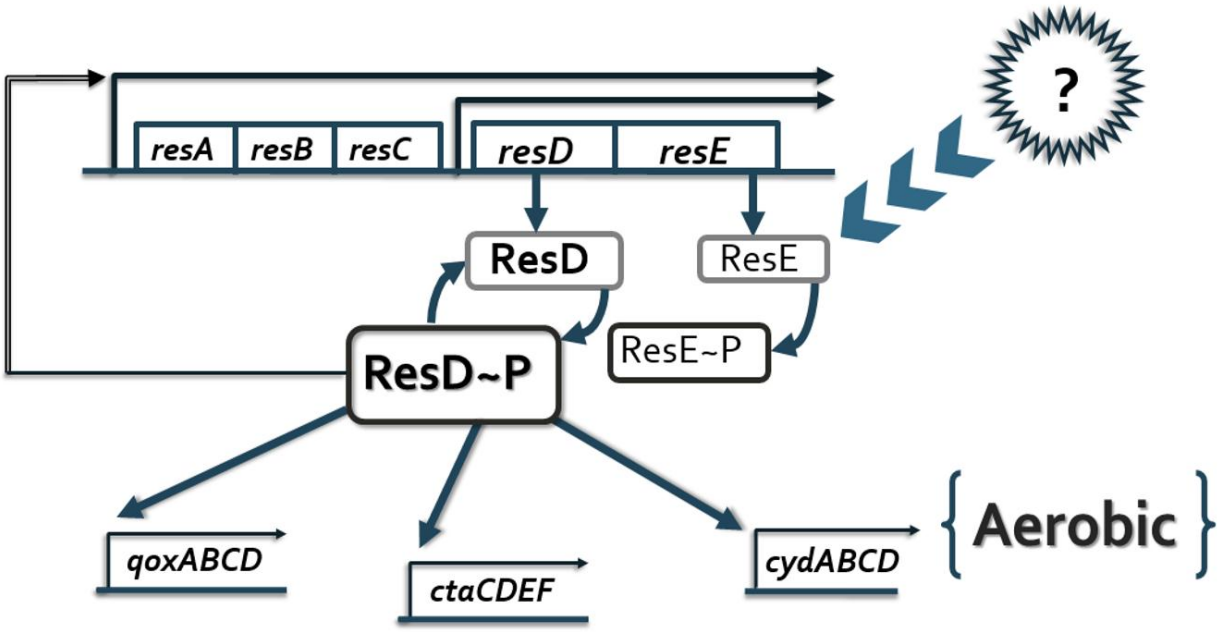

FIG. 1.2. The ResDE two-component regulatory system is required for aerobic respiration. *resD* and *resE* are transcribed from the *resA* promoter and a promoter located upstream of the *resD* gene. The membrane-bound ResE kinase likely senses reduced menaquinone levels, leading to autophosphorylation and transfer of phosphate to the cognate response regulator ResD. During anaerobic growth or after the end of aerobic exponential growth, transcription of *resDE f*rom *resA* promoter is activated by phosphorylated ResD (ResD~P), while transcription from the *resDE*-specific promoter is ResD-independent. *resABC* functions in cytochrome *c* biogenesis. Under aerobic conditions, ResD~P can turn on transcription of genes important for heme conversion and genes encoding oxidases.

mediate the Hfq (a putative RNA chaperon which affects mRNA transcript levels) influenced adaptation to anaerobic growth, as transcription of several ResD and Rex regulon genes is increased in the *hfq* mutant (Hammerle *et al.*, 2014).

1.1.4 Anaerobic growth of *B. subtilis*

When oxygen is not available, facultative anaerobes depend on the transmission of electrons to alternative reductases that reduce substrates such as nitrate (NO_3^-), nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), dimethylsulfoxide (DMSO), trimethylamine N-oxide (TMAO), sulfate, sulfite, and fumarate as final electron acceptors (Bueno *et al.*, 2012). The less energy-generating fermentation pathway is utilized in the absence of alternate electron acceptors. Nitrate respiration is the only anaerobic form of respiration known to be employed in *B. subtilis* (Nakano *et al.*, 1998b). *resDE* mutants are unable to grow anaerobically in the presence of nitrate (Sun *et al.*, 1996b). The ResDE pair, along with the [4Fe-4S]-cluster containing TFs, NsrR and Fnr, are important regulators of the genes involved in nitrate respiration (Fig. 1.3). More specifically, ResD has been shown to directly interact with promoter regions of the nitrate respiration genes *fnr* (anaerobic transcriptional regulator), *nasDEF* (nitrite reductase), and *hmp* (encodes flavohemoglobin) (Cruz Ramos *et al.*, 1995; LaCelle *et al.*, 1996; Nakano *et al.*, 1996; Nakano *et al.*, 1998a; Nakano *et al.*, 2000a; Nakano *et al.*, 2006; Ogawa *et al.*, 1995; Shaw *et al.*, 1983). *resD* and *resDE* mutations also have a moderate effect on fermentative growth, suggesting that nitrate respiration and fermentation are controlled by divergent regulatory pathways (Ye *et al.*, 2000).

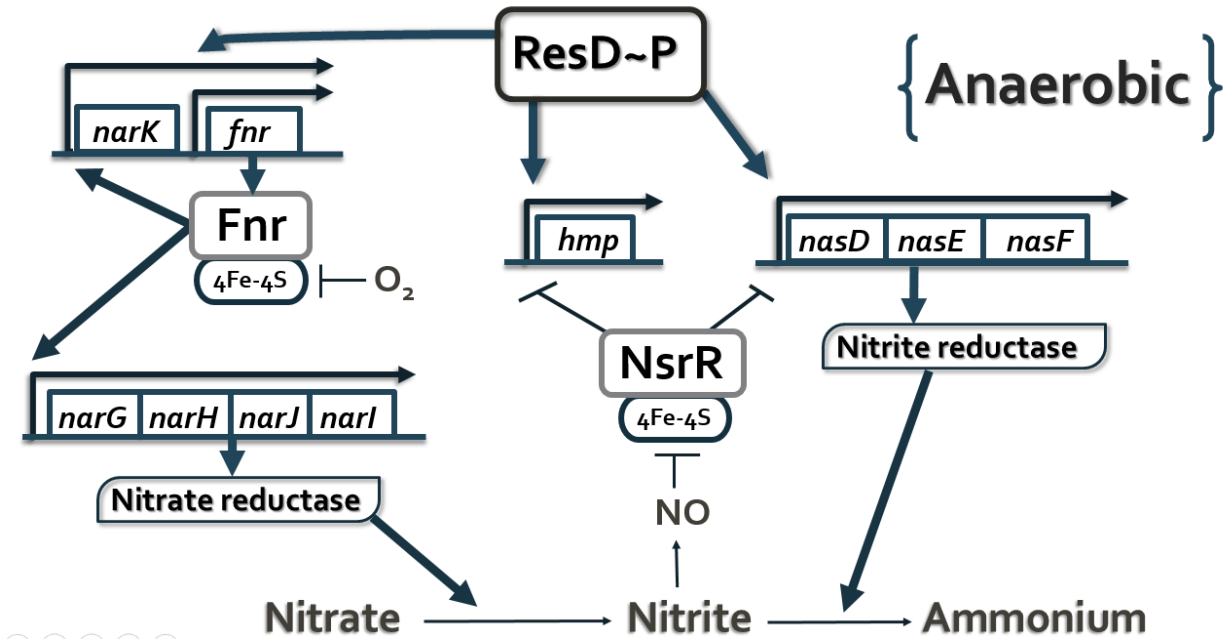

FIG. 1.3. The ResDE two-component regulatory system is required for anaerobic respiration. The ResDE pair, along with the [4Fe-4S]-cluster containing TFs, NsrR and Fnr, are important regulators of the genes involved in nitrate respiration. More specifically, ResD has been shown to directly interact with promoter regions of the nitrate respiration genes *fnr* (anaerobic transcriptional regulator), *nasDEF* (nitrite reductase), and *hmp* (encodes flavohemoglobin).

1.1.4.1 Fermentation

During fermentation, neither the respiratory chains linked to oxygen nor those linked to alternative electron acceptors are functional. While synthesis of pyruvate dehydrogenase (PDH) complex (pyruvate to acetyl-CoA conversion) is repressed during fermentation in *E. coli* (Yamamoto *et al.*, 1975), PDH functions in the conversion of pyruvate during fermentation in *B. subtilis* (Nakano *et al.*, 1997). *E. coli* and other bacteria are able to use mixed acid fermentation for glucose metabolism to form the end products such as ethanol, succinate, lactate, acetate, formate, hydrogen, and carbon dioxide (Clark, 1989). *B. subtilis* can grow anaerobically by fermentation either when both glucose and pyruvate are provided or when glucose and mixtures of amino acids are present. Nuclear magnetic resonance analysis has shown that lactate, acetate, acetoin, ethanol, and succinate are main products of fermentation in *B. subtilis* (Nakano *et al.*, 1997). Lactate formation is dependent on the *lctEP* locus (lactate dehydrogenase and a putative lactate permease). Anaerobic formation of 2,3-butanediol via acetoin involves the *alsSD* operon (acetolactate synthase and decarboxylase). The gene *alsR* (encoding a regulator potentially responding to changes of the intracellular pH and to acetate) is essential for anaerobic *lctEP* and *alsSD* expression, while anaerobic acetate synthesis from acetyl-CoA requires the phosphotransacetylase encoded by *pta* (Cruz Ramos *et al.*, 2000). The absence of formate amongst by-products of *B. subtilis* fermentation is perhaps due to the lack of a counterpart to the key enzyme in *E. coli* fermentation, pyruvate formate-lyase (Pfl) (Kunst *et al.*, 1997). Ultimately, *B. subtilis* prefers to utilize more energetically favorable processes if oxygen or the alternative electron acceptor, nitrate, are available (Nakano *et al.*, 1997).

1.1.4.2 Nitrate/nitrite respiration

Along with *E. coli* and related enteric bacteria, *B. subtilis* can couple the eight-electron reduction of nitrate to ammonium to growth by using the nitrate and nitrite reductases involved to energy-conserving respiratory electron transport systems. In contrast to nitrate assimilation, through which bacteria, archaea, and plants can assimilate nitrate for nitrogen source into cellular biomass under aerobic or anaerobic conditions, respiratory nitrate reduction is a strictly anaerobic process in which the expression of the nitrate and nitrite reductase genes is tightly repressed in the presence of oxygen, induced during anaerobic growth, and further regulated by the availability of nitrate and nitrite (Cole *et al.*, 2008; Hoffmann *et al.*, 1998). The reduction of nitrate to nitrite is catalyzed by nitrate reductase, while the reduction of nitrite to ammonium is undertaken by nitrite reductase.

1.1.4.2.1 Nitrate reductase

In *B. subtilis*, the nitrate reductases required for assimilation and respiratory nitrate reduction are encoded by two separate operons. The two nitrate reductases have different cellular localization and are induced in response to different environmental conditions (Nakano *et al.*, 1998b). Assimilatory nitrate reductase is a cytoplasmic protein encoded by the *nasBC* genes of the *nasBCDEF* operon. Under conditions of nitrogen limitation, the transcription of *nasBC* is activated by the global TnrA regulator for transcription of genes involved in nitrogen metabolism (Wray *et al.*, 1996). The *nasA* gene, which is divergently transcribed from the *nasBCDEF* operon, has been suggested to encode a nitrate transporter. *nasD* and *nasE* encode nitrite reductase. The *nasF* gene codes for a putative enzyme involved in the synthesis of siroheme, a cofactor of nitrite reductase (Ogawa *et al.*, 1995).

The respiratory nitrate reductase is encoded by *narGHJI* and is membrane-bound. A high degree of identity in amino acid sequence between the *E. coli* and *B. subtilis* NarG and NarH suggests that NarG is a catalytic subunit containing a molybdenum cofactor, and NarH contains iron-sulfur clusters necessary for electron transfer. The hydrophobic NarI contains conserved amino acid sequences implicated in heme cofactor binding which is essential for its cytochrome function. The *E. coli* analog of NarJ is a chaperone-like protein, functioning during the assembly of the enzyme (Hoffmann *et al.*, 1995). The mechanism of respiratory nitrate reduction is thought to originate with NarI receiving electrons from the quinones and transferring them sequentially to the iron-sulfur centers of the NarH subunit, and then to the molybdenum cofactor carried by the NarG protein, where nitrate is reduced to nitrite (Nakano *et al.*, 1998b). Fnr, the oxygen-sensing transcriptional regulator, activates the transcription of *narGHJI* under anaerobic conditions (Cruz Ramos *et al.*, 1995).

1.1.4.2.2 Fnr

The *narK-fnr* operon resides upstream of the *narGHJI* operon, with *fnr* transcriptional regulation exerted at either the Fnr-dependent *narK* operon promoter or the ResDE-dependent, *fnr*-specific intergenic promoter (Nakano *et al.*, 1996). *B. subtilis* NarK, like *E. coli* NarK, is involved in extrusion of nitrite produced during nitrate respiration and are both strongly induced by anaerobiosis (Cruz Ramos *et al.*, 1995; Rowe *et al.*, 1994). Similarly, *fnr* encodes a transcriptional regulator of nitrate respiration homologous to *E. coli* Fnr, a member of the catabolite gene activator protein (CAP) family of transcriptional regulators (Shaw *et al.*, 1983). Indeed, expression of *lacZ* driven from the *B. subtilis narK* promoter was activated 50-fold by the CAP-cAMP complex in *E. coli*, suggesting that CAP can recognize the *B. subtilis* Fnr-

binding site, and activate transcription at the *narK* promoter (Cruz Ramos *et al.*, 1995). However, in contrast to *E. coli* Fnr, gene expression of *B. subtilis* Fnr is strongly activated by oxygen limitation (Cruz Ramos *et al.*, 1995).

Structurally, *B. subtilis* Fnr contains an oxygen-labile $[4Fe\text{-}4S]^{2+}$ cluster as a cofactor. Cys-227, Cys-230, and Cys-235 are ligands of the $[4Fe\text{-}4S]^{2+}$ cluster of *B. subtilis* Fnr, and are essential for *in vivo* Fnr activity (Reents *et al.*, 2006a). Asp-141 was identified as the fourth iron-sulfur cluster ligand besides the three Cys residues. Compared to the fully cysteine-ligated cluster of *E. coli* Fnr, the aspartate-ligated $[4Fe\text{-}4S]^{2+}$ cluster would provide the dimeric *B. subtilis* Fnr protein with a highly redox-sensitive, fast reactive, and rearrangement-friendly sensory unit (Gruner *et al.*, 2011). The Fnr homologue in *Bacillus cereus* has been shown to function as an oxygen sensor via its Fe-S cluster (Esbelin *et al.*, 2012). *B. cereus* Fnr is active in both its apo-form and holo-form (Esbelin *et al.*, 2008), while the ability of *B. subtilis* Fnr to function as a TF ultimately depends on the integrity of its $[4Fe\text{-}4S]^{2+}$ cluster, which promotes a conformation that is necessary for site-directed DNA binding and transcriptional activation (Reents *et al.*, 2006a).

Four distinct groups of Fnr-dependent genes have been observed in *B. subtilis*. Group 1 genes (*narK-fnr*, *narGHJI*, and *arfM*) are generally induced by Fnr under anaerobic conditions. The promoter regions of all three Fnr-regulated genes carry the highly conserved potential *B. subtilis* Fnr-binding site (TGTGA-N_6-TCACA) centered 41.5/40.5 base pairs (bp) from the transcriptional start point (Cruz Ramos *et al.*, 1995; Marino *et al.*, 2001). Fnr mediates the nitrate-dependent repression of Group 2 genes (*alsSD*, *ldh lctP*, *ywcJ*, and *cydABCD*) by induction of nitrate reductase under anaerobic conditions. In response to the NADH/NAD+ ratio (as nitrate is used to reoxidize NADH to NAD+), Rex is activated and represses expression of

Group 2 genes. The effect of Fnr is also indirect on Group 3 genes (including *ykuNOP*, *dhbABCEF*, *hmp*, and *nasDE*), which all lack an Fnr-binding site in their promoters (Reents *et al.*, 2006b). Group 3 genes are directly repressed by NsrR (explained in more detail below). Fnr however plays a role in the activation of these genes, as induction of nitrate reductase leads to the conversion of nitrate->nitrite->NO. The repressor activity of NsrR is relieved in the presence of NO, thus leading to the upregulation of Group 3 genes (Kommineni *et al.*, 2012). The Group 4 operon *acoABCL*, also lacking an Fnr-binding site, requires Fnr-dependent nitrate reductase formation for its general anaerobic induction (Reents *et al.*, 2006b).

In a proposed regulatory cascade for differential anaerobic gene expression, ResDE directly activates *fnr* transcription. Fnr then directly activates the most efficient anaerobic mode of ATP generation, nitrate respiration, via induction of the nitrate reductase operon *narGHJI* and nitrate/nitrite transporter genes. Fnr also activates *arfM* transcription, which goes on to modulate the expression of genes encoding proteins important for sustaining nitrate respiration, such as *nasDE* and *hmp* (Marino *et al.*, 2001).

1.1.4.2.3 ArfM

ArfM, or the anaerobic respiration and fermentation modulator, is an important component of the redox regulatory system in *B. subtilis*. ArfM shows no significant similarity to any other protein of known function (Marino *et al.*, 2001). The observed role of ResDE in *arfM* expression is indirect, via ResDE-dependent *fnr* induction. ArfM regulates anaerobic *nasDE* and *hmp* expression, but not *narGHJI* expression. ArfM also stimulates expression of *lctEP* (conversion of pyruvate to lactate), *alsSD* (acetoin synthesis from pyruvate) under fermentative

conditions (Cruz Ramos *et al.*, 2000), and heme biosynthetic genes under nitrate respiratory conditions (Homuth *et al.*, 1999).

1.1.4.2.4 Nitrite reductase

Unlike the use of two distinct nitrate reductases to carry out anabolic and catabolic functions, a single NADH-dependent, soluble nitrite reductase encoded by the *nasDE* genes is utilized for both catabolic and anabolic processes by *B. subtilis* under aerobic or anaerobic conditions. Nitrite enhances anaerobic growth by serving as an electron sink, as nitrite reduction catalyzed by nitrite reductase does not result in a proton gradient and coupled ATP generation. While the nitrate reductase (*nasBC*) genes are activated only by nitrogen limitation, the nitrite reductase (*nasDEF*) genes are induced by oxygen limitation as well as by nitrogen limitation. *nasDEF* transcription is activated by the global nitrogen regulator, TnrA, during nitrogen-limited aerobic conditions, whereas ResDE is mainly responsible for *nasDEF* expression during anaerobic growth regardless of nitrogen sources (Nakano *et al.*, 1998a). Phosphorylation of ResD significantly stimulates binding of ResD to the promoter-regulatory regions of *nasD* and *hmp* in order to activate transcription (Nakano *et al.*, 2000a). The TnrA consensus sequence, with a dyad symmetry required for the activation of both *nasA* and *nasB* (Nakano *et al.*, 1995), is found in the intergenic region located upstream of the *nasD* gene. Mutational analysis and DNase I footprinting suggest that ResD directly binds to a partially overlapping site of where TnrA binds (Nakano *et al.*, 2000a; Wray *et al.*, 1998). The region covering TnrA/ResD-binding sites displays promoter activity. Thus, *nasDEF* is likely transcribed from the *nasD* promoter as well as from the *nasB* promoter. However, transcription of *nasDEF* from the *nasD* promoter ensures expression of nitrite reductase genes under anaerobic conditions (Nakano *et al.*, 1998b).

1.1.4.2.5 Flavohemoglobin

Another key contributor to long-term anaerobic survival in the presence of nitrate is the flavohemoglobin encoded by *hmp*. The Hmp protein belongs to the family of two-domain flavohemoproteins, homologs of which have been isolated from various organisms such as *E. coli*, *Alcaligenes eutrophus*, and *Saccharomyces cerevisiae*. These proteins consist of an N-terminal hemoglobin domain and a C-terminal redox active site domain with potential binding sites for NAD(P)H and FAD (LaCelle *et al.*, 1996). Nitric oxide (NO) exposure from an exogenous source or produced endogenously as a result of reduction of nitrite to NO during nitrate respiration (Ji *et al.*, 1988) can target Fe-S clusters of TFs such as Fnr, and NO also affects proper function of enzymes carrying Fe-S clusters (Stamler *et al.*, 1992). Induction of *hmp* by nitrite in *B. subtilis* (LaCelle *et al.*, 1996) and by NO in *E. coli* (Poole *et al.*, 1996) suggested a relationship between Hmp and the metabolism of nitrogen oxide compounds. NO dioxygenation to nitrate has been shown by flavohemoglobins in *E. coli* under oxic and microaerobic conditions, and reduction of NO to N_2O in the absence of oxygen, although the latter reaction is still controversial (Gardner *et al.*, 1998; Wu *et al.*, 2003). In *B. subtilis*, expression of the sRNA, RoxS, is induced by ResD in response to increasing NO levels, allowing cells to sense and respond to redox perturbations (Durand *et al.*, 2015). The flavohemoglobin encoded by the *hmp* gene also has a vital role in protecting against nitrosative stress by detoxifying NO. ResD co-regulates expression of *hmp* and *nasDEF* with the NO-responsive NsrR TF. Microarray analysis under aerobic conditions has suggested that ResDE is essential for the up-regulation of *nasD* but not *hmp* under nitrosative stress, while NsrR acts as a repressor of both *hmp* and *nasD* in the absence of nitrosative stress. Thus, even though *hmp* and

nasDEF are controlled by the same regulators, *hmp*, unlike *nasDEF*, can be expressed under conditions that do not activate ResD (Rogstam *et al.,* 2007).

1.1.4.2.6 NsrR

NsrR belongs to the NsrR subfamily of the Rrf2 family of putative transcriptional regulators found in diverse bacteria, including *E. coli* and pathogens such as *Neisseria gonorrhoeae* (Bodenmiller *et al.,* 2006; Isabella *et al.*, 2009). Rrf2 family proteins have a predicted helix-turn-helix motif in the N terminus, which is likely involved in DNA binding (Shepard *et al.,* 2011). Like Fnr, NsrR contains a $[4Fe\text{-}4S]^{2+}$ cluster that is important for efficient DNA binding as a repressor (Kommineni *et al.,* 2010). In *E. coli,* NO reacts with the $[4Fe\text{-}4S]^{2+}$ cluster of Fnr to generate a dinitrosyl-iron-cysteine complex. As a TF that can respond to both O_2 and NO, Fnr can then repress *hmp* expression in *E. coli* under anaerobic conditions (Cruz-Ramos *et al.,* 2002). The $[4Fe\text{-}4S]^{2+}$ cluster of *B. subtilis* NsrR is NO-sensitive (Yukl *et al.*, 2008). *nasDEF* and *hmp* activated by ResD are among the genes repressed by NsrR (Kommineni *et al.*, 2012). More recent single-nucleotide resolution analysis of GeF-seq (*in situ* DNase I digestion of genomic DNA followed by ChAP (Chromatin Affinity Precipitation) data has provided a more complete understanding of the genome-wide binding profile of ResD and NsrR. The study conducted in collaboration with members of our laboratory has revealed that NsrR can bind to the *hmp* promoter independently of ResD under fermentation conditions, but ResD only binds in the absence of NsrR (Chumsakul *et al.*, 2017). Reaction of NO (by an exogenous NO donor, such as spermine NONOate, or endogenous NO that is produced by nitrate respiration) with NsrR is required for derepression of target genes (Kommineni *et al.*, 2010; Nakano, 2002). Thus,

anaerobic conditions alone are insufficient for full induction of genes under ResDE regulation, and the presence of NO is required to attain full induction (Yukl *et al.*, 2008).

Two classes of regulation by NsrR have been suggested based on sequence specificity and NO sensitivity of NsrR-controlled genes. The Class I NsrR-binding site was predicted to be around the −35 element of the *nasD* and *hmp* promoters with binding enhanced by the presence of the NO-sensitive $[4Fe\text{-}4S]^{2+}$ cluster of NsrR. Class II binding sites are located in two overlapping regions within −93 to −63 and −71 to −40 of the *nasD* transcription start site, and binding is not enhanced by the presence of the $[4Fe\text{-}4S]^{2+}$ cluster of NsrR. Although NsrR recognizes a 17-bp partial dyad symmetry sequence in the class I cis-acting site containing critical residues, a similar sequence is not found in promoter regions of class II genes. Instead, nucleotide sequences of the class II binding sites are A+T rich and involve relaxed DNA sequence specificity (Kommineni *et al.*, 2010). More than 150 genes have been found to belong in this category, with NO moderately upregulating class II gene expression. Many of these genes were found to be under control of other TFs that also bind to A+T-rich regions, including ResD, Fur, AbrB, and Rok (Kommineni *et al.*, 2012).

1.2 TRANSCRIPTIONAL CONTROL EXERTED BY RESD

Binding of one transcriptional regulator to a promoter DNA can affect association of another regulator that targets the same DNA regulatory region (Henares *et al.*, 2014). For example, there are two types of ResD-controlled genes in the NsrR regulon: one is completely dependent on ResD regardless of NsrR (like *nasD*), and the other is conditionally dependent (like *sdpA*) in that the effect of the *resD* mutation is observed only when NsrR repression is relieved. Thus, ResD, in the absence of NsrR, likely acts as an antirepressor of AbrB-controlled *sdpA* and

Fur-controlled *ykuN* (Kommineni *et al.*, 2012). ResD positively affects binding of NsrR to both *nasD* and *ykuN*, whereas NsrR plays opposite roles by inhibiting ResD binding to *nasD* and stimulating ResD interaction with *ykuN* (Henares *et al.*, 2014). However, the repression mediated by NsrR is exerted only in the presence of ResD, where the two appear to act cooperatively in establishing promoter interaction. The interplay between the transcriptional regulators can be further analyzed in the case of *nasD* expression. In the absence of NO, NsrR occupies the −35 region, resulting in repression of *nasD* transcription. When NO is present, DNA binding affinity of NsrR is reduced and RNAP outcompetes NsrR for binding to the site (Henares *et al.*, 2014). As shown previously through EMSA (Electrophoretic mobility shift assay) experiments, the presence of RNAP enhances ResD binding to *nasD*, thus stabilizing the *nasD*-ResD-RNAP transcriptional initiation complex (Kommineni *et al.*, 2010). Thus, in response to various environmental signals including oxygen and NO, ResD displays altered transcriptional regulation depending on the presence of and interactions with RNAP and other TFs. A major aim of this work is to further current understanding of the mechanistic and functional interactions between ResD and another RNAP-associated TF, Spx, under nitrate respiration conditions.

1.2.1 Two-component signal transduction systems

resD and *resE* were identified in the *B. subtilis* genome sequencing project as genes encoding a response regulator and the cognate sensor histidine kinase of prokaryotic two-component signal transduction systems (Sorokin *et al.*, 1993). Two-component systems allow organisms to synergistically sense and respond to changes in environmental conditions. Two-component systems control many of the virulence factors required for pathogenic bacteria to survive in the foreign host, presenting a potential target in the development of therapeutic

interventions (Barrett *et al.*, 1998). Although the basic scheme is highly adaptable, most prokaryotic signal-transduction systems and a few eukaryotic pathways utilize a prototypical phosphotransfer scheme involving two conserved components, a histidine protein kinase and a response regulator (Fig. 1.4).

The histidine protein kinase, in response to some environmental stimuli, autophosphorylates at a histidine residue, creating a high-energy phosphoryl group that is subsequently transferred to an aspartate residue in the response regulator protein. The phosphorylation induces a conformational change in the N-terminal domain that results in activation of the C-terminal domain that affects the output response. Bacterial histidine kinases can be bifunctional, exhibiting both kinase and phosphatase activities toward the cognate response regulator (Igo *et al.*, 1989). Control in two-component pathways is thus accomplished through the ability of the histidine kinase to regulate the phosphorylation state of the downstream response regulator (Stock *et al.*, 2000). Most sensors and response regulators function as exclusive partners (cognate partners) that do not interact with components of other two-component systems. However, variations on the simple two-step scheme exist, in which multiple histidine kinases phosphorylate the same response regulator or a single histidine kinase controls several response regulators. Non-partner two-component system interactions are defined as cross-regulation or cross talk, and can either be detrimental or beneficial (Laub *et al.*, 2007). Cross talk between members of two distinct two-component systems in uropathogenic *E. coli* has been shown to play a role in the development of antibiotic resistance (Guckes *et al.*, 2017). The *B. subtilis* sporulation control system is an example of a variation of the typical two-component

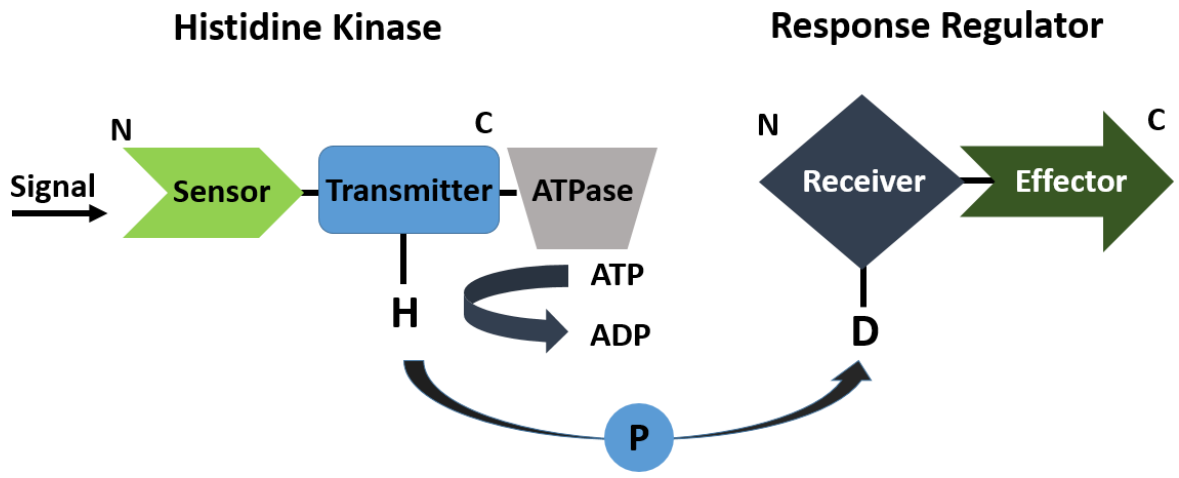

FIG. 1.4. The two-component signal transduction system. The prototypical phosphotransfer scheme involves two conserved components, a histidine protein kinase and a response regulator. The histidine protein kinase, in response to some environmental stimuli, autophosphorylates at a conserved histidine residue, creating a high-energy phosphoryl group that is subsequently transferred to an aspartate residue in the response regulator protein. The phosphorylation induces a conformational change in the N-terminal domain that results in activation of the C-terminal domain that affects the output response.

pathway, a His-Asp-His-Asp phosphorelay, where seven proteins, KinA (B, C or D), Spo0F, Spo0B, and Spo0A constitute the signal transduction system (Grimshaw *et al.*, 1998).

The first component of the two-component system, the histidine kinase, is composed of a sensory domain and transmitter domain, and is reminiscent of mammalian tyrosine kinase receptors in both its structure and mode of action (Besant *et al.*, 2003). Environmental stimuli are detected either directly or indirectly by the N-terminal sensing domain located on the outside of the plasma membrane. The transmitter domain contains two subdomains: a dimerization domain with a conserved His residue, and an ATP/ADP-binding phosphotransfer or catalytic domain that catalyzes the phosphorylation of the conserved His (Stock *et al.*, 2000). Instead of promoting a single phosphoryl transfer, hybrid kinases use multistep phosphorelay schemes. The *E. coli* hybrid kinase ArcB is composed of two N-terminal transmembrane regions followed by a kinase core, a domain similar to the regulatory domain of response regulators, and finally a second His-containing region termed a His-containing phosphotransfer (HPt) domain (Ishige *et al.*, 1994).

Most response regulators also consist of two domains: a conserved N-terminal regulatory domain, or phosphoryl group acceptor (receiver) domain, and a variable C-terminal output (effector) domain (Nguyen *et al.*, 2015). Structures of several single-domain response regulators and N-terminal phosphorylation or receiver domains, such as FixJ, NtrC, and PhoB, reveal a doubly wound five-stranded α/β fold, and the five-stranded parallel β-pleated sheet surrounded by 3 and 2 α helices on both sides (Birck *et al.*, 1999; Nohaile *et al.*, 1997; Sola *et al.*, 1999). The conserved Asp for phosphorylation lies at the beginning of the loop between β3 and α3 (Lβα3). Two other Asp residues involved in coordinating a Mg^{2+} ion reside in the Lβα1 loop. Additional catalytic residues are Thr and Ser, which interact with one of the oxygen atoms of the phosphoryl

group in the Lβα4 loop (Bourret, 2010). A conserved Lys residue plays a key role in phosphotransfer in the Lβα5 loop. The major differences in response regulator function arise from the subtle ways in which dimerization, auto-inhibition by the receiver domain, and activation by phosphorylation affect the output function (Kenney, 2002). Amongst response regulators, a variety of output domains that either bind DNA, RNA, protein, and other ligands or possess enzymatic activity allow for the regulation of diverse cellular processes (Galperin, 2010). However, the majority of response regulators are TFs with DNA-binding effector domains. These DNA-binding domains can be subdivided into three major families, represented by OmpR, NarL, and NtrC in *E. coli* (Mizuno, 1997).

The response regulator OmpR and the osmosensor histidine protein kinase EnvZ function in osmoregulation as a two-component signal transduction system in *E. coli* (Forst *et al.*, 1988). The N-terminal domain of OmpR contains the aspartate residue which is phosphorylated and other active-site residues that are highly conserved within the family of response regulator proteins, while the relatively small C-terminal domain (OmpRc) may accommodate numerous inter- and intramolecular interactions (Kato *et al.*, 1989; Martinez-Hackert *et al.*, 1997a; Pratt *et al.*, 1994).

1.2.2 The OmpR subfamily of response regulators

OmpR is one of the most extensively studied members of a subfamily of more than 50 response regulator proteins characterized by sequence similarity in their C-terminal DNA-binding domains. Members of this family include such diverse transcriptional regulators as *E. coli* PhoB (of the phosphate assimilation pathway), *Enterococcus faecium* VanR (controls resistance to the antibiotic vancomycin), and *Agrobacterium tumefaciens* VirG (involved in the

establishment of crown gall tumors in plants) (Martinez-Hackert *et al.*, 1997a). *B. subtilis* ResD also belongs to the OmpR/PhoB subfamily (Kenney, 2002). Members of the family exhibit between 20 and 65% sequence identity within the 98-amino acid OmpRc domain. The comparatively low level of sequence conservation is expected as each C-terminal domain must be involved in specific interactions, such as recognition of a particular DNA sequence, dimerization or perhaps oligomerization with itself, and contacts with either the N-terminal regulatory domain, the α subunit, or the σ^{70} subunit of RNAP. All members of the OmpR-family proteins for which DNA recognition sites have been determined appear to bind to direct repeat DNA sequences. However, there is variation in the arrangement of sites, both with respect to the number of recognition sites and the spacing between them (Martinez-Hackert *et al.*, 1997b). OmpR/PhoB members contain receiver and effector domains like other response regulators, but are characterized by a unique winged helix-turn-helix (wHTH) binding motif, followed by an additional hairpin in the C-terminal effector domain and a flexible linker between the receiver and effector domains. Differences in linker length between the N-terminal and C-terminal domains may play some role in inter-domain communication (Mattison *et al.*, 2002).

Secondary structure elements corresponding to α1 through β7 of OmpRc are common to most winged-helix domains and can ascribe putative functional roles (Fig. 1.5). The α2- α loop-α3 region forms a helix-turn-helix motif. Helix α3 corresponds to the recognition helix that interacts with the major groove of DNA and helix α2 corresponds to the positioning helix. The loop connecting β6 and β7 of the C-terminal hairpin has been shown in other winged-helix proteins to interact with the minor groove of DNA and has been termed recognition wing W1. In some winged-helix proteins, a second wing, W2, that is positioned adjacent to the recognition

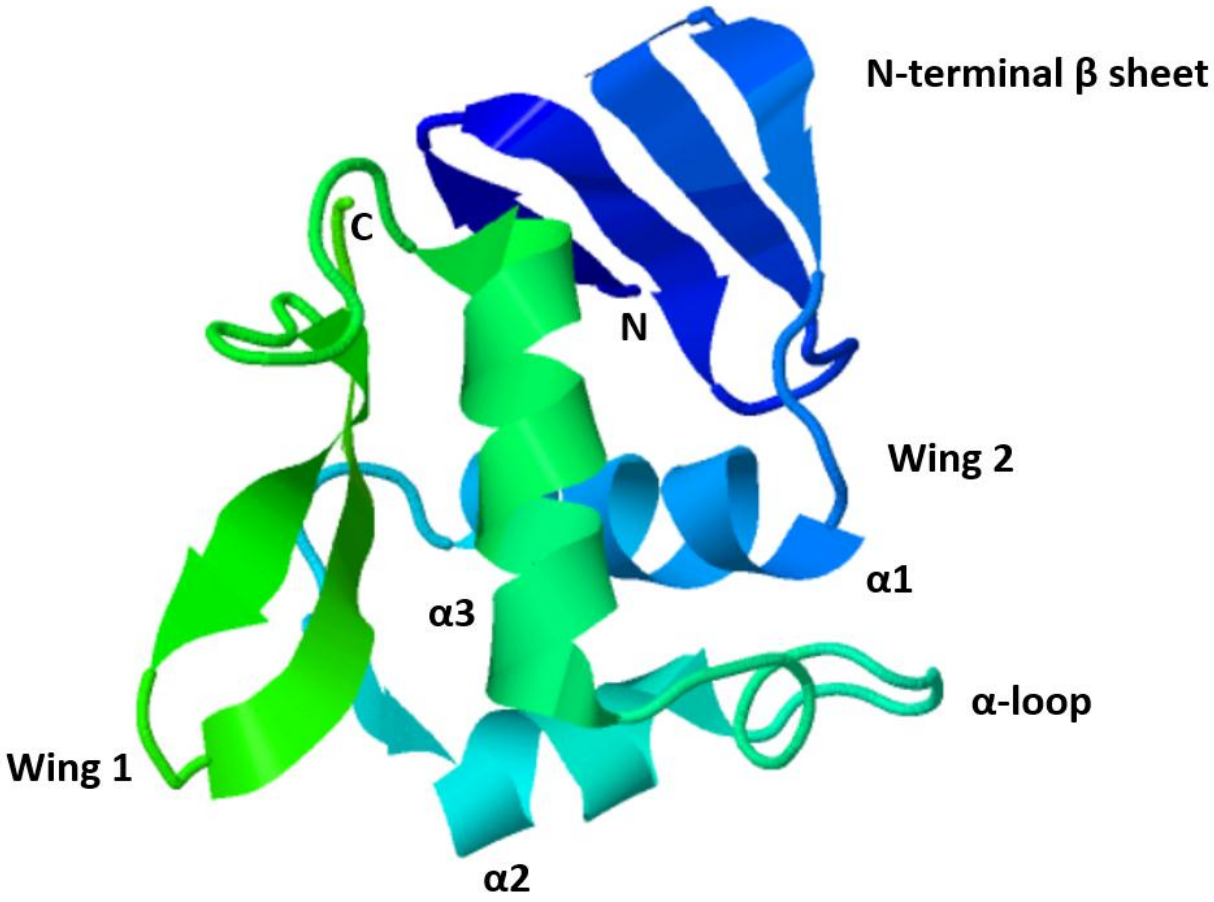

FIG. 1.5. The C-terminal domain of OmpR (OmpRc) structure. Secondary structure elements corresponding to α1 through β7 of OmpRc are common to most winged-helix domains and can ascribe putative functional roles (PDB code: 1OPC).

helix opposite to wing W1, contacts the minor groove (Clark *et al.*, 1993; Parkinson *et al.*, 1996; Schultz *et al.*, 1991). The wHTH of the OmpR/PhoB family is unique because it contains an additional 4-stranded antiparallel β-sheet before the classic wHTH motif (β1-β2-β3-β4-α1-β5-α2-α3-β6-β7). The antiparallel β sheet packs against helices α1 and α3, contributing six amino acids to the hydrophobic core of OmpRc, and is an integral part of the OmpRc DNA-binding fold (Martinez-Hackert *et al.*, 1997a). While the residues that form the hydrophobic core are the most highly conserved throughout the subfamily (Kenney, 2002), the amino acid sequence of the 4-stranded antiparallel β-sheet is not well conserved, which might indicate that the 4-stranded antiparallel β-sheet is involved in specific interactions with target genes (Martinez-Hackert *et al.*, 1997b). Another region of low conservation, the turn, or α-loop, is an unusually large loop (10 amino acids compared to the more common 3–5 in other HTH proteins) connecting helix α2 to helix α3. The α-loop was proposed to be the contact region between OmpR and the C-terminal domain of the RNAP α subunit (αCTD) (Pratt *et al.*, 1994). *E. coli* PhoB, along with many other OmpR/PhoB-related proteins, utilizes a different partner, the σ^{70} subunit, for interaction with RNAP (Makino *et al.*, 1993). The α-loop is often designated the transactivation loop, or "activation domain" since a number of OmpR mutants that fail to activate transcription lie within the loop (Pratt *et al.*, 1994). Mutations in PhoP, the phosphate deprivation response regulator of *B. subtilis*, that affect transcriptional activation have also been found to be broadly distributed throughout the transactivation loop (Chen *et al.*, 2004). On the other hand, mutations in the α-loop of *E. coli* PhoB have been shown to specifically affect DNA binding ability (Makino *et al.*, 1996).

OmpR binds to its high-affinity site as a monomer making contacts with T and G bases via residues in helix α3, allowing a second OmpR molecule to bind (Rhee *et al.*, 2008). The

effector domain of response regulators normally binds to a *cis*-acting element in the promoter region of regulons and controls gene expression. *E. coli* OmpR binds to three regions in the *ompF* promoter for regulated porin gene expression in an ordered fashion that requires increasing amounts of OmpR-phosphate (Rampersaud *et al.*, 1994). *B. subtilis* PhoP also shows increased DNA binding efficiency upon phosphorylation (Chen *et al.*, 2004). Studies indicating that OmpR can form stable multimers upon phosphorylation suggest that stabilization occurs through OmpR-OmpR interactions. OmpR-OmpR interactions may be greatly enhanced by DNA topology and factors that bring the bound OmpR molecules closer to each other, such as DNA looping (Rampersaud *et al.*, 1994). The ability of response regulators to access DNA is controlled by several factors, including dimerization of the effector domain, the binding motif, and DNA topology (Nguyen *et al.*, 2015). For example, relaxation of DNA supercoiling has been shown to increase OmpR binding to DNA in *Salmonella enterica* (Cameron *et al.*, 2012).

1.2.3 The ResDE two-component system

Similar to the DNA binding affinity of *E. coli* OmpR, *B. subtilis* ResD displays phosphorylation-enhanced binding. An *in vitro* phosphorylation assay suggested that Asp57 is the phosphorylation residue of ResD as a D57A mutant cannot be phosphorylated by ResE. Yet, ResD, even without phosphorylation, is able to respond to the redox state of cells and to activate transcription. ResD is thus an example of a response regulator that can be activated by a phosphorylation-independent mechanism to some extent and still respond to the same conditions that are sensed by the cognate sensor kinase (Geng *et al.*, 2004).

resD mutants (and to a lesser extent, *resE* mutants) exhibit pleiotropic phenotypes directly related to respiratory function, including streptomycin resistance, lack of production of

aa$_3$ or *caa*$_3$ terminal oxidases, acid accumulation when grown with glucose as a carbon source, and loss of ability to grow anaerobically on a medium containing nitrate. Colony morphology, carbon source utilization, sporulation, lysozyme sensitivity, and APase (alkaline phosphatase) productivity are also affected by a *resD* mutation (Sun *et al.*, 1996b). The induction of *resDE* under different growth conditions suggests that the ResDE regulatory system is involved in multiple cellular responses to diverse environmental signals. For example, ResDE, along with the Spo0A phosphorelay, act to regulate the activity of a third two-component system, PhoPR in *B. subtilis* (Sun *et al.*, 1996a). Conversely, the phosphorylated PhoP (PhoP~P) response regulator directly binds to and is essential for transcriptional activation of the *resABCDE* operon as well as being involved in repression of the internal *resDE* promoter during phosphate-limited, aerobic growth (Birkey *et al.*, 1998). Moreover, the ResDE system is important for anaerobic expression of the *nrd* operon, which encodes the ribonucleotide reductase essential for DNA synthesis (Hartig *et al.*, 2006).

ResDE orthologs have been found to play a role in virulence gene regulation in *Bacilli*, *Listeria*, and *Staphylococci*. The role the ResDE system in *B. anthracis*, BrrAB, plays in regulation of toxin gene expression under aerobic conditions remains unresolved due to conflicting observations (Vetter *et al.*, 2007; Wilson *et al.*, 2008). In the food-borne pathogen *B. cereus* F4430/73, Fnr has been shown to modulate enterotoxin gene expression through a mechanism central to the metabolism of several types of carbohydrates in a microaerobic environment (Messaoudi *et al.*, 2010). ResD~P and Fnr of *B. cereus* F4430/73 potentially co-regulate enterotoxin and fermentation gene expression (Esbelin *et al.*, 2009; Zigha *et al.*, 2007). Similar to the *B. subtilis* ResDE system, the *resDE* genes of *B. cereus* F4430/73 reside in the *resABCDE* operon and are transcribed from both the *resA* and *resD* promoters (Duport *et al.*,

2006). In contrast, the genome of *Listeria monocytogenes* lacks homologues of *resABC*, and *resD* is expressed as a monocistronic transcript (Larsen *et al.*, 2006). Similarly, the genes encoding SrrAB (or SrhSR), the ResDE orthologs of *Staphylococcus aureus*, are transcribed as a single gene pair and do not form part of a larger operon (Throup *et al.*, 2001). However, analysis of transcript lengths as determined by Northern analysis of *srrAB* transcription indicate that *srrA* can be transcribed independently of *srrB*, while *srrB* must be co-transcribed with *srrA* (Yarwood et al., 2001). ResD of *L. monocytogenes* modulates virulence gene expression in response to carbohydrates, while SrrAB of *S. aureus* participates in the differential regulation of virulence, biofilm formation, and energy transduction in response to changes in oxygen availability (Larsen *et al.*, 2006; Pragman *et al.*, 2007b; Throup *et al.*, 2001). During aerobic growth, SrrAB manages the mutually inclusive regulation of genes required for H_2O_2 resistance, aerobic respiration and virulence (Mashruwala *et al.*, 2017). Under low oxygen conditions, SrrA-SrrB functions as a repressor of exotoxins and staphylococcal superantigens such toxic shock syndrome toxin-1 (TSST-1) (Pragman *et al.*, 2007a). Under anaerobic growth conditions, SrrAB increases transcription of the intercellular adhesin (*ica*) cluster, leading to increased polysaccharide intercellular adhesin (PIA) production, and ultimately increased protection for *S. aureus* against non-oxidative defense mechanisms in human neutrophils (Ulrich *et al.*, 2007). Similarly, although the α and β chains of hemoglobin in human blood have been shown to affect SrrB and thus inhibit TSST-1 production, *S. aureus* has developed mechanisms to promote survival (Schlievert *et al.*, 2007). In response to nitrosative stress and hypoxia, the SrrAB system senses reduced electron flow through the respiratory chain and acts similarly to *B. subtilis* NsrR by regulating *hmp* expression (Kinkel *et al.*, 2013). SrrAB induction of the aa_3 and *bd* respiratory cytochromes (encoded by *qoxABCD* and *cydAB*, respectively), and consequently an increase in

the total number of terminal oxidases per cell, effectively raises the threshold of NO· required to wholly inhibit respiration, allowing Hmp to more quickly reduce NO· to respiration-permissive levels (Grosser *et al.*, 2016). SrrAB also plays a role in allowing *S. aureus* to coexist with H_2O_2-producing bacteria, including *Streptococcus sanguinis*, as *hmp* inactivation in *S. aureus* suppresses the generation of intracellular oxidative stress and supports less susceptibility to H_2O_2 (Oogai *et al.*, 2016).

1.2.3.1 ResE

How the *B. subtilis* two-component ResDE system activates aerobic or anaerobic respiration genes in response to oxygen availability still remains unresolved. β- Galactosidase activity assays have suggested that the cytoplasmic region, or the kinase domain, of ResE is at least partly, if not solely, responsible for perceiving the signal derived from oxygen limitation. However, the exact nature of the signal and the mechanism of signal perception by the sensor kinase ResE is still unknown. ResE is composed of an N-terminal signal input domain and a C-terminal catalytic (kinase) domain. The N-terminal domain contains two transmembrane subdomains (TM1 and TM2 transmembrane helices) and a large extracytoplasmic (periplasmic) loop flanked by the transmembrane subdomains. TM2, which is sufficient to anchor ResE to the membrane, is followed by a HAMP (histidine kinase, adenylyl cyclase, methyl-accepting chemotaxis protein, and phosphatase) linker and a PAS (Per-Arnt-Sim) subdomain. The HAMP linker is known to transmit signals between the input and output domains, while the PAS subdomain participates in signal reception and is essential for ResE activity. The kinase domain consists of ATPase subdomains and a conserved histidine residue at position 374 where ResE presumably undergoes autophosphorylation and subsequently promotes phosphorylation of ResD

(Baruah *et al.*, 2004). ResE is a bifunctional kinase: under anaerobic growth conditions the kinase activity is dominant, allowing for increased ResD phosphorylation, while under aerobic growth conditions, ResE mainly acts as a phosphatase (Nakano *et al.*, 2001b). Examination of ResD protein levels in wild type and a *resE* mutant during aerobic and anaerobic growth by Western blot analysis using an anti-ResD antibody followed by densitometric scanning showed the relative intensity of the ResD band in blots of proteins from anaerobically grown wild-type (100%) and *resE* cells (56%) and in blots of proteins from wild-type (42%) and *resE* (35%) cells of aerobically grown cultures. Consequently, robust induction of ResD-controlled genes can be attributed to the accumulation of higher amounts of ResD~P during anaerobic growth (Nakano *et al.*, 2000a).

1.2.3.2 ResD

Phosphorylated ResD of *B. subtilis* has been shown to directly bind to the promoter regions of target genes, such as *hmp* and *nasD*. More specifically, phosphorylation of ResD stimulates binding with DNA upstream of promoters having multiple target sequences for ResDE-dependent regulation, including the promoter regions of *hmp* and *nasD*. Conversely, with only a single target site, *fnr* requires a higher concentration of ResD for binding to the promoter region, and phosphorylation of ResD does not stimulate binding (Nakano *et al.*, 2000a). ResD is a monomer and does not oligomerize upon phosphorylation (Zhang *et al.*, 2000). DNase I footprinting experiments have indicated that five ResD monomers bind tandemly to the same face of the DNA helix except at the most proximal promoter binding site of *hmp* (Geng *et al.*, 2004). Mutational analysis of *B. subtilis* ResD-binding regions suggests $TTGTGAAN_3TTTN_4A$ as a consensus ResD box for ResD-controlled gene expression (Geng *et al.*, 2007). MEME motif

search conducted on GeF-seq data identified $TGANW_6$ or $TNTGANW_4$ (W=A or T), observed particularly in high-intensity ResD-binding sites, as the ResD consensus sequence (Chumsakul *et al.*, 2017). Mutations in ResD that could reduce ability to activate transcription can either affect DNA binding affinity (binding mutations), or affect the interaction of ResD with RNAP (interaction mutations). *In vitro* DNase I footprinting analysis of the *hmp* and *nasD* promoters showed that alanine substitution mutations in the α-loop (the transactivation loop identified with OmpR) at positions G198 and R201 affected DNA-binding ability of ResD (Geng, 2007). Amino acid substitution mutations of ResD were generated in the present study in order to elucidate the interaction surface of ResD with RNAP.

1.3 BACTERIAL TRANSCRIPTIONAL CONTROL

1.3.1 RNA polymerase

The principal enzyme responsible for transcription of genetic sequences is RNAP, which catalyzes the polymerization of ribonucleoside 5′-triphosphates as directed by a DNA template (Cooper, 2000). Transcription of genetic information ultimately into protein products is the first and most regulated step in gene expression (Ebright, 1998). In cellular regulatory networks, genetic activity is controlled by molecular signals that determine when and how often a given gene is transcribed. In genetically controlled pathways, the protein product encoded by one gene often regulates expression of other genes (McAdams *et al.*, 1997). Regulation of gene expression thus allows the cell to respond to variations in the environment, such as changes in the availability of nutrients and oxygen.

Transcriptional regulation has been intensively investigated in prokaryotes, especially in *E. coli*, and the first high-resolution X-ray crystal structure of any cellular RNAP, bacterial

Thermus aquaticus RNAP core enzyme, has contributed significantly to understanding structure-function relationships (Cooper, 2000; Zhang *et al.*, 1999). X-ray crystal structures of archaeal RNAP suggest that archaeal transcription is more of a hybrid, with eukaryotic-type transcription apparatus and bacterial-like regulatory mechanisms (Jun *et al.*, 2011). However, unlike eukaryotes that have three types of RNAP with different functions, archaea and prokaryotes have only one type of RNAP responsible for all transcription events (Ebright, 1998; Gehring *et al.*, 2016). During transcription in prokaryotes, the multi-subunit RNAP holoenzyme forms an initial closed promoter complex by recognizing specific promoter consensus sequences in the DNA template (the −10 Pribnow box and −35 element), melts a small region containing the transcription start site to form the open complex (transcription bubble), initiates RNA synthesis in the presence of nucleotide substrates, processively elongates the transcript after transitioning to a stable elongation complex and the σ subunit is discharged, and finally terminates and releases the RNA product when a specific termination signal is encountered (Darst *et al.*, 1989; deHaseth *et al.*, 1998).

The core RNAP is responsible for binding to template DNA to synthesize RNA and is comprised of distinct polypeptides, with the subunit composition differing between eubacteria. The core *E. coli* RNAP complex consists of two α subunits, single β, β′, and ω subunits. Core RNAP from *B. subtilis* and other gram-positive bacteria contains an additional subunit, δ, and a small auxiliary subunit, ε (Juang *et al.*, 1994; Keller *et al.*, 2014). Although the bacterial RNAP core is capable of transcription elongation, the σ factor is required to form the holoenzyme that recognizes the promoter sequence and initiates promoter-specific transcription (Gross *et al.*, 1998) (Fig.1.6.A).

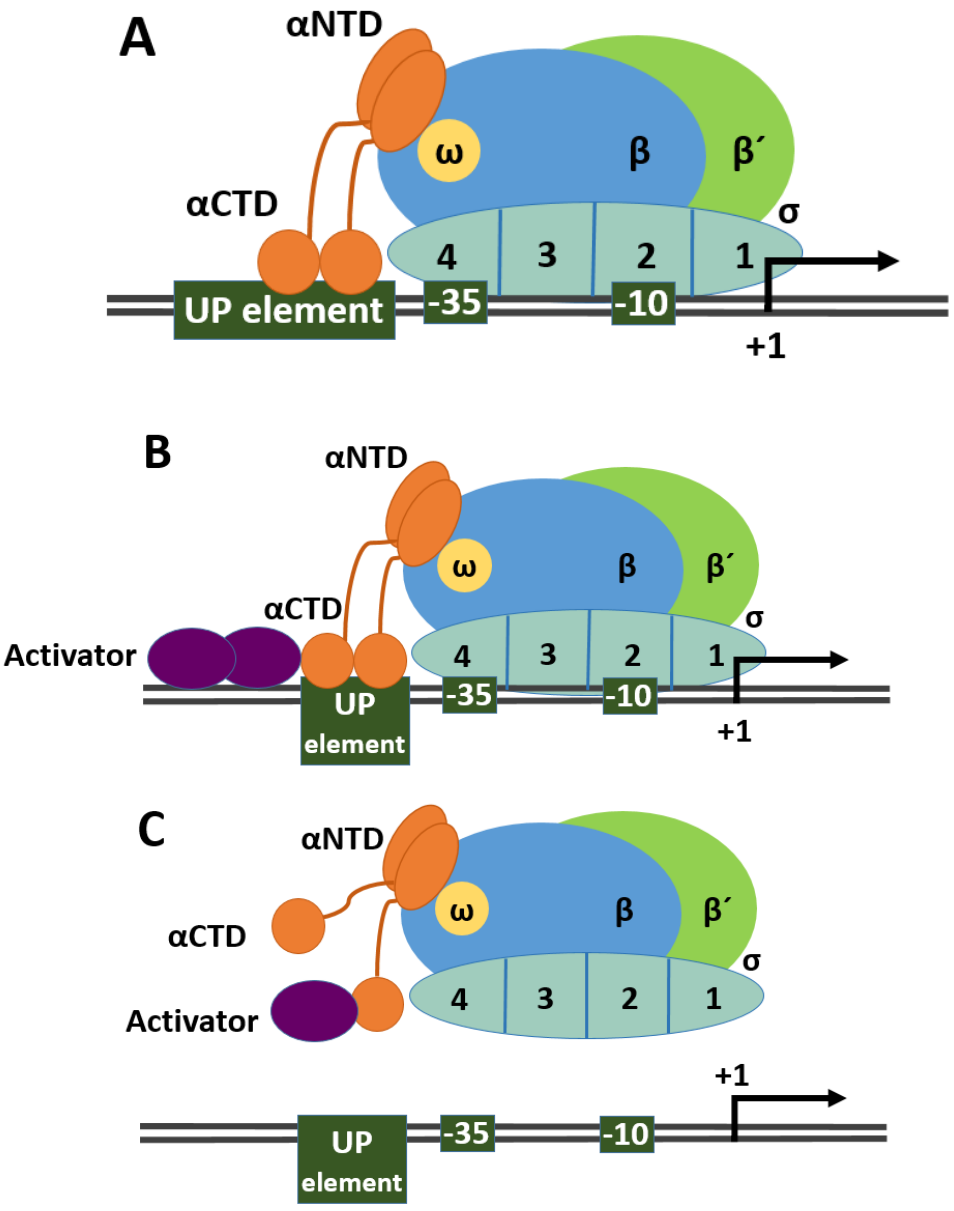

FIG. 1.6. RNA polymerase. A) The core *E. coli* RNAP complex consists of two α subunits, single β, β′, and ω subunits. Core RNAP from *B. subtilis* contains an additional subunit, δ (not shown), and a small auxiliary subunit, ε (not shown). The σ factor is required to form the holoenzyme that recognizes the promoter sequence and initiates promoter-specific transcription. DNA elements recognized by RNAP at bacterial promoters include the UP element, the −35 element, and the −10 element. The location of the transcription start site (+1) is indicated by the bent arrow. B) Recruitment mechanism of transcription activation: the activator binds to a sequence upstream of the promoter −35 element and recruits RNAP to the promoter by directly interacting with αCTD. C) Pre-recruitment mechanism: protein-protein interaction with RNAP is first established, then the binary complex scans the chromosome for promoters in order to activate transcription.

The shape of core RNAP is reminiscent of a crab claw, with an internal groove or channel running along the full length (between the claws) having a diameter sufficient to accommodate a double stranded nucleic acid and the active site Mg^{2+} at its base. One arm of the claw is primarily the β subunit, the other primarily β′ (Zhang *et al.*, 1999). The β and β' subunits form the catalytic center of RNA synthesis and also provide binding sites for double-stranded downstream DNA, the DNA/RNA hybrid formed during transcription, and RNA. Despite being highly conserved in bacteria, large insertions in the β and β' subunit sequences characterize specific evolutionary lineages of bacteria (Murakami, 2015). The α dimer serves as a platform for binding of β and β', with the α^{I} subunit interacting with β, and the α^{II} subunit interacting with β′. Each α subunit consists of two independent functional domains connected by a flexible linker, with the N-terminal domain of α (αNTD) responsible for interaction with β or β′, and the C-terminal domain of α (αCTD) facilitating protein-DNA interactions with upstream promoter DNA (UP elements) and protein-protein interactions with upstream activators and repressors (Busby *et al.*, 1994; Ebright, 2000). αCTD plays an important role in regulating transcription by allowing for communication between RNAP and associated TFs.

Studies with *E. coli* have shown that the ω subunit and its N-terminal domain bind to full length β′ and assist the final step of RNAP core assembly when β′ associates with the $\alpha_2\beta$ sub-complex, and can also facilitate adjustment of transcriptional profiles through interactions with the stringent response-inducing molecule ppGpp (Ghosh *et al.*, 2001; Ross *et al.*, 2013). In cyanobacteria, the ω subunit facilitates the association of the primary σ factor with the RNAP core, allowing for efficient transcription of highly expressed genes (Gunnelius *et al.*, 2014). In *S. aureus*, ω is important for RNAP complex stabilization, influences σ factor recruitment to the RNAP complex, is required for biofilm formation, and allows the cell to circumvent multiple

forms of environmental stress (Weiss *et al.*, 2017). The δ protein acts as an allosteric modulator of RNAP conformation, functions together with σ as an initiation subunit of RNAP, and enhances core enzyme recycling (Juang *et al.*, 1994). In *B. subtilis*, δ has been shown to act synergistically with the helicase HelD in order to stimulate transcription and promote more efficient cycling of RNAP (Wiedermannova *et al.*, 2014). Single-particle analysis suggests that the ε subunit of *B. subtilis* RNAP binds to the β′ jaw and secondary channel of RNAP, potentially preventing access of phage proteins to the transcriptional machinery (Keller *et al.*, 2014).

The σ factor transiently associates with and guides the core RNAP to recognize specific promoter elements and initiate transcription. Two main promoter elements recognized by RNAP holoenzyme are the −10 hexamer and the −35 hexamer, located 10 and 35 base pairs upstream from the transcription start site, respectively. Two other important promoter elements, the extended −10 element (a 3–4 bp motif located immediately upstream of the −10 hexamer), and the UP element (a ~20 bp sequence located upstream of the promoter −35 hexamer), contribute to the initial binding of RNAP to a promoter (Busby *et al.*, 1994; Keilty *et al.*, 1987; Rosenberg *et al.*, 1979). The synthesis of a variety of σ factors with unique promoter preferences provides bacteria with the opportunity to maintain basal gene expression as well as regulate gene expression in response to altered environmental or developmental signals. Based on sequence similarity, the bacterial σ factors can be grouped into two families, the σ^{70} and σ^{54} families. The majority of σ factors belong to the σ^{70} family, and can be divided into three different functionally and structurally related groups (Wosten, 1998). The transcription of housekeeping genes expressed during log-phase growth depends on the group 1 σ factor (σ^{70} in *E. coli* and σ^{A} in *B. subtilis* and other bacteria). Primary (or group 1) σ factors consist of four functional regions

called regions 1, 2, 3, and 4 (Gribskov *et al.*, 1986). Each region has been further subdivided, for example, sub-region 1.1, found only in the primary σ factors, modulates DNA binding. Region 2 is the most conserved region, with sub-region 2.4 implicated in the recognition of the –10 promoter element. Region 3 recognizes the extended –10 element, and region 4 (specifically region 4.2) participates in recognition of the –35 promoter sequence (Lonetto *et al.*, 1992). Group 2 σ factors bear sequence similarity in their DNA-binding regions to group 1 σ factors, but are non-essential for exponential cell growth. The third group of σ factors include alternative factors that control the transcription of specific regulons during special physiological or developmental conditions, such as sporulation. Members of the σ^{54} family play a role in the expression of genes involved in diverse processes such as nitrogen fixation and levanase production (Haldenwang, 1995; Helmann *et al.*, 1988; Murakami, 2015; Wosten, 1998).

1.3.2 Regulation of bacterial transcription initiation

In response to growth signals or environmental conditions, TFs also play a crucial role in the exquisitely complex regulation of gene expression. Indeed, just seven regulatory proteins (CRP, FNR, IHF, FIS, ArcA, NarL and Lrp) are sufficient for directly modulating the expression of 51% of genes in *E. coli*. Global regulators control operons that belong to different metabolic pathways, and frequently work together with more specific local regulators (Martinez-Antonio *et al.*, 2003). Some TFs function solely as activators or repressors, whereas others can function as either according to the target promoter. Three general mechanisms are used for activation: (i) the activator binds to a sequence upstream of the promoter −35 element and recruits RNAP to the promoter by directly interacting with αCTD, (ii) the activator binds to a target that overlaps the promoter −35 element and contacts region 4 of σ, recruiting RNAP to the promoter, or (iii) the

activator alters the conformation of the target promoter, enabling RNAP interaction with the promoter at the -10 and -35 elements. Three general mechanisms of repression include steric hindrance of RNA polymerase binding to promoter DNA, repression by DNA looping, and repression by the modulation of an activator protein [reviewed in (Browning *et al.*, 2004; Lee *et al.*, 2012)]. Although recruitment is commonly accepted as the primary mechanism of transcription activation in *E. coli*, SoxS (activator of superoxide regulon) and related proteins have been shown to utilize a pre-recruitment pathway, where protein-protein interaction with RNAP is first established, and then the binary complex scans the chromosome for promoters in order to activate transcription (Griffith *et al.*, 2002) (Fig.1.6.B,C). Spx found in low-GC-content Gram-positive bacteria belongs to the TFs that control transcription through pre-recruitment mechanism (Nakano *et al.*, 2010).

1.3.3 Spx

Spx, a global transcriptional regulator in *B. subtilis*, exerts both positive and negative control over transcription initiation of genes (Nakano *et al.*, 2010). As a pleiotropic global TF, Spx directly regulates genes involved in cysteine and bacillithiol biosynthetic pathways, acts as a modulator of other stress response regulons such as the heat or salt stress response, and is required for the basal activity of some promoters even in the absence of external disulfide stress (Rochat *et al.*, 2012; Runde *et al.*, 2014). Several Gram-positive bacterial species including *Enterococcus faecalis* encode a single version of Spx, a member of the ArsC (arsenate reductase) family of proteins (Kajfasz *et al.*, 2012; Zuber, 2004). Conversely, many streptococci, including *Streptococcus mutans*, have two or more orthologues of Spx that regulate virulence traits along with the oxidative stress response (Kajfasz *et al.*, 2009; Kajfasz *et al.*, 2010). While SpxA1 and

SpxA2 of *S. sanguinis* may have different functions, SpxA1 and SpxA2 of *Streptococcus pyogenes* coordinate toxin expression, pathogenesis, and stress responses (Chen *et al.*, 2012; Port *et al.*, 2017). *S. aureus* Spx plays a key role in growth under both stress and nonstress conditions, and may impact biofilm formation by modulating the expression of *icaR* in response to glucose (Pamp *et al.*, 2006). In contrast to *B. subtilis*, where members of the Spx regulon are synthesized at a higher rate after NO stress, *S. aureus* responds to NO by increasing synthesis of enzymes involved in anaerobic metabolism (Hochgrafe *et al.*, 2008).

By a single monomer of Spx-RNAP interaction followed by contact with a conserved AGCA upstream promoter sequence element, Spx functions in controlling the *B. subtilis* response to thiol-specific oxidative stress by inducing the expression of genes such as *trxA* and *trxB*, which encode thioredoxin and thioredoxin reductase, respectively (Lin *et al.*, 2012; Lin *et al.*, 2013; Nakano *et al.*, 2003a; Nakano *et al.*, 2005). Genetic studies, biochemical data, and structural studies all suggest that residue G52 of Spx and the αCTD residue Y263 are important for Spx/α complex formation (Lamour *et al.*, 2009; Nakano *et al.*, 2000b; Nakano *et al.*, 2001a; Newberry *et al.*, 2005). Spx-dependent repression and transcriptional activation also require contact between the K267 residue of αCTD and residue R47 of Spx (Zhang *et al.*, 2006). When wild-type cells are not undergoing disulfide stress, the Spx protein is present at very low levels due to the rapid degradation of the protein by the ClpXP protease and its Spx-specific proteolysis enhancing adaptor, YjbH (Garg *et al.*, 2009; Larsson *et al.*, 2007), allowing for transcriptional activation of target genes. Upon disulfide stress or in either a *clpX* or *clpP* mutant, Spx accumulates in a manner dependent on YjbH aggregation (Engman *et al.*, 2015), binds to αCTD, and blocks the activator-RNAP interaction. The direct interaction of Spx with αCTD interferes with transcriptional activation that is mediated by certain response regulators, such as ResD. For

example, *in vitro* run-off transcription analysis showed that increasing amounts of Spx lead to a decrease in the ResD-dependent *hmp* transcription (Nakano *et al.*, 2003b). In effect, genes whose products either are unnecessary or might exacerbate the damage caused by oxidative stress are repressed by Spx globally. Thus, Spx has been classified as an "anti-alpha" protein since its primary target is αCTD (Nakano *et al.*, 2003b). However, Spx could function as an extension of αCTD, introducing new activator binding surfaces to the RNAP holoenzyme, and essentially serving as a mediator through which activators can gain contact with RNAP to stimulate transcription at activator-controlled promoters (Zuber, 2004).

Together, previous studies suggest that the two RNAP-associated regulators, ResD and Spx, interact with RNAP competitively. My research investigates the interaction surface of RNAP with ResD in *B. subtilis* under anaerobic nitrate respiration conditions in order to understand the transcriptional profile governed by ResD and another RNAP-associated TF, Spx.

CHAPTER 2

INVESTIGATION OF THE RESD-RNAP INTERACTION SITE

2.1 INTRODUCTION

2.1.1 ResD interacts with RNAP

Found in organisms of all domains, Eubacteria, Archaea, and Eukarya, two-component signal transduction pathways allow for the detection of chemical and/or physical stimuli and coordination of appropriate responses [reviewed in (Stock *et al.*, 2000)]. In the Gram-positive model organism *B. subtilis*, the ResD-ResE two-component regulatory system responds to a signal presumably derived from oxygen limitation and exerts control globally over gene expression at the transcriptional level in order to support diverse cellular processes including aerobic or anaerobic metabolism. ResE, a membrane-bound sensor histidine kinase undergoes autophosphorylation and subsequently donates a high energy phosphoryl group to its cognate response regulator, ResD (Sun *et al.*, 1996b). Affinity purification of RNAP complex followed by LC-MS/MS analysis and more recent GeF-seq (*in vivo* genome-wide DNA binding analysis) suggested that ResD, like Spx, associates with RNAP (Chumsakul *et al.*, 2017; Delumeau *et al.*, 2011).

GeF-seq analysis revealed that a population of ResD, which binds to the promoter region of genes positively controlled by ResD, does not dissociate from the core RNAP and travels through the gene with the RNAP elongation complex, with the trail of binding ending at the ρ-independent terminator of the operon. A majority of genes directly activated by ResD identified by this study, including *nasD*, encode proteins that play a role in aerobic or anaerobic respiration (Chumsakul *et al.*, 2017; Nakano *et al.*, 1998a). On the other hand, at another class of ResD-

bound loci, ResD acts as a repressor (including *yjlC* which encodes an uncharacterized protein), with ResD only binding to the promoter region (Chumsakul *et al.*, 2017; Larsson *et al.*, 2005) (Fig. 2.1).

Although the high affinity of ResD to RNAP could explain why ResD travels with RNAP in coding regions, the mechanistic and functional interactions of ResD and RNAP still remain to be elucidated. Proteins in the OmpR/PhoB subfamily, of which ResD is a member, utilize different mechanisms of transcriptional activation (Kenney, 2002). Indeed, the activation loop of both PhoB and OmpR has been implicated as the region that directly interacts with RNAP, yet OmpR interacts with αCTD (Pratt *et al.*, 1994) and PhoB interacts with σ^{70} (Makino *et al.*, 1993). Since DNase I footprinting analysis of the *hmp* and *nasD* promoters has shown that the α-loop of ResD is important for DNA-binding (Geng, 2007), the region(s) of ResD directly involved in RNAP interaction have remained unidentified. In order to gain insight on the interaction surface of ResD with RNAP, previous genetic and crystallization work utilizing another RNAP-associated TF (Delumeau *et al.*, 2011), Spx, was considered.

2.1.2 Spx-RNAP interaction inhibits activator-dependent transcription

Under nonstress conditions, cellular levels of Spx are kept low by the protease ClpP, which works synergistically with the ATPase ClpX, in order to unfold and degrade targeted proteins (Wojtkowiak *et al.*, 1993). Genetic competence development, sporulation, and growth are severely impeded when *clpP* or *clpX* is mutated, highlighting the importance of the removal of Spx by ClpXP from cells during unperturbed growth conditions (Gerth *et al.*, 1998; Msadek *et al.*, 1998). Genetic studies led to the identification of the *spx* gene as the site of mutations that suppress *clpX* and *clpP* mutations. Suppressor mutations of *clpX* were mapped to two loci, and

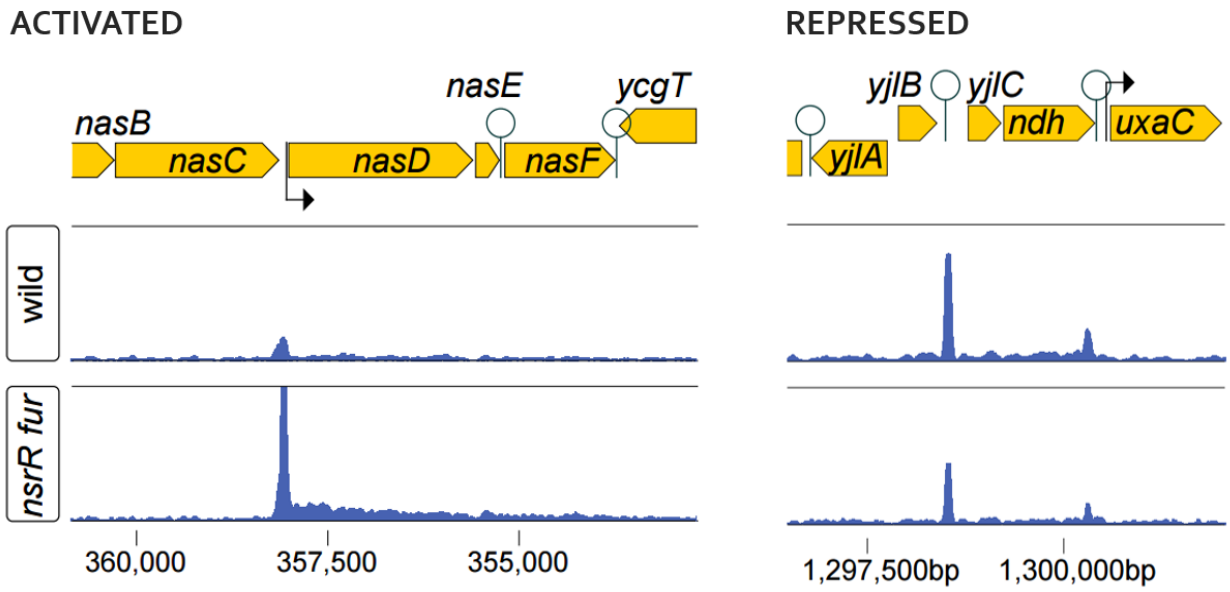

FIG. 2.1. Comparison of ResD binding profiles: activated and repressed genes. GeF-seq was carried out using double mutant strains in order to analyze the binding of ResD in the absence of other TFs (NsrR and Fnr), which often bind overlapping sequence where ResD binds; ResD binding was determined in the *nsrR fur* mutant cells under anaerobic fermentation conditions. The ResD binding patterns strongly correlate to how these genes are controlled by ResD and the function of the genes. ResD interacts with both the promoter and coding regions of genes activated by ResD, such as *nasD*, while ResD binds to only the promoter region of genes repressed by ResD, such as *yjlC*. Arrows indicate the direction of transcription. Lollipops show locations of ρ-independent transcription terminators. Numbers shown at Y-axis are x 1/1000 of normalized protein-binding intensities. Genome positions are indicated at the bottom (Chumsakul *et al.*, 2017).

given the designation *cxs* (*clpX* suppressor). Mutations were first detected in the *rpoA* gene encoding the RNAP α subunit. More specifically, these point mutants cause amino acid substitutions V260A and Y263C, in helix α1 of αCTD (Nakano *et al.*, 2000b). *In vivo* and *in vitro* genetic and biochemical techniques have shown that helix α1 plays a role in activator-stimulated transcription and RNAP binding to UP elements (Gaal *et al.*, 1996; Savery *et al.*, 2002). The $rpoA^{cxs}$ mutations partially alleviated defects in competence and growth in minimal medium characteristic of a *clpX* null mutation, while the heat sensitivity of the *clpX* mutant was retained (Nakano *et al.*, 2000b).

Suppressor mutations of *clpX* also mapped to the *yjbD* gene, which was renamed *spx* (suppressor of *clpP* and *clpX*). Similar to the $rpoA^{cxs}$ mutations, the *spx* loss-of-function mutations partially suppressed *clpX*, and the heat sensitivity of the *clpX* mutant was retained in *clpX spx* double mutants. Mutations in the *spx* gene that suppressed *clpP* also alleviated defects in competence development and growth on minimal medium (Nakano *et al.*, 2001a).

Apart from activating genes whose products function in maintaining thiol homeostasis, Spx negatively regulates developmental programs and energy-consuming growth-related functions, effectively allowing the cell to cope with stress. Spx blocks transcription of the *srfA* operon, which plays an important role in surfactin production, competence, and sporulation (Nakano *et al.*, 1991). ComA~P (of the ComPA two component regulatory system)-dependent transcription of *srf* is severely impaired in *clpP* and *clpX* mutants (Nakano *et al.*, 2001a). Since the pleiotropic effects on growth and development conferred by mutations in *clpP* and *clpX* could be suppressed by the elimination of Spx or by the *cxs* mutations in the *rpoA* gene, Spx must exert its negative effect on ComA-mediated transcription by interaction with αCTD (Nakano *et al.*, 2003b). The "anti-α" mode of repression by Spx, whereby Spx directly disrupts

complex formation between activator proteins and promoter-bound RNAP, was also observed for ResD-activated transcription. *In vitro* run-off transcription analysis showed that the activity of both ComA and ResD was inhibited when increasing amounts of Spx was added to reactions containing RNAP and promoter DNA of ComA- or ResD-controlled genes (Nakano *et al.*, 2003b).

The Spx global transcriptional regulator thus exerts both positive and negative control over transcription initiation in response to oxidative stress by primarily targeting αCTD (Nakano *et al.*, 2003b; Zuber, 2004). Tyr-263, identified as the site of suppressor mutation (Nakano *et al.*, 2000b), is a highly conserved *rpoA* residue among Gram-positive homologues (Murakami *et al.*, 1996). On the other hand, the Arg substitution of Spx at Gly-52 led to the *cxs* phenotype, a phenocopy of the Y263C mutation in *rpoA*. Based on the result, the hypothesis was raised that RpoA Y263 and Spx G52 are likely involved in α-Spx interaction, which was later confirmed by crystal structure (Lamour *et al.*, 2009; Newberry *et al.*, 2005). The G52R substitution introduces steric hindrance within the αCTD-Spx interface, resulting in loss of transcriptional activation and repression by Spx (Nakano *et al.*, 2003b; Newberry *et al.*, 2005) (Fig. 2.2a). Crystal structure studies followed by *in vitro* transcription analysis also showed that the K267 residue of αCTD forms a hydrogen bond with R47 of Spx (Newberry *et al.*, 2005) (Fig. 2.2b). Sensitivity to the thiol-oxidizing agent, diamide, is a phenotype associated with defective RNAP-Spx interaction, as seen in *B. subtilis* strains bearing a K267A or Y263C codon substitution of the *rpoA* allele (Nakano *et al.*, 2003a; Zhang *et al.*, 2006).

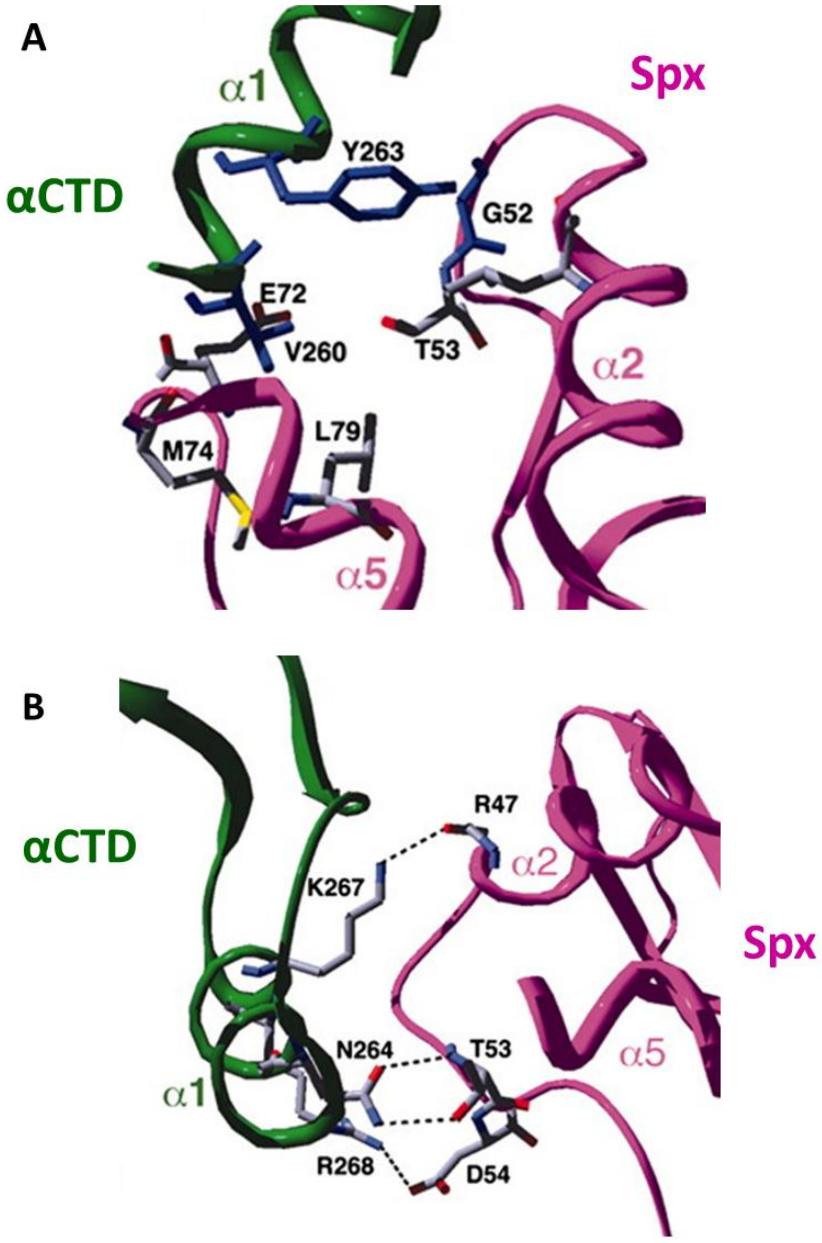

FIG. 2.2. The Spx–αCTD heterodimer interface. The backbones of Spx and αCTD are colored pink and green, respectively. Interacting residues are depicted as sticks and are colored according to atom type. Hydrogen bonds are depicted as dashes. (A) Interactions of *cxs* mutants of αCTD (V260 and Y263) and Spx (G52). The G52R substitution introduces steric hindrance within the αCTD-Spx interface, resulting in loss of transcriptional activation and repression by Spx. These residues cluster and are shown as blue sticks. (B) Hydrogen bonding interactions at the dimer interface. The K267 residue of αCTD forms a hydrogen bond with R47 of Spx (Newberry et al. 2005).

2.1.3 Search for the sites in ResD that play roles in RNAP interaction

DNase I footprinting analysis of the wild-type *fnr* promoter carried out using the αCTD (K267A) protein and ResD~P suggested that the K267 residue of αCTD is essential for interaction with ResD~P at the *fnr* promoter and that the interaction accelerates transcription initiation of *fnr* (Geng *et al.*, 2007). Subsequently, the effect of αCTD mutations on diamide sensitivity (for a lack of Spx activity) and anaerobic growth (for ResD activity) was assessed using plate assays. Compared to a *B. subtilis* K267A mutant, a Y263A mutant is more sensitive to diamide. ResD-controlled anaerobic growth by nitrate respiration showed that there is no significant difference in growth of *B. subtilis* Y263A, K267A, or Y263A K267A mutant strains, suggesting that both Y263 and K267 play roles in ResD activity (M.M. Nakano, unpublished results) (Fig 2.3). These results suggested that the αCTD residues Y263 and K267 are required for ResD activity.

Previous attempts to identify the residues of ResD involved in interaction with RNAP in light of ResD's status as a member of the OmpR/PhoB subfamily of response regulators (Kenney, 2002) instead uncovered residues important for ResD to bind to DNA (Geng, 2007). A region of low conservation amongst response regulators of the OmpR/PhoB subfamily, the α-loop connecting helix α2 and helix α3, was proposed to be the contact region between OmpR and αCTD in *E. coli* (Pratt *et al.*, 1994). Mutations in PhoP (the phosphate deprivation response regulator of *B. subtilis*), which affect transcriptional activation, are also broadly distributed throughout the α-loop (Chen *et al.*, 2004). On the other hand, mutations in the α-loop of *E. coli* PhoB specifically affect DNA binding ability (Makino *et al.*, 1996). Alanine scanning mutagenesis of the residues in ResD corresponding to the α-loop (V191-R201) and conserved residues in the OmpR/PhoB subfamily revealed that the α-loop in ResD serves as

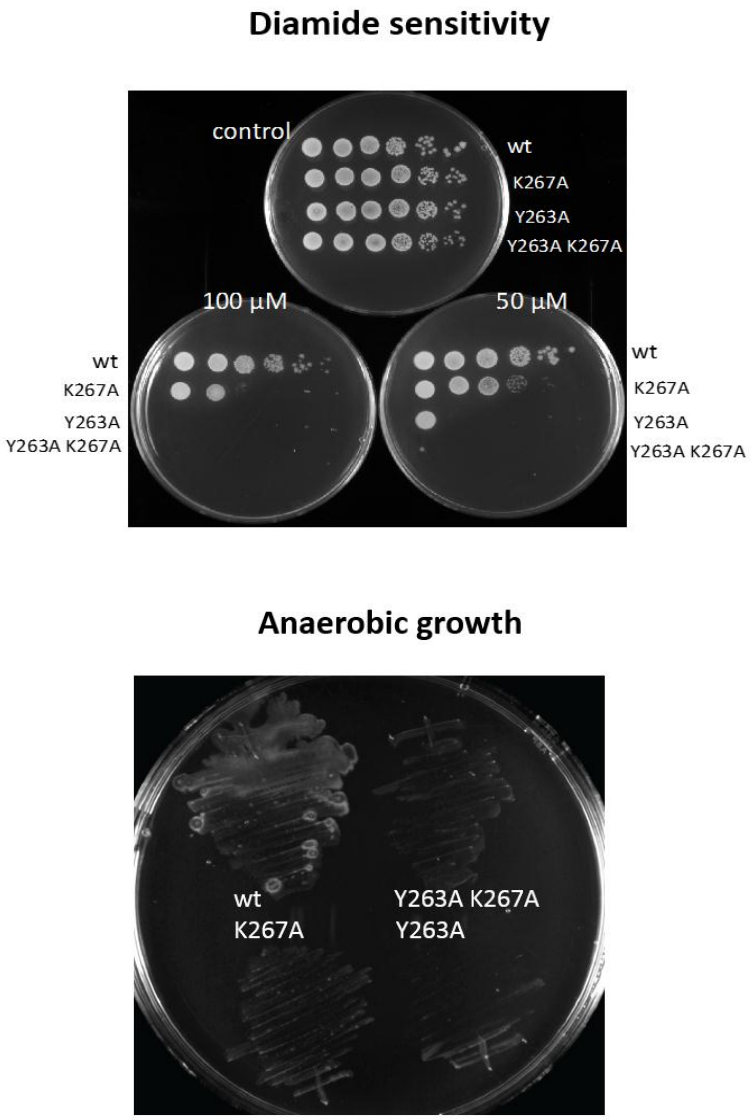

FIG. 2.3. Effect of αCTD interaction mutations on diamide sensitivity (for Spx activity) and anaerobic growth (for ResD activity). The αCTD residues Y263 and K267 play a role in ResD activity. To assess diamide sensitivity, wild-type and mutant strains were serially diluted and spotted on agar plates with or without 50 µM or 100 µM diamide. For growth assays, strains were cultured under anaerobic conditions on LB with glucose/nitrate and given a 36 h incubation time. (M.M. Nakano, unpublished results).

a DNA-binding site (Geng, 2007). Expression of *hmp*, *nasD*, and *fnr* was reduced by more than 50% in the R211A mutant, suggesting that R211 of ResD, like the corresponding and conserved residue in OmpR and PhoB, is involved in DNA binding. The alanine substitution in G198 and R201 of the putative transactivation α-loop reduced *nasD* transcription to 20% and less than 5% of the wild-type level, respectively. Conversely, *nasD* expression of a D199A mutant increased by about 60% compared to the wild-type level.

To distinguish whether the G198A, D199A, and R201A are binding or interaction mutations, DNase I footprinting analysis of the *hmp* and *nasD* promoters was carried out in the presence of mutant and wild-type ResD (Geng, 2007). Corresponding well to the ResD activity for *nasD* transcription *in vivo*, the *in vitro* assay showed that the G198A and R201A mutations lead to a complete loss of ResD binding activity to the *nasD* promoter, while the D199A mutant protein showed a higher binding affinity than the wild-type ResD. Thus, instead of playing a role in transcriptional activation, the α-loop of ResD likely serves as a DNA-binding site, and the D199A mutation might be important for reducing charge repulsion with DNA.

In the present study, the hypothesis is tested that N-terminal β-sheet regions in the C-terminal domain of ResD (ResDc), which shows a remarkable similarity to the αCTD interaction surface of Spx, might function in ResD-RNAP complex formation. Amino acid substitutions of the ResDc region were carried out by site-directed mutagenesis. Transcriptional *lacZ* fusions to the promoters of *nasD* (a gene directly activated by ResD) and *yjlC* (a gene directly repressed by ResD) were generated in order to examine the effect of ResD amino acid substitutions on transcriptional control. The phenotypic and physiological effects caused by the ResD mutations were investigated, and genetic analysis was undertaken to test the possibility that selected ResD mutants are defective in αCTD interaction.

2.2 MATERIALS AND METHODS

2.2.1 Strains and plasmids

Strains and plasmids are listed in Table 2.1, while primers are listed in Table 2.2. All *B. subtilis* strains used in this study are derivatives of *B. subtilis* 168 *E. coil* Top10 was used as the host for plasmid construction. *rpoA* with alanine substitutions at the αCTD residues Y263, K267, and at both Y263 and K267 had been previously generated as described (Nakano *et al.*, 2000b; Sheng *et al.*, 2007; Zhang *et al.*, 2006). The transcriptional *lacZ* fusions used are those integrated at the native loci or ectopic loci (*thrC* or *amyE*). The native *resD* gene was disrupted by an insertion of *cat* (chloramphenicol acetyl transferase) (in ORB8520) or *tet* (tetracycline-resistance gene) (in ORB10086). *nasD-lacZ* and *yjlC-lacZ* constructs were integrated into the *thrC* locus of the *B. subtilis* 168 chromosome. ORB9850 (*nasD-lacZ*) chromosomal DNA was used to transform ORB8520 (*resD*::*cat*), generating ORB9985 (*nasD-lacZ, resD*::*cat*). The antibiotic-resistance marker replacement plasmid JM103(pCm::Tc) (Steinmetz *et al.*, 1994) was used to transform ORB8520 (*resD*::*cat*), generating ORB10086 (*resD*::*tet*), which was then used for transformation with ORB9900 (*yjlC-lacZ*) chromosomal DNA, resulting in ORB10088 (*yjlC-lacZ, resD*::*tet*). ORB9900 chromosomal DNA was also used for transformation with ORB9558 [*rpoA* (Y263A K267A)], resulting in ORB10090.

Construction of the strain ORB10015 is described here as an example for the process of generating *resD* mutant strains by two-step PCR site-directed mutagenesis. Two sets of primers, o*spacup* and oMN16-765, and oMN16-764 and o*spacdown* were used in the first PCR reaction to generate and amplify the partially overlapping fragments “A” and “B” containing the mutated residue R153A of *resD*. oMN16-764 and oMN16-765 are mutagenic primers to generate the substitution of codon CGC with GCC in Arg153. The first PCR template was pMMN546,

pDR111 carrying wild-type *resD*. pDR111 is an ectopic integration vector that allows for the expression of *resD* from a LacI-repressible isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible P*hyperspank* (P*spank-hy*) promoter (Britton *et al.*, 2002). After each PCR product was purified from agarose gel, the second PCR reaction was used to anneal and extend fragments "A" and "B," using o*spacup* and o*spacdown* as the outside oligonucleotides. PCR products were then purified, digested with SalI and SphI, and ligated with pDR111 digested with the same enzymes. The inserted *resD* gene was verified by nucleotide sequencing. Each resulting plasmid was used to transform ORB9985 with selection for spectinomycin (75 µg/ml), erythromycin (1 µg/ml)/lincomycin (25 µg/ml), and chloramphenicol (5 µg/ml) resistance, and to transform ORB10088 with selection for spectinomycin (75 µg/ml), tetracycline (12.5 µg/ml), and chloramphenicol (5 µg/ml) resistance. Transformants were screened for amylase-negative phenotype that is indicative of a double recombination event at the *amyE* locus. The double mutant strains, for example ORB10067 (D148A G158R), were constructed similarly. To construct pHJ7, pMMN901 (*resD* with G158R mutation) was used as the first PCR template, and primers oHJ3 and oHJ4 were used to generate the D148A mutation. The previously generated pHG23 (*resDE* with the G198A mutation) was used as a PCR template, with primers oMN02-205 and oMN02-206, in order to amplify the *resD* fragment carrying the G198A mutation, leading to the construction of pHJ5.

Spx mutagenesis and strain construction followed a similar protocol. For mutagenesis, pSN56 was used as a template with a combination of mutagenic primers and o*spacup* and o*spacdown* in the first PCR. pSN56 (pDR111 with wt spx^{DD}) was previously constructed to allow Spx to resist ClpXP proteolysis under nonstress conditions (Nakano *et al.*, 2003a). HindIII and SalI were used for cloning each PCR product into pDR111. After the insert into pDR111

was confirmed by sequencing, each plasmid was used to transform ORB10107 with selection for spectinomycin (75 µg/ml), erythromycin (1 µg/ml)/lincomycin (25 µg/ml), and neomycin (5 µg/ml) resistance. ORB10107 was constructed by transforming ORB9357 (*spx*::*neo*) with pSN78 carrying *PtrxB-lacZ* at the *thrC* locus.

pMMN631 (*nasD-lacZ*) was used to transform ORB9543 [*rpoA* (K267A]] and ORB9544 [*rpoA* (Y263A)], generating ORB10035 and ORB10036, respectively. ORB8520 (*resD*::*cat*) chromosomal DNA was then used to transform ORB10035 and ORB10036, resulting in ORB10080 and ORB10081, respectively.

2.2.2 Measurement of β-galactosidase activity

To measure ResD-controlled transcription, *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 for nitrate respiration conditions, or 0.5% glucose and 0.5% pyruvate for fermentation conditions with relevant antibiotics. 1mM IPTG was added into cell cultures to express *resD*. The starting optical density at 600 nm (OD_{600}) was 0.02 using fresh overnight culture. Samples were taken at 1-h intervals, and β-galactosidase activity was measured as described previously (Miller, 1972). For measurement of Spx-controlled transcription, *B. subtilis* cells were grown aerobically in DSM (Difco sporulation medium). When OD_{600} of cultures reached around 0.3-0.4, 0.5 mM IPTG was added to induce *spx* expression and samples were taken at 0.5-h intervals until 3 h after the induction.

2.2.3 Western blot analysis

resD mutant and wild type strains (all carrying *nasD-lacZ*) were cultured anaerobically in 2xYT supplemented with 1% glucose, 0.2% KNO_3, spectinomycin (75 µg/ml), erythromycin (1 µg/ml)/lincomycin (25 µg/ml), chloramphenicol (5 µg/ml), and -/+ 1 mM IPTG. Cells were harvested at Time 0 (the end of exponential growth), and the pellet was frozen at -80°C after centrifugation and removal of supernatant. Pellets were resuspended in 150 µL lysis buffer (30 mM TrisHCl-1 mM EDTA pH 8.0) before lysis by the glass beads method. 100 µL of glass beads were added to the cell suspension, the tube was then vortexed for 2 min, set on ice for 2 min, vortexed again for 2 min, and set on ice for 5 min before lysate was taken to a new tube. After measuring protein concentration using a Bradford Assay, 5 µg of total protein were applied to a SDS polyacrylamide (12%) gel for electrophoresis. Western blot analysis was carried out using alkaline phosphatase detection method with anti-ResD antibody as described (Nakano *et al.*, 2000a).

2.2.4 Anaerobic Growth assay

resD mutant strains ORB9986, ORB9987, ORB10018, ORB10065, ORB1066, ORB10067, ORB10076, ORB10094, and *resD* wild-type strain ORB9435, were grown for 36 h on LB agar supplemented with 1% glucose and 0.2% KNO_3, -/+ 1 mM IPTG along with the control ORB8520 (*resD::cat*) at 37°C under anaerobic conditions. To generate an anaerobic atmosphere, the GasPak EZ Anaerobe Container System was used (Benton, Dickinson and Company).

2.2.5 Cytochrome caa_3 assay

The *resD* null mutant ORB8520 carrying an empty vector (pDR111 at *amyE*), *resD* mutant strains ORB9986, ORB9987, ORB10018, ORB10065, ORB1066, ORB10067, ORB10076, ORB10094, and *resD* wild-type strain ORB9435 were streaked onto DSM plates -/+ 1 mM IPTG and incubated under aerobic conditions at 37°C for 24 h. Afterward, plates were incubated at -20°C for 12 min. Immediately upon removal of plates from -20°C, plates were tested for N, N, N′, N′-tetramethyl-p-phenylene diamine (TMPD) oxidation as previously described (Le Brun *et al.*, 2000). The staining solution (50 ml) was prepared by mixing, at 55°C in the given order, 8 ml 10% (v/v) Triton X-100 in 0.1 M potassium phosphate buffer, pH 7.0; 2 ml 10% (w/v) sodium deoxycholate, pH 8.0; 10 ml 95% (v/v) ethanol; 10 ml 1% (w/ v) TMPD in water; and, 20 ml 1.2% (w/v) melted agar. 5 ml of the pre-warmed staining solution was added per plate and allowed to set. For TMPD-oxidase positive colonies, blue color formation occurred within 5 min.

Table 2.1 Bacterial Strains and plasmids

Strain/ Plasmid	Genotype	Source/ Reference
ORB8520	168 *trpC2 resD*::*cat*	Nakano, M
ORB9357	168 *trpC2 spx*::*neo*	Nakano, M
ORB9435	168 *trpC2 resD*::*cat amyE*::P*hs-resD*(wild type) *thrC*::*nasD-lacZ*	Quirke, T
ORB9543	168 *trpC2 rpoA*(K267A)	Nakano, M
ORB9544	168 *trpC2 rpoA*(Y263A)	Nakano, M
ORB9558	168 *trpC2 rpoA*(Y263A K267A)	Nakano, M
ORB9567	168 *trpC2 thrC*::*glpF-lacZ*	Nakano, M
ORB9568	168 *trpC2 thrC*::*glpF-lacZ resD*::*cat*	Nakano, M
ORB9834	168 *trpC2 yxiE*::pMutin	Nakano, M
ORB9845	168 *trpC2 rpoA*(Y263A K267A) *thrC*::*glpF-lacZ*	Nakano, M
ORB9867	168 *trpC2 sboA-lacZ*	Nakano, M
ORB9868	168 *trpC2 rpoA*(Y263A K267A) *sboA-lacZ*	Nakano, M
ORB9869	168 *trpC2 hmp*::*pML67(hmp-lacZ)*	Nakano, M
ORB9870	168 *trpC2 rpoA*(Y263A K267A) *hmp*::pML67(*hmp-lacZ*)	Nakano, M

ORB9885	168 *trpC2 rpoA*(Y263A K267A) *yxiE*::pMutin	Nakano, M
ORB9897	168 *trpC2 thrC*::pMMN929*(yozB-lacZ)*	Nakano, M
ORB9899	168 *trpC2 rpoA*(Y263A K267A) *thrC*::pMMN929(*yozB-lacZ*)	Nakano, M
ORB9900	168 *trpC2 thrC*::*cDA5 (thrC*::*ndh-lacZ)*	Nakano, M
ORB9985	168 *trpC2 thrC*::*nasD-lacZ resD*::*cat*	This study
ORB9986	168 *trpC2 thrC*::*nasD-lacZ resD*::*cat amyE*::P*hs-resD*(H152A)	This study
ORB9987	168 *trpC2 thrC*::*nasD-lacZ resD*::*cat amyE*::P*hs-resD*(G158R)	This study
ORB9988	168 *trpC2 thrC*::*nasD-lacZ resD*::*cat amyE*::P*hs-resD*(T159A)	This study
ORB9989	168 *trpC2 thrC*::*nasD-lacZ resD*::*cat amyE*::P*hs-resD*(E160A)	This study
ORB10005	168 *trpC2 thrC*::*nasD-lacZ resD*::*cat amyE*::P*hs-resD*(T155I)	This study
ORB10006	168 *trpC2 thrC*::*nasD-lacZ resD*::*cat amyE*::P*hs-resD*(D157A)	This study
ORB10015	168 *trpC2 thrC*::*nasD-lacZ resD*::*cat amyE*::P*hs-resD*(R153A)	This study
ORB10018	168 *trpC2 thrC*::*nasD-lacZ resD*::*cat amyE*::P*hs-resD*(G198A)	This study
ORB10035	168 *trpC2 rpoA*(K267A) *thrC*::*nasD-lacZ*	This study
ORB10065	168 *trpC2 thrC*::*nasD-lacZ resD*::*cat amyE*::P*hs-resD*(A156E)	Halow, N
ORB10066	168 *trpC2 thrC*::*nasD-lacZ resD*::*cat amyE*::P*hs-resD*(I147A)	Halow, N
ORB10067	168 *trpC2 thrC*::*nasD-lacZ resD*::*cat amyE*::P*hs-resD*(D148A G158R)	This study
ORB10075	168 *trpC2 thrC*::*nasD-lacZ resD*::*cat amyE*::P*hs-resD*(S146A)	This study
ORB10076	168 *trpC2 thrC*::*nasD-lacZ resD*::*cat amyE*::P*hs-resD*(L145A)	This study
ORB10077	168 *trpC2 thrC*::*nasD-lacZ resD*::*cat amyE*::P*hs-resD*(F142A)	This study
ORB10080	168 *trpC2 rpoA*(K267A) *thrC*::*nasD-lacZ resD*::*cat*	This study
ORB10081	168 *trpC2 rpoA*(Y263A) *thrC*::*nasD-lacZ resD*::*cat*	This study
ORB10086	168 *trpC2 resD*::*tet*	This study
ORB10088	168 *trpC2 resD*::*tet thrC*::*yjlC-lacZ*	This study
ORB10089	168 *trpC2 resD*::*tet thrC*::*yjlC-lacZ amyE*::P*hs-resD*(G198A)	This study
ORB10090	168 *trpC2 resD*::*tet thrC*::*yjlC-lacZ rpoA*(Y263A K267A)	This study
ORB10093	168 *trpC2 resD*::*tet thrC*::*yjlC-lacZ amyE*::P*hs-resD*(wt)	This study
ORB10094	168 *trpC2 thrC*::*nasD-lacZ resD*::*cat amyE*::P*hs-resD*(D148A)	This study
ORB10095	168 *trpC2 thrC*::*nasD-lacZ resD*::*cat amyE*::P*hs-resD*(F142A G158R)	This study
ORB10097	168 *trpC2 resD*::*tet thrC*::*yjlC-lacZ amyE*::P*hs-resD*(A156E)	This study
ORB10099	168 *trpC2 resD*::*tet thrC*::*yjlC-lacZ amyE*::P*hs-resD*(S146A)	This study
ORB10100	168 *trpC2 resD*::*tet thrC*::*yjlC-lacZ amyE*::P*hs-resD*(D148A G158R)	This study
ORB10101	168 *trpC2 resD*::*tet thrC*::*yjlC-lacZ amyE*::P*hs-resD*(H152A)	This study
ORB10102	168 *trpC2 resD*::*tet thrC*::*yjlC-lacZ amyE*::P*hs-resD*(G158R)	This study
ORB10107	168 *trpC2 spx*::*neo thrC*::*trxB-lacZ*	Nakano, M
ORB10109	168 *trpC2 spx*::*neo thrC*::*trxB-lacZ amyE*::P*hs-spx*(L37A)	Nakano, M
ORB10110	168 *trpC2 spx*::*neo thrC*::*trxB-lacZ amyE*::P*hs-spx*(S38A)	Nakano, M
ORB10111	168 *trpC2 spx*::*neo thrC*::*trxB-lacZ amyE*::P*hs-spx*(I39A)	Nakano, M
ORB10112	168 *trpC2 spx*::*neo thrC*::*trxB-lacZ amyE*::P*hs-spx*(R47A)	Nakano, M
ORB10113	168 *trpC2 spx*::*neo thrC*::*trxB-lacZ amyE*::P*hs-spx*(E50A)	Nakano, M
ORB10114	168 *trpC2 spx*::*neo thrC*::*trxB-lacZ amyE*::P*hs-spx*(D40A)	Nakano, M
ORB10115	168 *trpC2 spx*::*neo thrC*::*trxB-lacZ amyE*::P*hs-spx*(wt)	Nakano, M
ORB10116	168 *trpC2 rpoA*(K267A) *thrC*::*nasD-lacZ resD*::*cat amyE*::P*hs-resD*(wt)	This study
ORB10117	168 *trpC2 rpoA*(K267A) *thrC*::*nasD-lacZ resD*::*cat amyE*::P*hs-resD*(G158R)	This study
ORB10118	168 *trpC2 rpoA*(K267A) *thrC*::*nasD-lacZ resD*::*cat amyE*::P*hs-*	This study

	resD(D148A)	
ORB10120	168 *trpC2 rpoA*(Y263A) *thrC*::*nasD-lacZ resD*::*cat amyE*::P*hs-resD*(wt)	This study
ORB10121	168 *trpC2 rpoA*(Y263A) *thrC*::*nasD-lacZ resD*::*cat amyE*::P*hs-resD*(G158R)	This study
ORB10122	168 *trpC2 rpoA*(Y263A) *thrC*::*nasD-lacZ resD*::*cat amyE*::P*hs-resD*(D148A)	This study
Plasmids		
pDG793	*thrC* integration plasmid	(Guerout-Fleury *et al.*, 1996)
pMMN631	pDG793 carrying *nasD-lacZ*	Nakano, M
JM103	pCm::Tc antibiotic resistance marker replacement	(Steinmetz *et al.*, 1994)
pDR111	pDR111 empty vector	(Britton *et al.*, 2002)
pMMN546	pDR111 carrying *resD*(wt)	Nakano, M
pMMN549	pDR111 carrying *resD*(H152A)	Nakano, M
pMMN901	pDR111 carrying *resD*(G158R)	Nakano, M
pMMN903	pDR111 carrying *resD*(T159A)	Nakano, M
pMMN904	pDR111 carrying *resD*(E160A)	Nakano, M
pMMN932	pDR111 carrying *resD*(T155I)	Nakano, M
pMMN933	pDR111 carrying *resD*(D157A)	Nakano, M
pHJ2	pDR111 carrying *resD*(D148A)	This study
pHG23	pDR111 carrying *resD*(G198A) *resE*	Geng, H
pHJ4	pDR111 carrying *resD*(R153A)	This study
pHJ5	pDR111 carrying *resD*(G198A)	This study
pHJ7	pDR111 carrying *resD*(D148A G158R)	This study
pHJ8	pDR111 carrying *resD*(S146A)	This study
pHJ9	pDR111 carrying *resD*(L145A)	This study
pHJ10	pDR111 carrying *resD*(F142A)	This study
pHJ11	pDR111 carrying *resD*(F142A G158R)	This study
pNH1	pDR111 carrying *resD*(A156E)	Halow, N
pNH2	pDR111 carrying *resD*(I147A)	Halow, N
pSN56	pDR111 carrying *spx*(wt) (DD)	(Nakano *et al.*, 2003a)
pSN78	pDG793 carrying *trxB-lacZ*	(Nakano *et al.*, 2003a)
pMMN944	pDR111 carrying *spx*(L37A) (DD)	Nakano, M
pMMN945	pDR111 carrying *spx*(S38A) (DD)	Nakano, M
pMMN946	pDR111 carrying *spx*(I39A) (DD)	Nakano, M
pMMN947	pDR111 carrying *spx*(R47A) (DD)	Nakano, M

pMMN948	pDR111 carrying *spx*(E50A) (DD)	Nakano, M
pCB134	pDR111 carrying *spx*(D40A) (DD)	Birch, C

Table 2.2 Oligonucleotides

Primer	Sequence (5´ to 3´)	Purpose
o*spacup*	GACTTTATCTACAAGGTGTG	pDR111 with *resD* or *spx*
o*spacdown*	AAATGATGACCTCGTTTCCA	pDR111 with *resD* or *spx*
oMN03-239	ATAGACCATGATGCTGCCCGCGTAACAGCTGAT	pMMN549
oMN03-240	ATCAGCTGTTACGCGGGCAGCATCATGGTCTAT	pMMN549
oMMN15-709	CACCGCGTAACAGCTGATCGAACAGAAGTGAGCCTGA	pMMN901
oMMN15-710	TCAGGCTCACTTCTGTTCGATCAGCTGTTACGCGGTG	pMMN901
oMN15-713	CACCGCGTAACAGCTGATGGAGCAGAAGTGAGCCTGACGC CAAAA	pMMN903
oMN15-714	TTTTGGCGTCAGGCTCACTTCTGCTCCATCAGCTGTTACGC GGTG	pMMN903
oMN15-715	CGCGTAACAGCTGATGGAACAGCAGTGAGCCTGACGCCAA AAGAG	pMMN904
oMN15-716	CTCTTTTGGCGTCAGGCTCACTGCTGTTCCATCAGCTGTTA CGCG	pMMN904
oHJ3	TTTTCACATCTGTCAATAGCCCATGATGCTCACCGCGTA	pHJ2
oHJ4	TACGCGGTGAGCATCATGGGCTATTGACAGATGTGAAAA	pHJ2
oMN16-764	ATAGACCATGATGCTCACGCCGTAACAGCTGATGGAACA	pHJ4
oMN16-765	TGTTCCATCAGCTGTTACGGCGTGAGCATCATGGTCTAT	pHJ4
oMN16-766	CATGATGCTCACCGCGTAATAGCTGATGGAACAGAAGTG	pMMN932
oMN16-767	CACTTCTGTTCCATCAGCTATTACGCGGTGAGCATCATG	pMMN932
oMN16-767	GCTCACCGCGTAACAGCTGCTGGAACAGAAGTGAGCCTG	pMMN933
oMN16-767	CAGGCTCACTTCTGTTCCAGCAGCTGTTACGCGGTGAGC	pMMN933
oMN02-205	AGATAAGTCGACAGAAGGAAAGCAGGG	*resD* up
oMN02-206	GCATAAGCATGCCTACTACGCTTTTCC	*resD* down
oMN16-773	GATGCTCACCGCGTAACAGAAGATGGAACAGAAGTGAGC	pNH1
oMN16-774	GCTCACTTCTGTTCCATCTTCTGTTACGCGGTGAGCATC	pNH1
oMN16-775	GTGTTTTCACATCTGTCAGCAGACCATGATGCTCACCGC	pNH2
oMN16-776	GCGGTGAGCATCATGGTCTGCTGACAGATGTGAAAACAC	pNH2
oMN16-777	CTCGTGTTTTCACATCTGGCAATAGACCATGATGCTCAC	pHJ8
oMN16-778	GTGAGCATCATGGTCTATTGCCAGATGTGAAAACACGAG	pHJ8
oMN16-779	GTGCTCGTGTTTTCACATGCGTCAATAGACCATGATGCT	pHJ9
oMN16-780	AGCATCATGGTCTATTGACGCATGTGAAAACACGAGCAC	pHJ9
oMN16-781	ACGAAAAATGTGCTCGTGGCTTCACATCTGTCAATAGAC	pHJ10
oMN16-782	GTCTATTGACAGATGTGAAGCCACGAGCACATTTTTCGT	pHJ10
oMN17-786	ATTTTCTCTGAACCTGCATCAATTGATGAAAT	pMMN944

oMN17-787	ATTTCATCAATTGATGCAGGTTCAGAGAAAAT	pMMN944
oMN17-788	TTCTCTGAACCTTTAGCAATTGATGAAATAAA	pMMN945
oMN17-789	TTTATTTCATCAATTGCTAAAGGTTCAGAGAA	pMMN945
oMN17-790	TCTGAACCTTTATCAGCTGATGAAATAAAACA	pMMN946
oMN17-791	TGTTTTATTTCATCAGCTGATAAAGGTTCAGA	pMMN946
oMN17-792	ATAAAACAAATTCTTGCAATGACTGAGGACGG	pMMN947
oMN17-793	CCGTCCTCAGTCATTGCAAGAATTTGTTTTAT	pMMN947
oMN17-794	TTCTTCGAATGACTGCGGACGGAACAGATGAA	pMMN948
oMN17-795	TTCATCTGTTCCGTCCGCAGTCATTCGAAGAA	pMMN948

2.3 RESULTS

2.3.1 αCTD residues Y263/K267 are required for ResD-dependent activation

Our genome-wide GeF-seq, *in vivo* DNA binding analysis, uncovered genes directly activated or repressed by ResD. The study further indicated that the ResD-RNAP complex travels through genes activated by ResD during transcription (Chumsakul *et al.*, 2017). We previously showed that ResD-dependent *nasD* transcription is much lower in the Y263A mutant or the K267A mutant, and that the Y264 K267 double mutant nearly abolishes *nasD* expression, indicating that the effect of the two substitutions is additive (Geng, 2007). In order to confirm that the αCTD residues are universally required for ResD-dependent activation, transcription of these genes was analyzed using transcriptional *lacZ* fusions in the wild-type and αCTD(Y263A K267A) mutant backgrounds under both anaerobic nitrate respiration and fermentation conditions (Fig. 2.4). The four genes analyzed, *sboA*, *yozB*, *yxiE* and *hmp*, displayed a binding profile where ResD binds both promoter and gene-coding regions (Chumsakul *et al.*, 2017). Transcription of *sboA*, which encodes the antibiotic peptide subtilosin A, was significantly affected in the Y263A K267A mutant under both anaerobic nitrate respiration and fermentation conditions (Zheng *et al.*, 1999). A similar effect was observed for *yozB-lacZ* and *yxiE-lacZ*

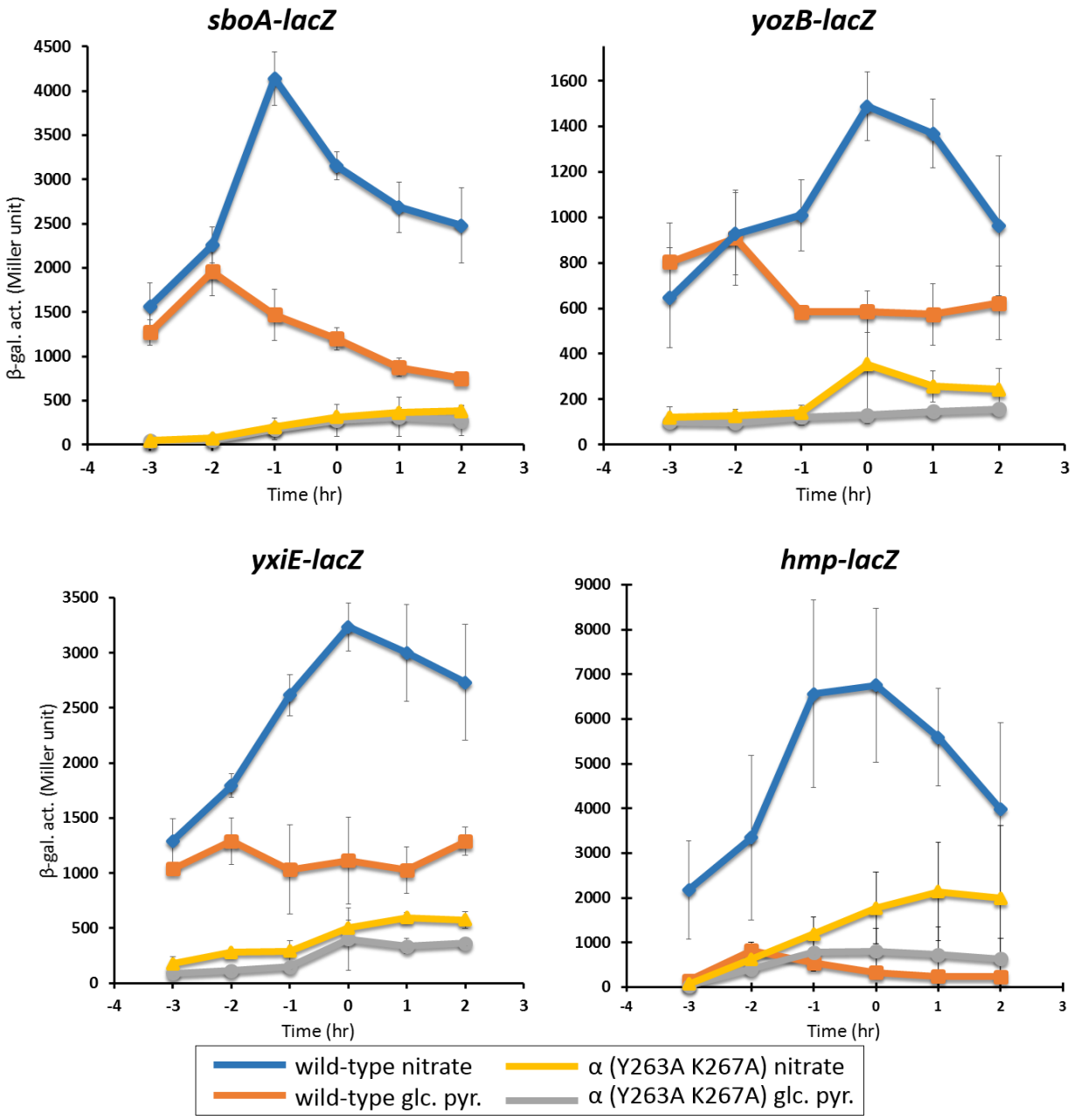

FIG. 2.4. The effect of the Y263A K267A double amino acid substitution of *rpoA* on expression of *sboA, yozB, yxiE,* and *hmp* compared to wild type. Cells were grown under anaerobic conditions in 2xYT supplemented with 1% glucose and 0.2% KNO_3 (for nitrate respiration conditions), or 0.5% glucose and 0.5% pyruvate (for fermentation conditions). Spectinomycin (75 µg/ml) was added for *sboA* experiment, erythromycin (1 µg/ml)/lincomycin (25 µg/ml) for *yozB* and *yxiE* experiments, and chloramphenicol (5 µg/ml) for *hmp*. Time 0 indicates the end of exponential growth. Each experiment was repeated with three independent isolates and the data points are shown as the average with standard deviation.

expression. Although their physiological functions are currently unknown, *yozB* encodes a putative membrane protein, while *yxiE* is induced by an unknown mechanism under phosphate starvation conditions (Antelmann *et al.*, 2000; Wilson *et al.*, 2009). Meanwhile, *hmp-lacZ* expression did not follow the same pattern. Although the expression was significantly reduced by the αCTD mutation under nitrate respiration conditions, there is no significant difference between wild type and the mutant under fermentation conditions. Furthermore, *hmp* transcription is higher in the mutant cells grown in the presence of nitrate than that in the wild-type cells without nitrate. This is likely due to derepression of *hmp* transcription by NsrR during nitrate respiration. In summary, the results strongly suggested that interaction between ResD and residues Y263 and K267 of αCTD is required for ResD-dependent activation.

Next, we examined whether residues Y263 and K267 of αCTD are required for direct ResD-dependent repression recently identified (Chumsakul *et al.*, 2017). To this end, the effect of the Y263A K267A double mutation was examined on the expression of *glpF* and *yjlC*. *glpF* encodes a membrane protein which facilitates diffusion of glycerol across the cell membrane (Beijer *et al.*, 1993). Although *yjlC* encodes a protein of unknown function, the gene belongs to the *yjlC-ndh* operon, which plays a role in maintaining the NADH/NAD^+ ratio in *B. subtilis* cells. The *yjlC-ndh* operon is negatively regulated by Rex, yet ResD is capable of carrying out its role as a repressor in a Rex-independent manner (Chumsakul *et al.*, 2017; Gyan *et al.*, 2006; Larsson *et al.*, 2005). Under anaerobic nitrate respiration conditions, the Y263A K267A mutation showed only a minor effect on expression of both *glpF-lacZ* and *yjlC-lacZ* (Fig. 2.5), as expected from its role in transcription repression. The results showed that possible interaction between ResD and Y263 and/or K267 of the α subunit is essential for ResD-dependent activation but not repression.

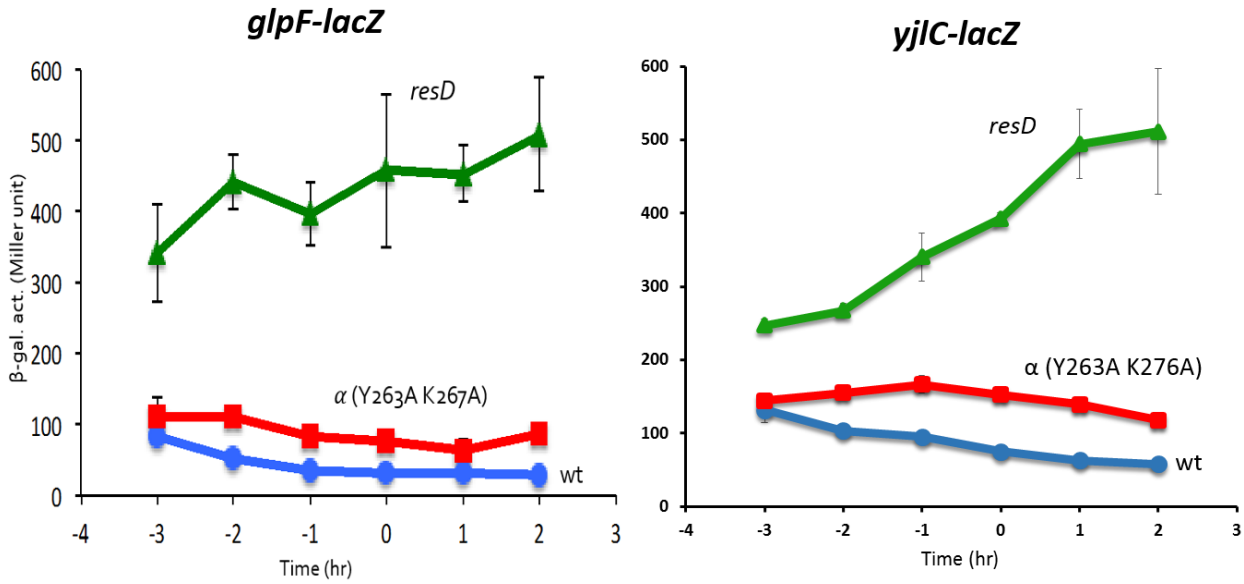

FIG. 2.5. The effect of a Y263A K267A double amino acid substitution mutation of *rpoA* on expression of *glpF* and *yjlC* compared to wild type and a *resD* mutant. Cells were grown under anaerobic nitrate conditions in 2xYT supplemented with 1% glucose, 0.2% KNO_3, and chloramphenicol (5 µg/ml). Time 0 indicates the end of exponential growth. Each experiment was repeated with three independent isolates and the data points are shown as the average with standard deviation (*glpF* data-Michiko Nakano).

2.3.2 Effect of ResD amino acid substitutions on ResD-dependent transcription

The next questions we asked are whether the Y263/K267 residues directly interact with ResD to activate transcription and which region of ResD serves as an interaction surface. As described earlier, our studies showed that the α-loop utilized for OmpR to interact with αCTD is the DNA-binding site for ResD, suggesting the presence of a yet-unidentified ResD region for αCTD interaction. Zuber's group previously uncovered the unique anti-α mechanism of repression utilized by Spx. Overproduction of the wild-type Spx, not αCTD-interaction mutants, abrogates ResD-dependent activation. The anti-α mechanism is likely caused by direct competition of αCTD interaction between Spx and ResD, based on *in vitro* work demonstrating that increased concentrations of Spx disrupts complex formation between ResD and promoter-bound RNAP. Furthermore, overproduction of wild-type Spx has been shown to inhibit activity of ResD for *in vitro* transcription of ResD-controlled genes (Nakano *et al.*, 2003b).

To our surprise, the αCTD interaction site of Spx shows a high sequence similarity with a region of the N-terminal four-stranded antiparallel β-sheet in ResDc (Fig. 2.6). Based on these findings, we speculated that the β-sheet is involved in αCTD interaction. Aforementioned genetic and crystal structure studies had identified that residues 47-54 of Spx (region B in Fig. 2.6) constitute the interaction surface with αCTD. MgsR, a paralogue of Spx regulating a subregulon within the general stress response controlled by σ^B in *B. subtilis*, displays sequence similarity around region B including a conserved glycine (G55), that when mutated to an arginine, results in loss of function of MgsR. Furthermore, MgsR, like Spx, was shown to exert both positive and negative control similar to Spx (Reder *et al.*, 2008). We have shown that MgsR interacts with αCTD using yeast two-hybrid system, although ResD does not (M. Nakano, unpublished). Together, these results indicate that region B of MgsR directly interacts with αCTD.

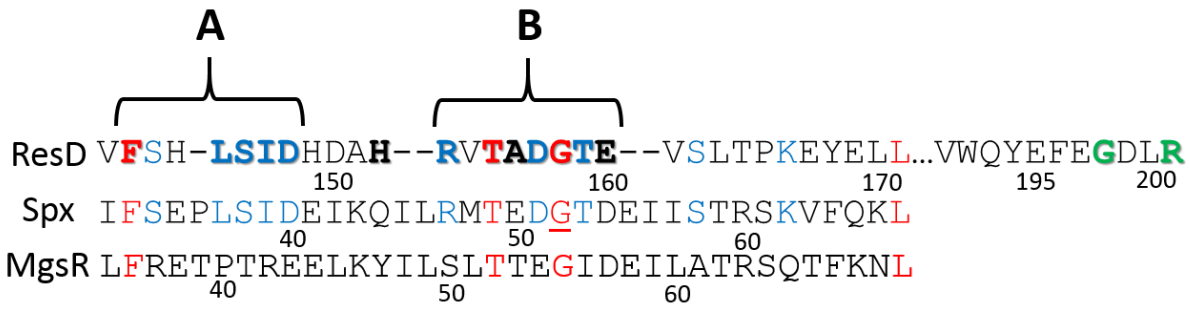

FIG. 2.6. Alignments of ResD (residues 141-201) with Spx (residues 32-67) and MgsR (residues 35-70). The amino acid numbers are shown below each sequence. G52, the residue of Spx required for interaction with Y263 of αCTD, is underlined. Residues conserved between all three TFs are in red. Residues conserved between ResD and Spx are in blue. Residues targeted for mutagenesis in this study are in bold. Residues identified as the DNA-binding site for ResD are in green. Region A consists of residues 33-40 in Spx and residues 142-148 of ResD. Region B consists of residues 47-54 of Spx and residues 153-160 of ResD.

Interestingly, compared to Spx and MgsR, ResD has two amino acid omissions on both sides of region B. On the other hand, residues F32-D39 upstream of region B (region A in Fig. 2.6) show sequence similarity between ResD and Spx. Particularly LSID (residues 37-40 in Spx) has a perfect match with the corresponding residues 145-148 in ResD, whereas no sequence similarity is detected in MgsR.

We postulated that either region A or B (or both) is the site that interacts with the Y263/K267 surface of αCTD, hence this study focused on amino acid substitutions in these regions. As the α (Y263A K267A) mutation strongly affects ResD-dependent activation of genes such as *nasD*, but does not significantly affect ResD-dependent repression of genes such as *yjlC* (Fig. 2.5), *nasD* and *yjlC* transcriptional *lacZ* fusions were used to monitor ResD-dependent transcriptional activation or repression, respectively. We anticipated that an amino acid substitution mutation would affect *nasD* expression, but not *yjlC* expression, if it were truly a site of ResD-αCTD interaction.

Mutant strain construction was described in more detail in Materials and Methods. In brief, mutant *resD* genes were generated by two-step PCR reactions and were integrated by an ectopic integration vector into the *amyE* locus of the *B. subtilis* 168 chromosome. *nasD-lacZ* and *yjlC-lacZ* constructs were integrated into the *thrC* locus (Fig. 2.7). The native *resD* gene was disrupted by chloramphenicol-resistance gene or tetracycline-resistance gene. The integrated wild-type or mutant *resD* gene was expressed under an IPTG-inducible promoter so that ResD was produced solely from the IPTG-inducible *resD* construct.

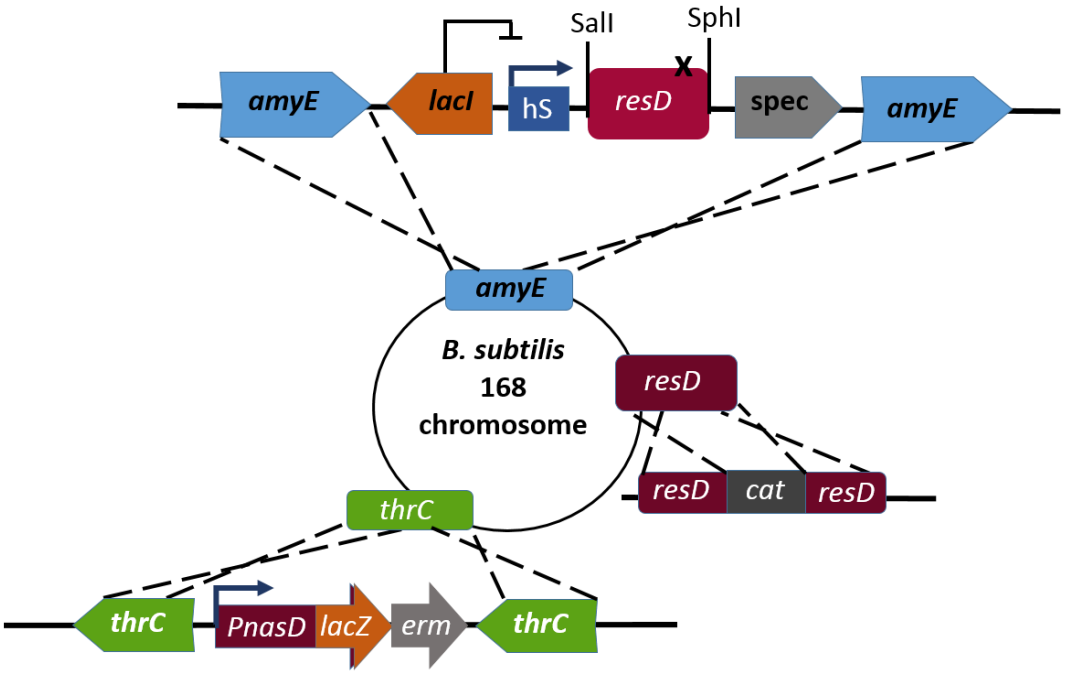

FIG. 2.7. Representative scheme of mutant strain construction. The native *resD* gene in the *B. subtilis* 168 chromosome is disrupted by an antibiotic resistance gene (in this case *cat*). The integrated *resD* gene is expressed under an IPTG-inducible promoter (hS-hyper spac) at the *amyE* locus. Mutations were generated by 2-step PCR mutagenesis and residue(s) mutated is shown by x. SalI and SphI sites were used to clone mutated *resD* into the pDR111 integration plasmid. *nasD-lacZ* fusion (bearing *erm* gene in this case) is integrated into the *thrC* locus.

2.3.2.1 DNA-binding G198A mutation

First, the effect of the G198A DNA-binding mutation on *nasD-lacZ* and *yjlC-lacZ* expression was determined in order to establish and differentiate between the expression profiles of ResD mutations that affect DNA binding affinity and those that affect the interaction with RNAP. If ResD repressor activity remains intact, *yjlC* transcription would be lower in the presence of IPTG than in the absence of IPTG. As expected, an alanine substitution at G198 nearly abolished *nasD-lacZ* expression and increased *yjlC-lacZ* expression compared to wild-type in the presence of IPTG (Fig. 2.8). We thus confirmed that the ResD G198A mutant is unable to either activate or repress transcription, as expected for a DNA-binding mutant.

2.3.2.2 Region B mutations

Crystal structural analysis indicated that the phenolic moiety of Y263 is involved in van der Waals contact with G52 of Spx. The side chain of Y263 lies within 3.8 Å of G52, thus the long Arg side chain introduced by the Spx G52R substitution is unable to form the αCTD/Spx complex (Newberry *et al.*, 2005) (Fig. 2.2). Therefore, we examined whether introduction of the same amino acid substitution at the corresponding ResD residue, G158, would produce a similar adverse effect on ResD-dependent transcription. Although *nasD-lacZ* expression was significantly affected in the G158R mutant, the effect was surprisingly not as severe as the Spx G52R mutation (Fig. 2.9). If Y263 were indeed involved in direct interaction with ResD, the results could indicate that either G158 of ResD is not in close vicinity with Y263 unlike G52 of Spx, and/or the long Arg side chain in G158R does not cause steric interference in the case of αCTD/ResD interaction. Next, whether the G158R mutation affects ResD-dependent repression was investigated using a *yjlC-lacZ* transcriptional fusion. The G158R mutant repressed

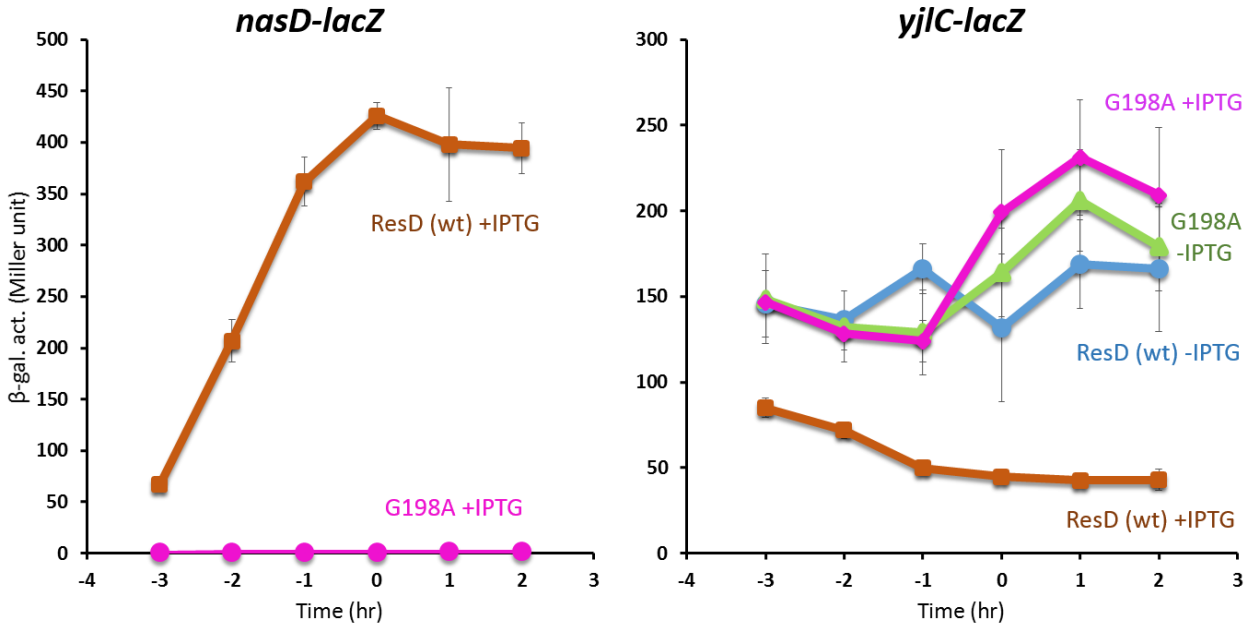

FIG. 2.8. The DNA-binding mutant (G198A) is unable to repress *yjlC* transcription. *nasD-lacZ* and *yjlC-lacZ* expression of the DNA-binding G198A mutant. Cells were grown under anaerobic conditions in 2xYT supplemented with 1% glucose, 0.2% KNO_3, and +/- 1 mM IPTG. spectinomycin (75 µg/ml), erythromycin (1 µg/ml)/lincomycin (25 µg/ml), and chloramphenicol (5 µg/ml) were added for the *nasD* experiment. Spectinomycin (75 µg/ml), chloramphenicol (5 µg/ml), and tetracycline (12.5 µg/ml) were added for the *yjlC* experiment. Time 0 (T_0) indicates the end of exponential growth. Each experiment was repeated with three independent isolates and the data points are shown as the average with standard deviation.

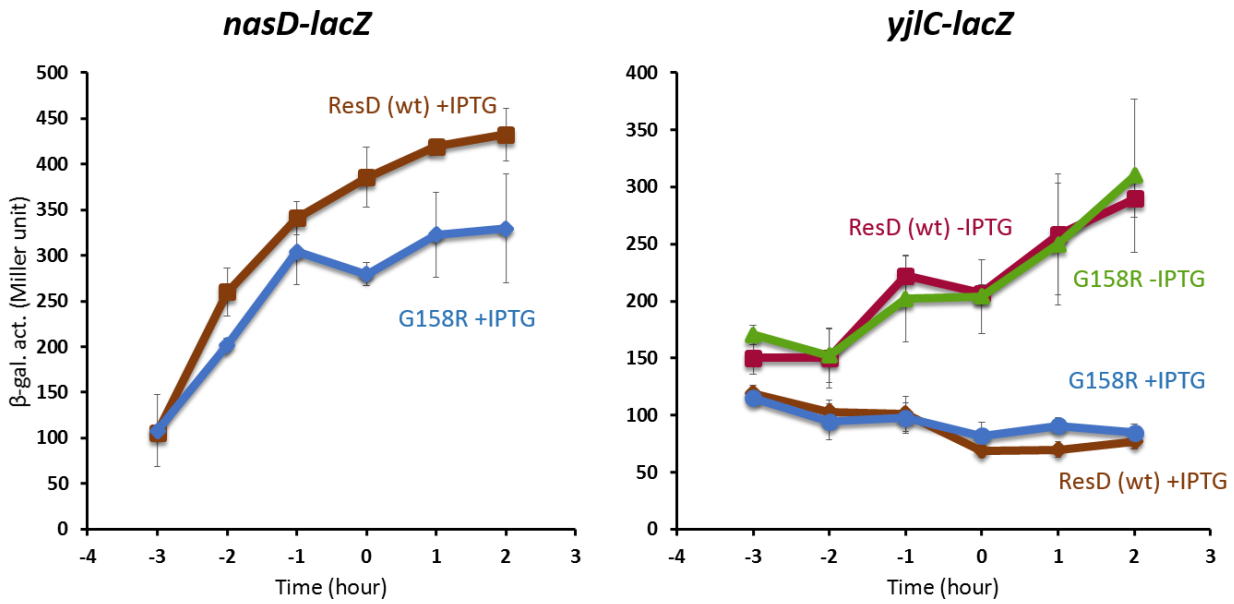

FIG. 2.9. *nasD-lacZ* and *yjlC-lacZ* expression of the G158R mutant. Cells were grown as described in FIG. 2.8.

transcription similar to the wild-type ResD only in the presence of IPTG, indicating that G158R has no effect on ResD activity as a repressor.

In addition to G52, structural studies had shown that residues T53, R47, and D54 of Spx form hydrogen bonds with αCTD residues N264, K267, and R268 (Newberry *et al.*, 2005), although extensive search of suppressor mutations by our group and the Wachenfeldt group (Larsson *et al.*, 2007) did not identify any amino acid substitution in these Spx residues. Alanine substitutions were made in corresponding ResD residues T159, R153, and E160. *nasD* transcription was moderately reduced in the R153A mutant, while the expression was higher in the T159A and E160A mutants than wild-type (Fig. 2.10). These results showed that T159 and E160 unlikely play important roles in ResD/αCTD and ResD/RNAP interaction.

The Wachenfeldt group identified Spx (T49I) results in loss of Spx function probably due to disruption of the interaction between Spx and αCTD (Larsson *et al.*, 2007). Therefore, we introduced an isoleucine substitution at the corresponding T155 of ResD to determine if we could see a similar effect on activation by ResD. In addition, another conserved residue, D157, corresponding to Spx D51, underwent alanine substitution. *nasD* transcription was not significantly affected in either the T155I or D157A mutants (Fig. 2.11).

At this point, out of the amino acid substitutions of residues conserved between ResD and Spx in region B, only R153A and G158R have moderate but reproducible effect on *nasD* expression. In this region, ResD carries an alanine at residue 156, whereas the corresponding residue in Spx is glutamate. This is the only amino acid that is not conserved nor even similar between ResD (residues 153-160) and Spx (47-54). As the substitution of a small Ala to acidic larger Glu might have a drastic effect on ResD activity, the A156E mutant was generated. Interestingly, *nasD-lacZ* expression was severely affected in the A156E mutant; however, to our

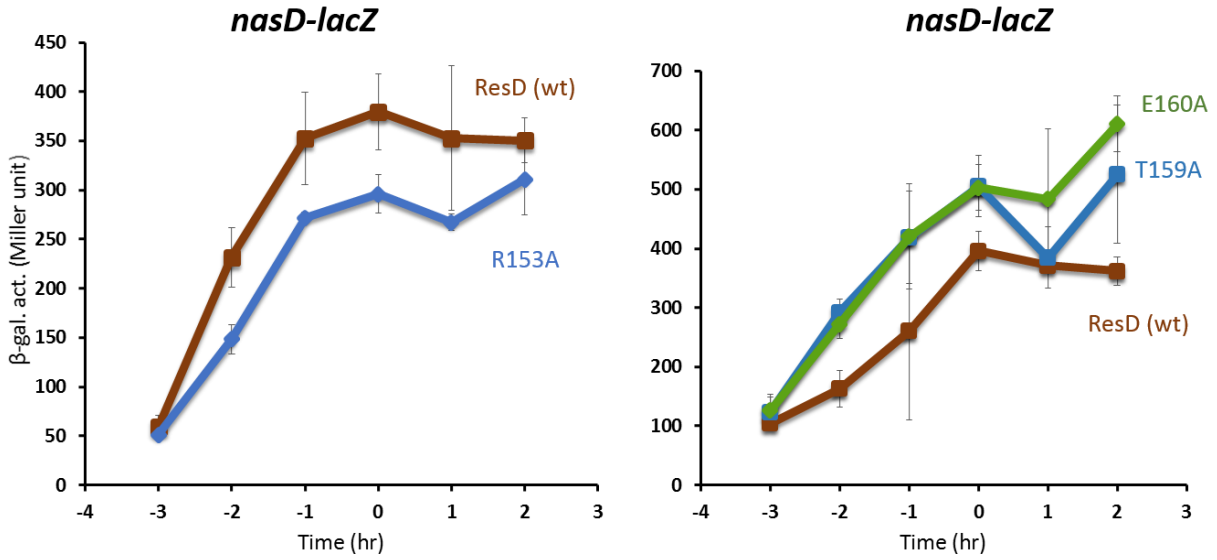

FIG. 2.10. *nasD-lacZ* expression of the R153A, T159A, and E160A mutants. Cells were grown as described in FIG. 2.8.

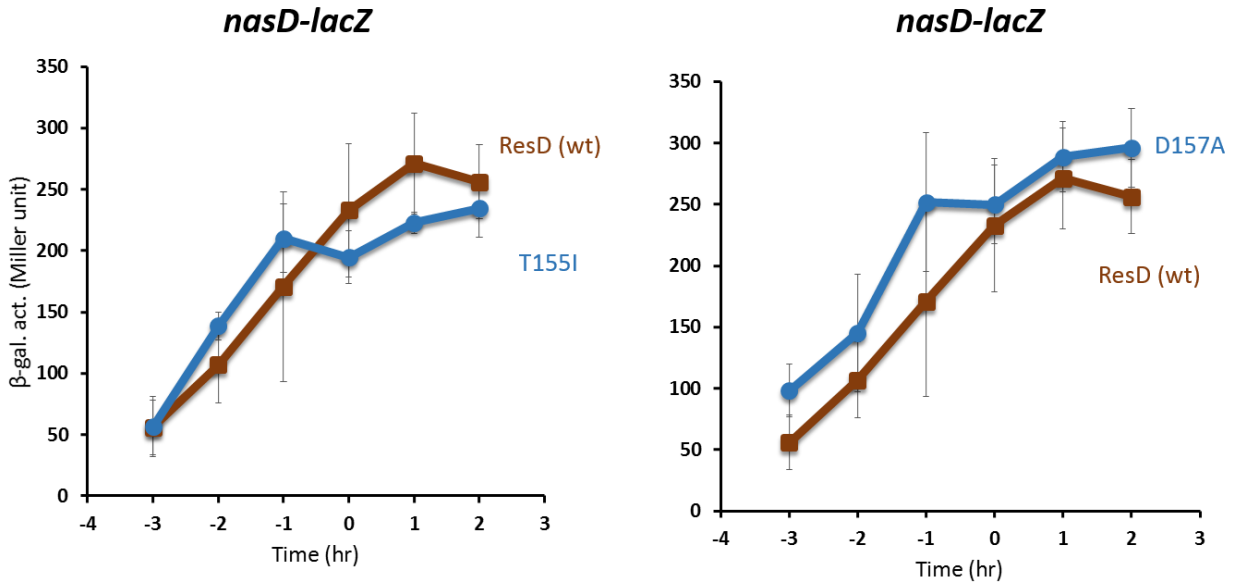

FIG. 2.11. *nasD-lacZ* expression of the T155I and D157A mutants. Cells were grown as described in FIG. 2.8.

surprise, the mutant ResD was unable to repress *yjlC-lacZ* expression as well (Fig. 2.12). Overall, amino acid substitutions in the ResD region (residues 153-160) that correspond to the Spx-αCTD interaction surface resulted in only a moderate decrease, except A156E, in *nasD-lacZ* expression compared to wild type.

2.3.2.3 H152A mutation

H152 is located between regions A and B and next to one of the two amino acid gaps found in ResD compared to Spx and MgsR (Fig. 2.6). The region is well conserved between Spx and MgsR, but no similarity was detected in ResD. The H152A mutant generated previously for another purpose had severely reduced *nasD* expression. It remained unknown how the amino acid substitution causes defective ResD-dependent transcription activation. As the mutation was generated in a different *B. subtilis* strain background, the H152A mutation was introduced in the 168 strain background with which the current work has been conducted. While *nasD-lacZ* expression was severely reduced as expected, *yjlC-lacZ* expression of the mutant was also significantly affected compared to wild type (Fig. 2.13).

2.3.2.4 Region A mutations

While region B is well conserved in ResD, Spx, and MgsR, region A is only conserved between ResD and Spx (Fig. 2.4). To determine whether the conserved sequence is important for ResD activity, alanine substitutions were introduced at each residue in the LSID sequence and F142 in ResD (corresponding to Spx F33) (Fig. 2.14-2.16). *nasD* transcription was significantly reduced in the F142A and D148A mutants and was nearly abolished in the L145A, S146A, and

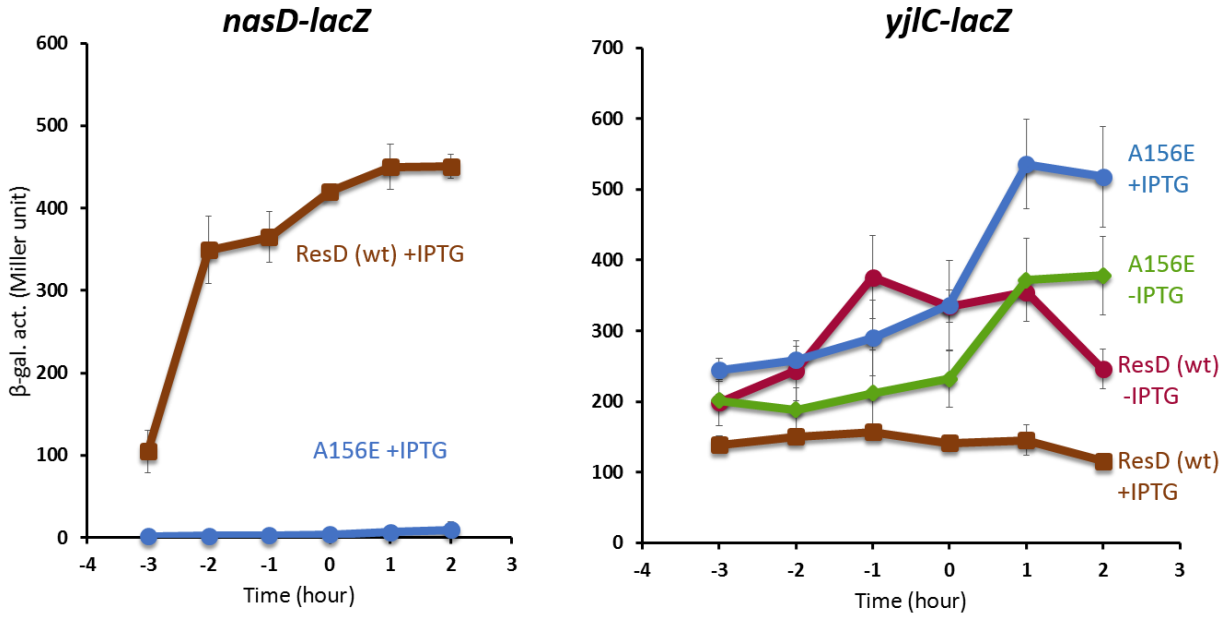

FIG. 2.12. *nasD-lacZ* and *yjlC-lacZ* expression of the A156E mutant. Cells were grown as described in FIG. 2.8.

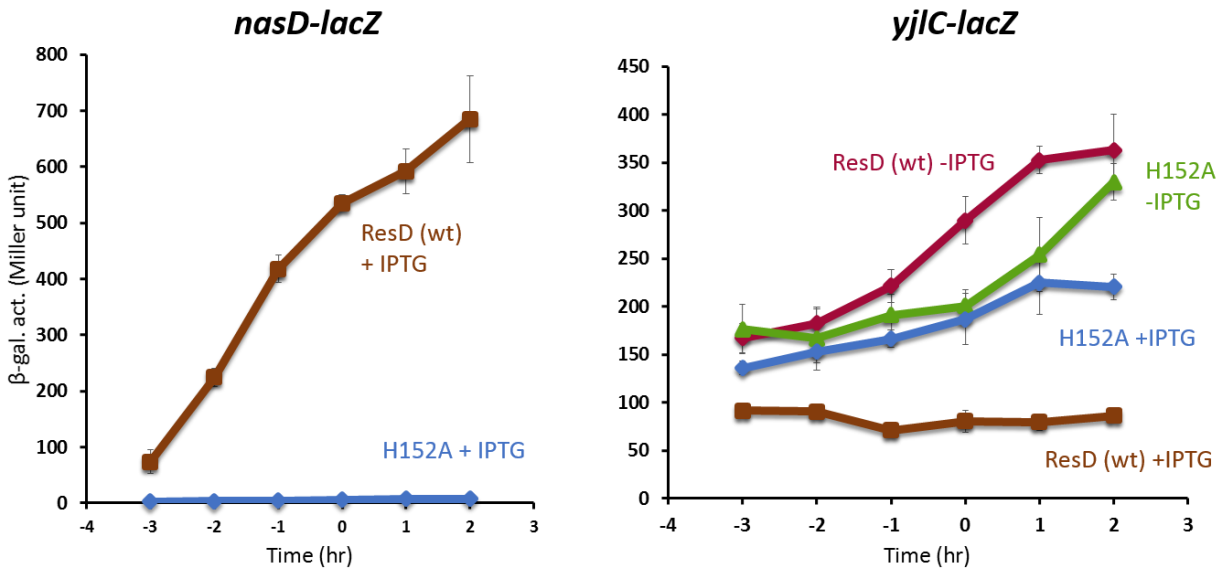

FIG. 2.13. *nasD-lacZ* and *yjlC-lacZ* expression of the H152A mutant. Cells were grown as described in FIG. 2.8.

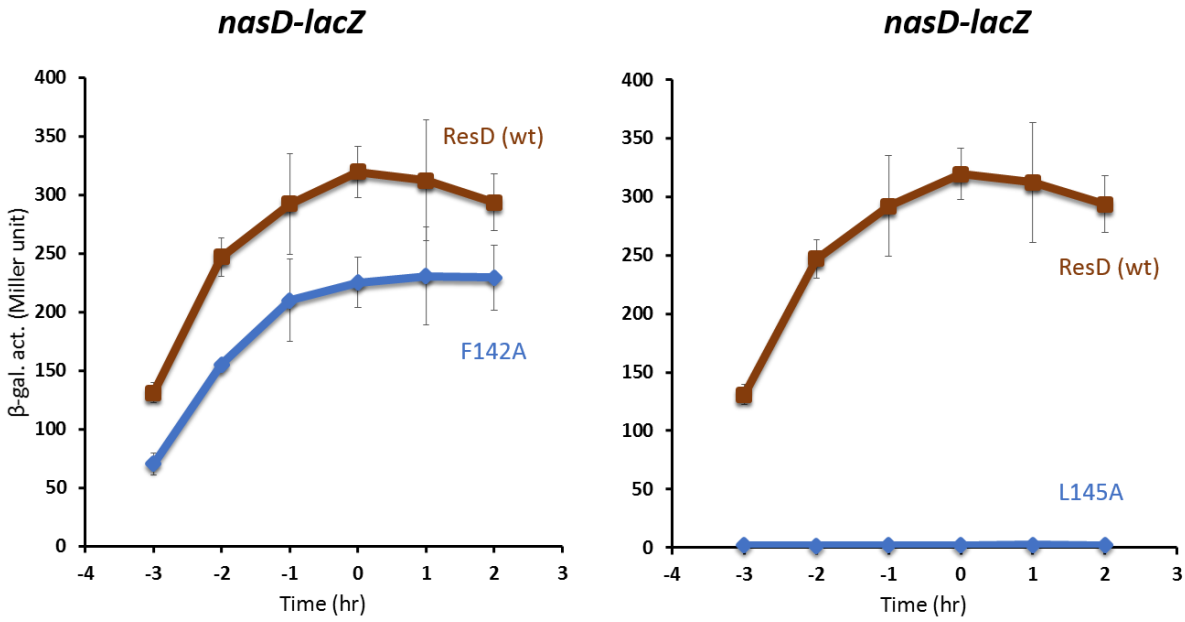

FIG. 2.14. *nasD-lacZ* expression of the F142A and L145A mutants. Cells were grown as described in FIG. 2.8.

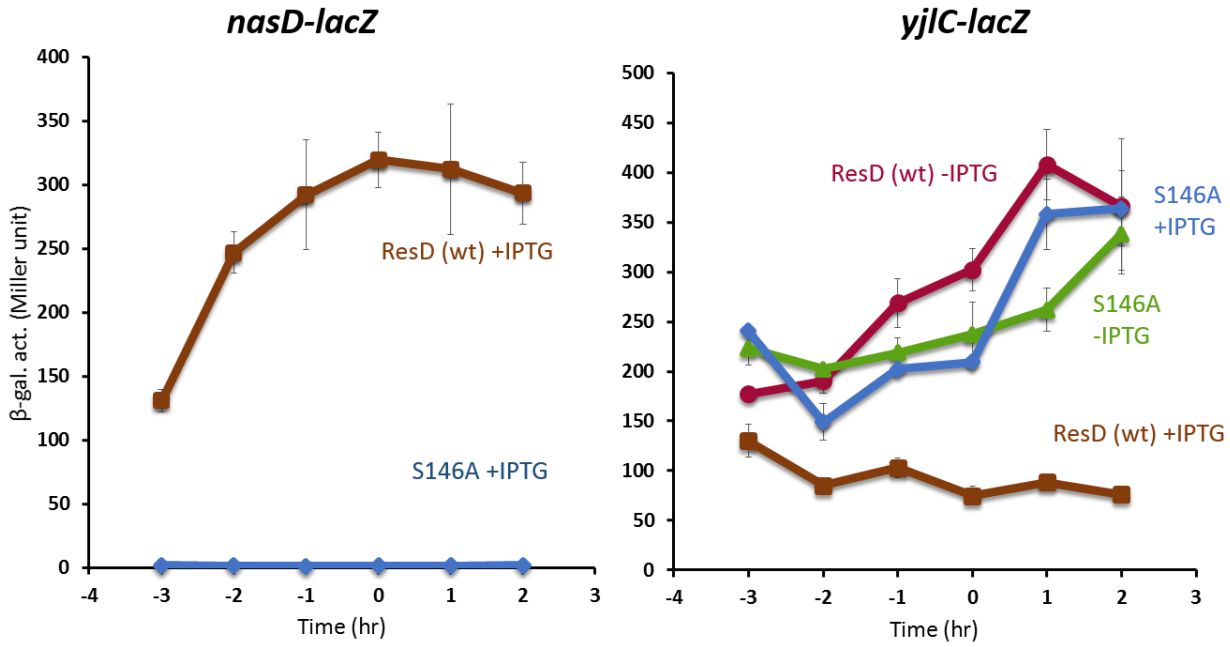

FIG. 2.15. *nasD-lacZ* and *yjlC-lacZ* expression of the S146A mutant. Cells were grown as described in FIG. 2.8.

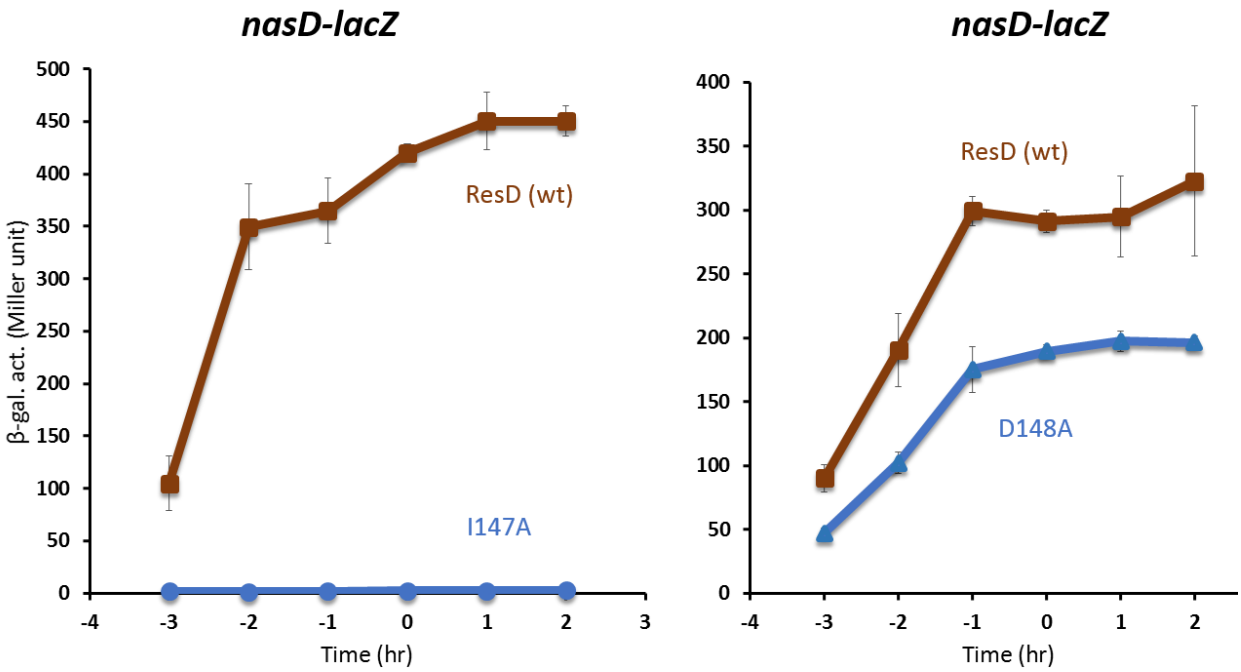

FIG. 2.16. *nasD-lacZ* expression of the I147A and D148A mutants. Cells were grown as described in FIG. 2.8.

I147A mutants. Since amino acid substitutions in the ResD region A displayed a more severe effect on *nasD* expression compared to wild type, region A might be involved in interaction with another subunit of RNAP or with another part of α outside of the Y263/K267 surface.

In order to investigate whether mutations in region A and B show synergistic effect, the D148A G158R double mutant, with one residue mutated from each region, was generated. Although both single mutants exhibited moderate effects (Fig. 2.9, Fig. 2.16), the double mutant nearly abolished *nasD* expression, indicating that the effect of the two substitutions is additive (Fig. 2.17). The F142A G158R double mutant was constructed according to similar rationale. An additive effect was also detected, as *nasD-lacZ* expression was nearly abolished in the F142A G158R double mutant (Fig. 2.18). These results suggest that regions A and B (corresponding to residues 142-148 and 153-160, respectively) of ResD likely play distinctive roles in transcriptional control. Summarized in Fig. 2.19 is the effect of single amino acid substitution mutants on *nasD* transcription shown as a ratio of each mutant to the wild-type expression.

2.3.3 Stability of ResD mutant proteins

In order to determine whether the adverse effect observed in ResD amino acid substitutions is attributed to their loss of stability, the presence of ResD mutant proteins *in vivo* was examined by western blot analysis using an anti-ResD antibody as described in Materials and Methods (Fig. 2.20). The concentration of the G198A protein is comparable to the wild-type ResD protein, supporting that the impaired transcription activity by the G198A mutation is due to the loss of DNA-binding activity. The G158R, R153A, H152A, D148A, and F142A mutant proteins all appear to be stable *in vivo*, with cellular expression levels comparable to wild-type levels. The protein of the A156E and L145A mutants, as well as the D148A G158R double

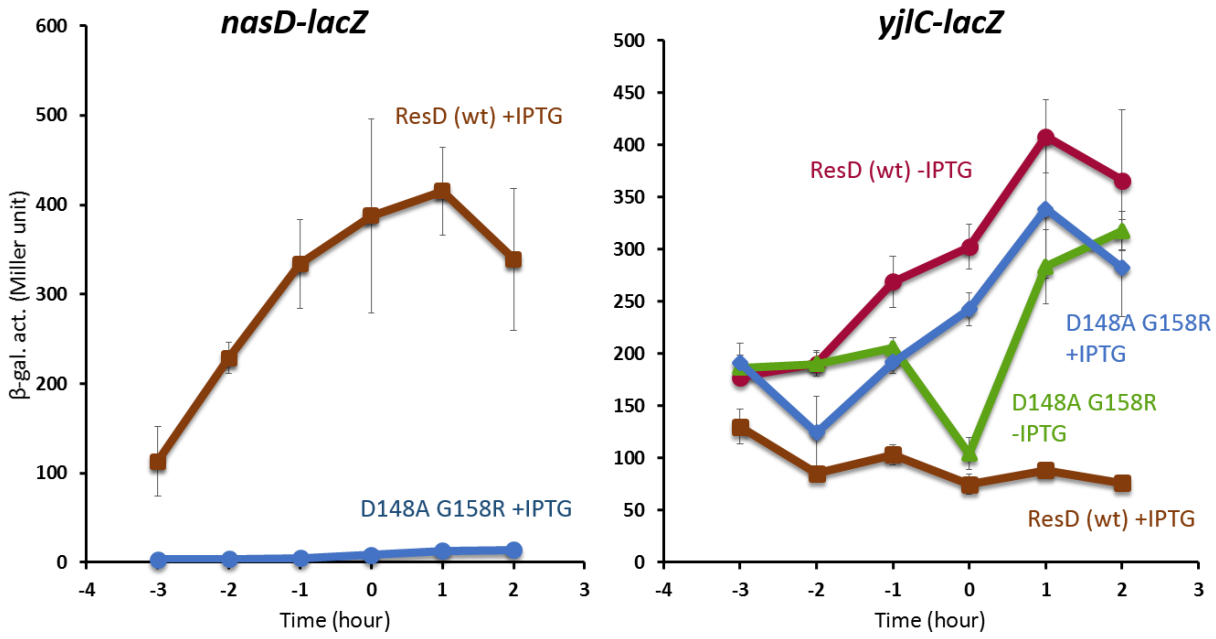

FIG. 2.17. *nasD-lacZ* and *yjlC-lacZ* expression of the D148A G158R double mutant. Cells were grown as described in FIG. 2.8.

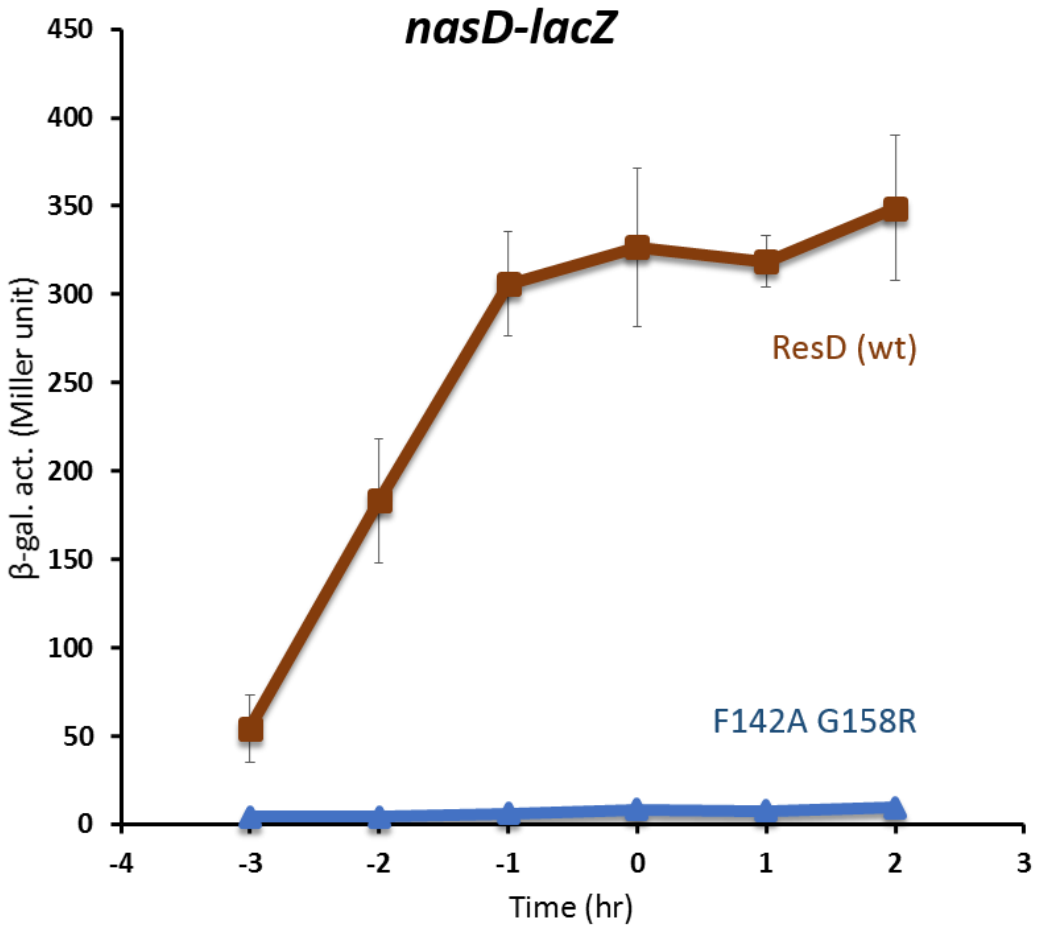

FIG. 2.18. *nasD-lacZ* expression of the F142A G158R double mutant. Cells were grown in the presence of 1 mM IPTG, as described in FIG. 2.8.

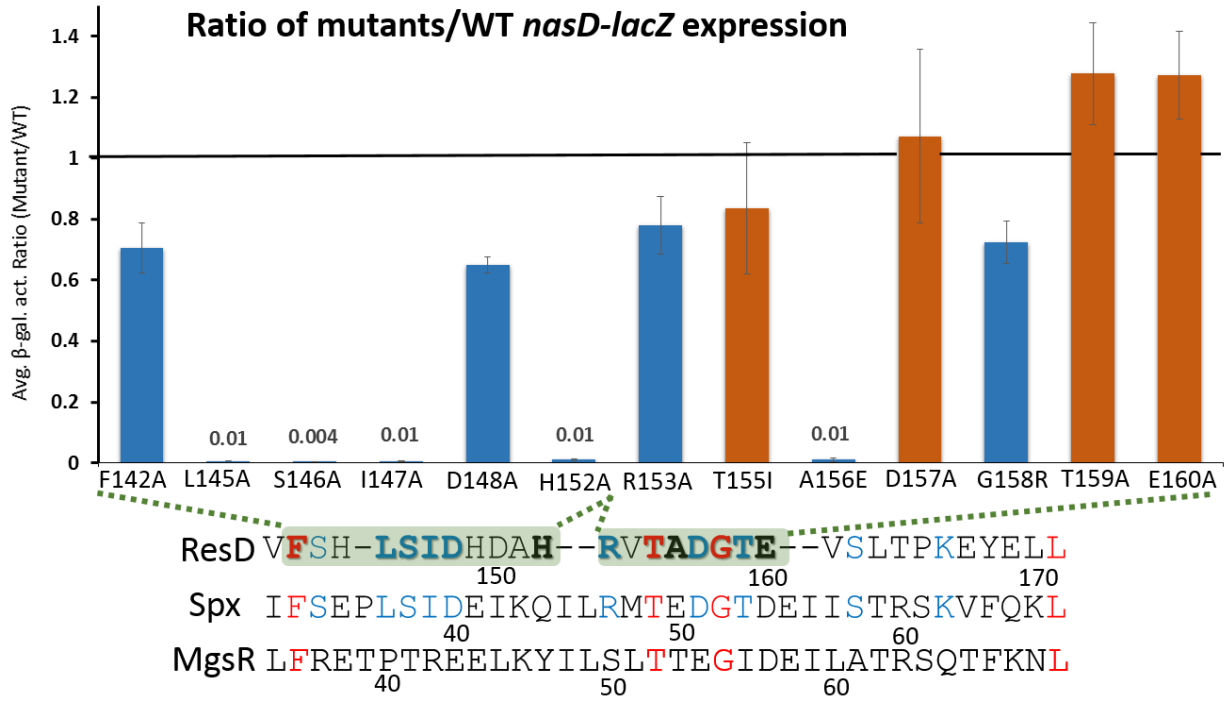

FIG. 2.19. Summary of the effect of ResD mutations on *nasD-lacZ* expression. The ß-galactosidase activities of *nasD-lacZ* at T_0 were chosen and ratio of mutants/WT *nasD-lacZ* expression is shown. Blue bars represent significantly affected residues, while orange bars represent residues not significantly affected. Each experiment was repeated with three independent isolates and the data points are shown as the average ratio with standard deviation.

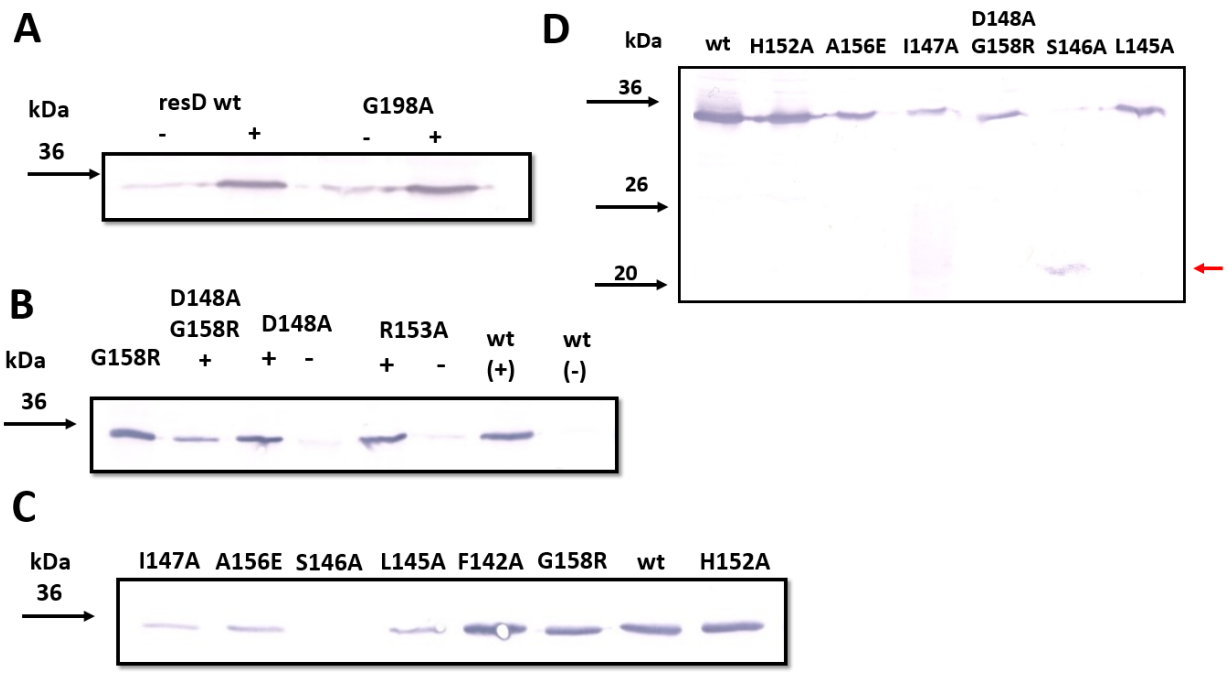

FIG. 2.20. Western immunoblot analysis using anti-ResD antibody. Cells were grown anaerobically in 2xYT with 1% glucose, 0.2% KNO_3 and -/+ 1 mM IPTG. Cells were harvested at T_0. Equal amounts of the cell extract (5 µg total protein) were applied to 12% SDS polyacrylamide gels. Position of molecular weight markers are shown by arrows on the left. + and – indicate that cells were cultured in the presence or absence of IPTG. Cells were only cultured in the presence of IPTG for C and D. Red arrow on D shows S146A reacting with anti-ResD antibody as a smaller molecular weight.

mutant are produced *in vivo* but reproducibly lower than the wild type ResD. The I147A mutant also displayed lower cellular expression levels than wild-type, but seems to be unstable after freeze-thaw of cell lysates unlike other mutant proteins that were stable after multiple cycles of freeze-thaw processes. All ResD mutants were strongly induced in the presence of IPTG. The S146A mutant protein was consistently undetected and produced a clear band with a smaller size than the full ResD that cross-reacts with anti-ResD antibody, suggesting that the S146A mutant protein is subject to proteolytic cleavage, and perhaps misfolding due to the mutation.

2.3.4 Phenotype of ResD amino acid substitution mutants

This study mostly used transcription of *nasD* and *yjlC* to examine mutant ResD activities as an activator and repressor, respectively. However, it is important to assess how these mutations affect overall phenotype under aerobic and anaerobic culture conditions. Thus, phenotypic changes caused by amino acid substitutions of ResD were evaluated next by anaerobic growth supported by nitrate respiration and cytochrome caa_3 activity under aerobic conditions.

2.3.4.1 Anaerobic growth by nitrate respiration

Our previous study demonstrated that the majority of genes directly activated by ResD function in aerobic/anaerobic respiration (Chumsakul *et al.*, 2017). If ResD-RNAP complex formation is essential for transcription activation, one would expect that nitrate respiration is impaired by the loss of ResD/RNAP interaction. We determined whether ResD mutations constructed in this study affect anaerobic growth in the presence of nitrate. To generate anaerobic atmosphere, the GasPak EZ Anaerobe Container System (Benton, Dickinson and

Company) was used. The system produces an anaerobic atmosphere within 2.5 h with less than 1% oxygen and around or over 13% CO_2 within 24 h according to the manufacturer. Figure 2.21 shows anaerobic growth in the presence of 1 mM IPTG after 36-h incubation. When ectopic *resD* is absent in the *resD* mutant, *B. subtilis* was unable to grow on LB agar supplemented with 1% glucose and 0.2% KNO_3. Cells grew when the wild-type *resD* expression was induced by the addition of IPTG, whereas mutant ResD did not fully support the growth. Particularly, H152A, G198A, A156E, I147A, D148A G158R, S146A, and L145A showed severely retarded growth, although S146A mutant protein was shown to be unstable, thus the effect of S146A is inconclusive. The anaerobic growth phenotype mirrors the effect of the mutations on *nasD* expression.

2.3.4.2 Cytochrome *caa*$_3$ activity in aerobic cultures

The ResDE system is required for transcription of *ctaCDEF* (encodes subunits of the heme-A copper oxidase *caa*$_3$) (Liu *et al.*, 1998). The physiological effects of the ResD amino acid substitutions on aerobic respiration were investigated by conducting a cytochrome *caa*$_3$ oxidase assay with the artificial electron donor N, N, N′, N′-tetramethyl-p-phenylene diamine (TMPD) as described in Materials and Methods. The assay is used to detect cytochrome *c* activity, thus bacterial colonies with an intact *caa*$_3$ are able to oxidize TMPD and turn blue upon exposure to TMPD. Conversely, if TMPD is not oxidized, colonies remain their natural color upon exposure to TMPD (Mueller *et al.*, 1989) (Fig. 2.22). Fig. 2.22A shows that the H152A mutant was still functional, although displaying reduced *caa*$_3$ activity compared to wild type. *caa*$_3$ activity for the L145A and D148A G158A double mutant were more affected than the H152A mutant. The S146A, I147A, and A156E mutations severely affected *caa*$_3$ activity, which

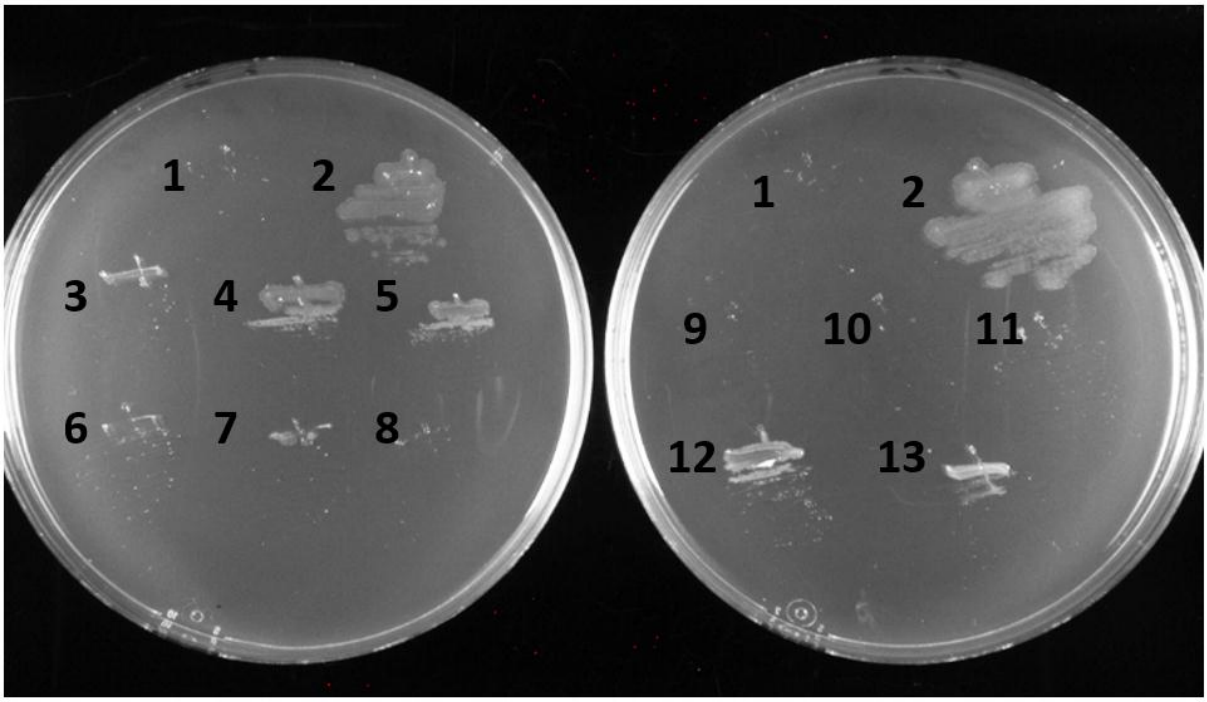

Fig. 2.21. The ResD mutations constructed in this study affect anaerobic growth in the presence of nitrate. Anaerobic growth on LB agar supplemented with 1% glucose and 0.2% KNO_3 in the presence of 1 mM IPTG after 36 h incubation in the GasPak EZ Anaerobe Container System. Strains: (1) *resD*::*cat*, (2-13) *resD*::*cat* strains producing ResD of (2) wild type, (3) H152A, (4) G158R, 5) R153A, 6) G198A, 7) A156E, 8) I147A, 9) D148A G158R double mutant, 10) S146A, (11) L145A, (12) F142A, and (13) D148A.

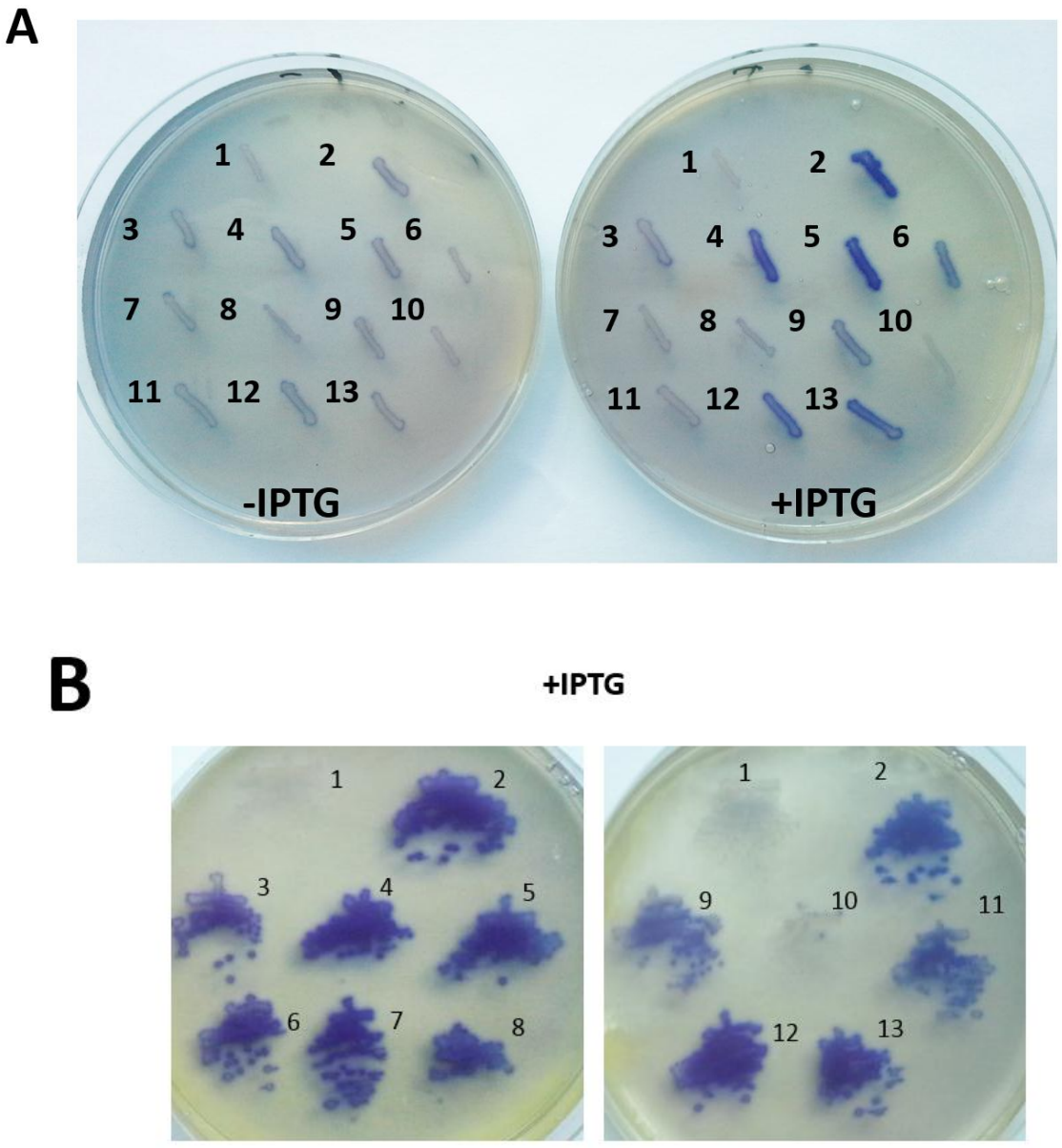

FIG. 2.22. Cytochrome *caa*$_3$ activity in aerobic cultures of ResD mutants. Strains were streaked onto DSM plates -/+ 1 mM IPTG and incubated at 37°C for 24 h. Afterward, plates were incubated at -20°C for 12 min. Immediately upon removal of plates from the freezer, plates were tested for N, N, N′, N′-tetramethyl-p-phenylene diamine (TMPD) oxidation. TMPD-oxidase positive clones turned blue within 5 min. A) 1) *resD*::*cat* (no *resD* integrated at *amyE*). The *resD* null mutant with 2) wt ResD, 3) H152A, 4) G158R, 5) R153A, 6) G198A, 7) A156E, 8) I147A, 9) D148A G158R double mutant, 10) S146A, 11) L145A, 12) F142A, 13) D148A. B) same numbering, expect only in the presence of IPTG 1) pDR111 empty vector integrated at *amyE* as control.

is attributed to highly reduced transcription of the *cta* operon. When cell density increases at later growth stage where caa_3 activity is the highest (Figure 2.22B), all mutants show stronger activity, while activity is hardly detected in the negative control (pDR111 vector at *amyE* -#1) and the S146A mutant that was shown to be unstable by western blot analysis. Although the results indicated a similar tendency of ResD activity between aerobic and anaerobic conditions, a more quantitative assay is needed to draw a solid conclusion.

2.3.5 Investigating the ResD-αCTD interface using genetic analysis

Since an additive effect on ResD-dependent activation was seen in the D148A G158R double mutant, strains carrying a single residue substitution of αCTD (Y263A or K267A) together with a ResD (D148A in region A or G158R in region B) mutation were constructed to examine whether one of the sites around G158 or D148, interacts with Y263 or K267 of αCTD. ResD-dependent transcriptional activation was monitored using a β-galactosidase activity assay of *nasD-lacZ* (Fig. 2.23). The ResD G158R mutation hardly exhibited an additive effect to the αCTD Y263A mutation on *nasD* expression, which suggests that G158 likely interacts with Y263. The D148A mutation resulted in synergistic negative effect with either K267A or Y263A mutation, which is a similar phenotype seen by combining D148A and G158R. Hence, the result suggests that D148, and likely region A, play a role different from the interaction with the Y263/K267 region of αCTD in ResD-controlled transcriptional activation.

2.3.6 Mutational analysis of Spx residues corresponding to ResD affected residues

A major question brought about by this study is whether ResD and Spx use similar but not identical residues to interact with RNAP. Since amino acid substitutions in region A of ResD

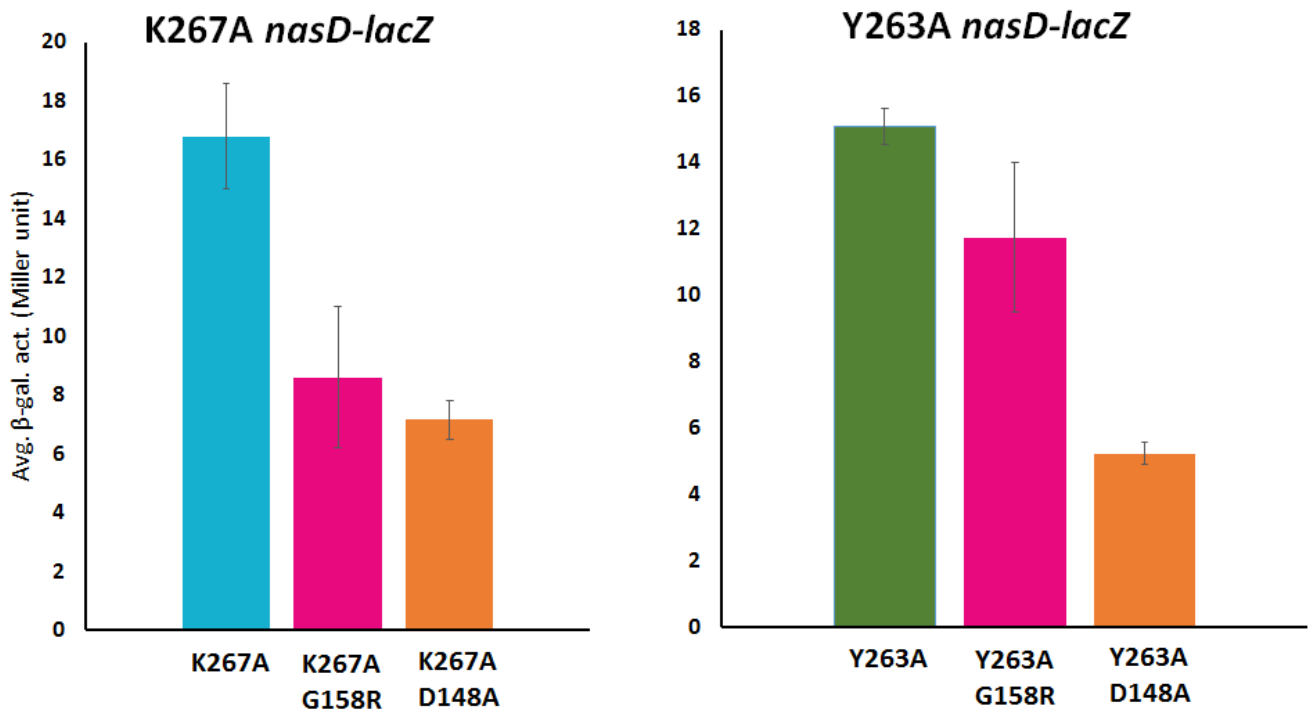

FIG. 2.23. Investigation of the ResD-αCTD interface. Mutant strains carrying a single residue substitution of αCTD (Y263A or K267A) together with a ResD (G158R or D148A) mutation were constructed to examine whether one of the sites around G158 or D148, interacts with Y263A or K267A of αCTD. The average ß-galactosidase activities of *nasD-lacZ* at T_0 were chosen. Each experiment was repeated with three independent isolates and the data points are shown as the average ratio with standard deviation.

(the LSID sequence at residues 142-148) cause severe effects on *nasD* transcription, protein stability (for S146A), and/or physiological function of ResD, whether corresponding mutations in Spx (L37 to D40) affect its activity was determined. In addition, alanine substitutions of R47 and E50 (corresponding to R153 and A156 in ResD, respectively) were undertaken. Mutant strains were constructed in a manner similar to the *resD* mutant construction (Fig. 2.7), with the process described in more detail in Materials and Methods. A transcriptional *lacZ* fusion to the *trxB* promoter was used to monitor Spx-dependent transcriptional activation. Since we are unable to measure β-galactosidase activity under stress conditions such as after diamide treatment, we used the previously constructed Spx^{DD} that is resistant to ClpXP proteolysis (Nakano *et al.*, 2003a). On IPTG induction, the Spx^{DD} protein accumulates, allowing for the detection of Spx-dependent activation of *trxB* under nonstress conditions.

One of the many residues identified by crystal structure studies as constituting Spx-αCTD interaction surface, R47, was shown to form a hydrogen bond with the K267 residue of αCTD, and some diamide sensitivity was detected in the K267A strain (Newberry *et al.*, 2005; Zhang *et al.*, 2006), suggesting that the R47-K267 interaction might be important for Spx function. However, *trxB-lacZ* was not significantly affected in the R47A mutant compared to wild-type, although alanine substitution of the corresponding ResD residue, R153, showed significantly decreased *nasD-lacZ* expression (Fig. 2.24). This result is in good agreement with the observation that the K267A mutation has no effect on *trxB* transcription (Lin, 2013), and suggests that R47A-K273 interaction is not essential at least for Spx to activate *trxB* tanscription. *trxB-lacZ* expression was significantly decreased in the L37A and I39A mutants, but not as severely affected as observed in the ResD L145A or I147A mutants. *trxB-lacZ* expression was hardly affected in the S38A and D40A mutants. As ResD (A156E) is defective in *nasD*

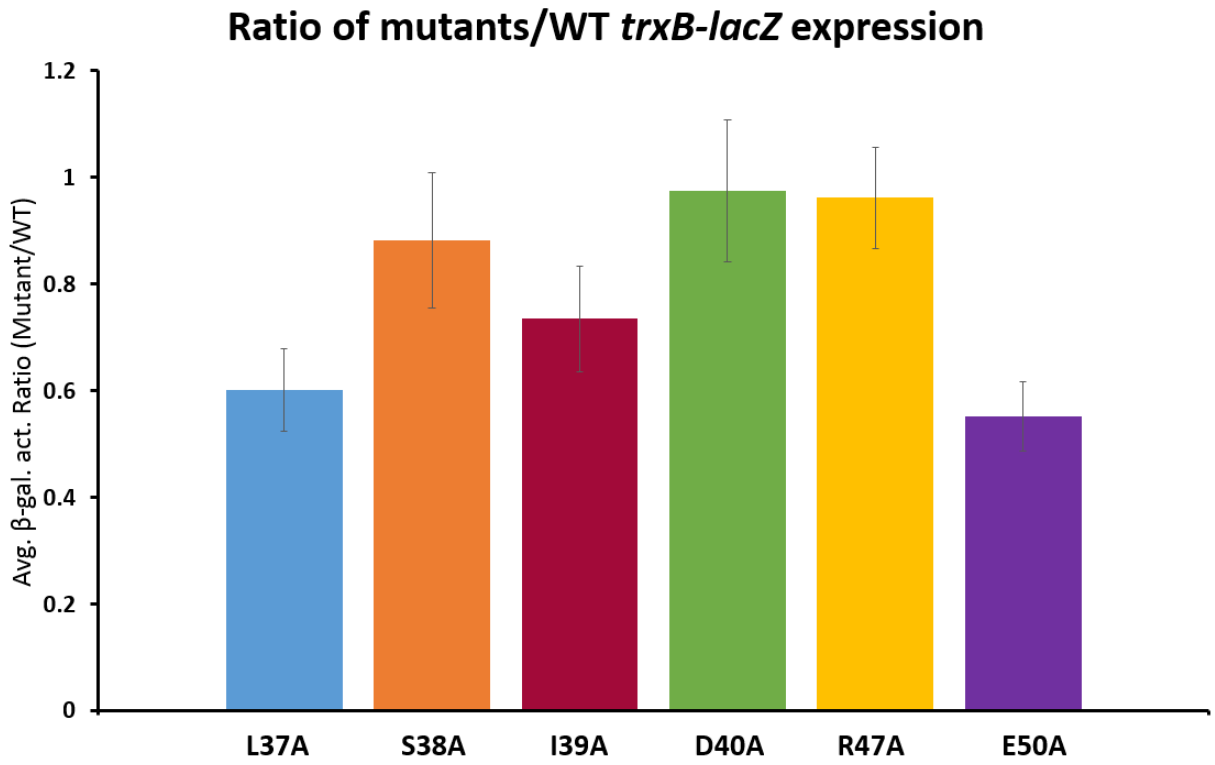

FIG. 2.24. Mutational analysis of Spx residues corresponding to ResD affected residues. Mutational analysis of Spx residues was carried out corresponding to ResD residues where amino acid substitutions severely affected ResD-dependent transcription. Graph shows the ratio of mutants/WT *trxB-lacZ* expression. Cells were grown under aerobic conditions in 2xYT supplemented with 0.5% glucose, 0.5% pyruvate, neomycin (5 mg/ml). Ratio is of values taken 1.5 hrs after 0.5 mM IPTG added. Each experiment was repeated with three independent isolates and the data points are shown as the average ratio with standard deviation.

transcription (Fig. 2.12), the converse Spx (E50A) mutation was constructed. The mutation significantly reduced *trxB-lacZ* expression; however, the effect is much weaker than the effect of A156E on *nasD* expression. The ground work laid by these experiments suggests that the two conserved regions in Spx and ResD may contribute in different ways to their activities as TFs and interaction with RNAP, if ResD does indeed interact with RNAP through the region(s).

2.4 DISCUSSION

Previous alanine-scanning study of αCTD demonstrated that the Y263A and K267A mutations show the highest negative effect on ResD-dependent activation of *fnr* and *nasD* as well as anaerobic growth supported by nitrate respiration (Geng, 2007; Geng *et al.*, 2007). A major aim of this work was to examine whether the αCTD residues Y263 and/or K267 directly interact with ResD to activate ResD-dependent transcription and to identify which region of ResD serves as an interaction surface by using amino acid substitutions of ResD. Two regions, namely residues 142-148 (region A) and 153-160 (region B) in ResD (Fig.2.6), show a high sequence similarity with Spx, the latter of which corresponds to the site engaged in Spx-αCTD interaction. We postulated that either region A or B (or both) is the site that interacts with the Y263/K267 surface of αCTD; thus the amino acid substitutions are focused on these regions. *nasD* and *yjlC* transcriptional *lacZ* fusions were used to monitor ResD-dependent transcriptional activation or repression, respectively. We anticipated that a mutation in the site of ResD-αCTD interaction would affect *nasD* activation, but not *yjlC* repression.

Overall, amino acid substitutions in region B resulted in only a moderate decrease in *nasD-lacZ* compared to wild type. The G52R substitution in Spx resulted in a complete loss of

transcriptional activation of *trxB* (Nakano *et al.*, 2003b), whereas the corresponding substitution in ResD (G158R) reproducibly but only moderately affected *nasD* expression. The only exception in region B is the A154E mutation that caused severe defect in *nasD* transcription. Amino acid substitutions in region A (residues 142-148) overall displayed a more severe effect on *nasD* than region B. *nasD* transcription was moderately reduced in the F142A and D148A mutants and was nearly abolished in the L145A, S146A, and I147A mutants, although the S146A mutant protein is easily susceptible to proteolysis based on western blot analysis.

Genetic analysis examining the phenotype of the double mutants was conducted to determine whether region A and region B of ResD execute different functions, and which region likely interacts with αCTD (Y263 and/or K267), if it does. By evaluating the additive effect of ResD mutants D148A (or F142A) and G158R, as well as D148A (or G158R) and αCTD mutants Y263A (or K267A), the study indicated that the role of ResD G158, but not D148, is similar to the role of αCTD Y263 in ResD-dependent *nasD* expression. A similar approach will be used in the future to confirm the separate function of region A and region B using double mutants in region A (F142A D148A) or region B (R153A G58R) as well as F142A R153A and D148A R153A. In addition, the additive effect of ResD R153A to αCTD Y263A or K267A is worth investigating. As R47 of Spx forms a hydrogen bond with K267 of αCTD, ResD R153A likely shows an additive effect to αCTD Y263A (the effect similar to G158R and Y263A).

The αCTD mutations E254A, V260A, and A269I, in addition to Y263A and K267A, were shown to reduce *nasD* expression (Geng, 2007) and *fnr* expression (Geng *et al.*, 2007). The crystal structure of *B. subtilis* αCTD indicates that all of these residues, including Y263 and K267, constitute the same surface (Newberry *et al.*, 2005). Thus, it is possible that ResD-RNAP

interaction requires α residues in addition to/outside of the Y263/K267 surface, or other subunits of RNAP interact with ResD at region A.

Our original premise was a RNAP interaction mutant will affect ResD activation but not ResD repression. However, the study showed that all mutations that strongly affect *nasD* activation also lost repressor activity. On the other hand, the αCTD (Y263A K267A) mutant still represses transcription of *yjlC* and *glpF*. The result indicates that any of the residues L145 to I147, H152, A156, and G198, do not interact with Y263 or K267. If the mutant effect of some of these residues were caused by the loss of interaction with RNAP, ResD-dependent repression would likely require interaction of ResD with other residues of RNAP except Y263 or K267. At the moment, it is unknown if and how ResD-RNAP interaction is needed for repression. ResD alone might not affect repression as binding of ResD to these genes is too weak to cause roadblock or outcompete RNAP in binding to these promoters, thus transcription proceeds. The RNAP-ResD complex might be able to bind more stably to the promoter regions, but RNAP would be trapped in the RNAP-ResD-DNA complex and RNAP release from the promoters is inhibited, and thus transcription initiation could be blocked.

Another possibility is that these ResD mutations are not RNAP interaction mutations. Both regions A and B of ResD belong to the N-terminal four-stranded antiparallel β sheet in the CTD unique to the OmpR subfamily. Since the function of the β sheet remains unknown, and is not well conserved in winged-helix TFs other than the OmpR-response regulator family, it has been suggested to play a role specific to response regulators, such as mediating interactions with the regulatory domain. The structure of the DrrB response regulator supports this proposal, as the entire surface of the β sheet interfaces with the N-terminal regulatory domain (Robinson *et al.*, 2003). The antiparallel β sheet of OmpRc is also parallel to α-helix 3 in OmpRc, which positions

two DNA contact loops on the same side of the DNA contact α-helix 3 (Rhee *et al.*, 2008). Since the L145A, S146, I147A, H152A, and A156E mutations abolished *yjlC* repression, it remains as a possibility that these residues play important structural roles to modulate ResD activity.

ResD amino acid substitutions in region A and region B affect aerobic caa_3 activity although the assay is not quantitative. To evaluate activity of ResD mutations under aerobic conditions more accurately, *ctaA* and *ctaC* transcription need to be measured in the wild-type and mutant cells cultured aerobically in DSM. The result will uncover if the effect of the mutations differs between aerobic and anaerobic conditions.

CHAPTER 3

SUMMARY AND FUTURE DIRECTIONS

3.1 SUMMARY

The ResD mutagenesis analysis brought about unexpected and more complicated outcomes than we imagined. Despite a high sequence similarity of the ResD region to the αCTD-interaction surface of Spx, critical residues between Spx and ResD for their TF activities are largely different. While ResD activity is drastically reduced by many amino acid substitutions in region A, H152A, and A156E in region B, the only mutation tested so far that severely affects Spx activity is G52R in region B.

Although Spx-dependent *trxB* transcription is almost abolished in the Y263A mutant, all other mutations in αCTD show moderate (around 50%) or no effect (Lin, 2013). On the other hand, ResD-dependent *nasD* expression is highly reduced (5% to 20%) by the Y263A, K267A, and A269I mutations (Geng, 2007). Taken together, Spx-RNAP complex formation relies on the strong interaction between a single residue of Spx and αCTD. In contrast, successful ResD-RNAP complex formation seems to be supported by multiple weaker interactions. The difference might explain how Spx is able to efficiently compete with ResD for association with RNAP.

3.2 FUTURE DIRECTIONS

3.2.1 Does ResD interact with RNAP?

Although ResD was identified as a RNAP-binding protein along with Spx (Delumeau *et al.*, 2011), the question remains whether ResD directly interacts with RNAP, and if so, through which subunit? A preliminary experiment done by our laboratory had shown that ResD and Spx co-purify with RNAP carrying His_{10}-tagged β on Ni-NTA column chromatography; however, we

were unable to make a solid conclusion from the experiment as ResD, like Spx, has some affinity to Ni-NTA. Unlike Spx and MgsR, ResD does not interact with αCTD (or α) judging from a result using yeast two-hybrid system. We hypothesized that ResD interacts with α in the context of RNAP based on previous genetic studies and the presence of ResD sequence similar to the Spx site that interacts with the Y263/K267 αCTD surface. Furthermore, the Y263A K267A mutation adversely affects both ResD-dependent anaerobic growth and transcription. Nevertheless, the current work suggested the alternative possibility that interaction of ResD and αCTD, even in the context of RNAP, is very weak at best. As a result, we need to use another approach to assess ResD interaction with RNAP. EMSA will be carried out using RNAP purified from *B. subtilis* and ResD (both wild type and mutant proteins) purified from *E. coli*. *nasD* and *yjlC* promoter DNA will be used as a probe. As a negative control, we will use the G198A DNA-binding mutant and RNAP carrying the Y263A or K267A mutation. To determine whether ResD and RNAP associate in the absence of DNA, we will carry out pull-down experiment. The use of another tag will be considered if the His-tag turns out to be problematic for this approach.

In addition to Y263A and K267A, the E254A and A269I mutations of αCTD significantly affect *nasD* and *fnr* transcription (Geng, 2007; Geng *et al.*, 2007). Since Y263, E254, K267, and A269 locate on the same surface patch, the effect of E254A and A269I on ResD-RNAP interaction is also worth investigating. A genetic approach looking for suppressor mutants may also expose the interaction site. The αCTD (K267A) mutant strain carrying *nasD-lacZ* will be plated onto LB agar supplemented with glucose, nitrate, and Xgal, and cultured under anaerobic conditions (GasPak EZ anaerobic system). Of the suppressor mutants that grow and express *nasD-lacZ*, we will amplify and sequence the *resD* gene to determine whether the compensatory mutation resides in *resD*. An IPTG-inducible *resD* construct would allow us to

test whether the suppressor effects are dependent on IPTG, and allele-specific suppression could be confirmed using the Y263A mutant carrying *nasD-lacZ*.

3.2.2 Is another subunit of RNAP involved in ResD interaction?

The possibility of ResD interaction with another subunit of RNAP will be considered next if the αCTD mutations that affect ResD-dependent transcription mentioned in 3.2.1 are able to interact with ResD, and some of the ResD mutations, particularly in region A, lack RNAP association ability. Region 4 of σ is a frequent target for DNA-bound activators of transcription initiation regulators (Dove *et al.*, 2003). Direct contact of ResD with one or more subunits of RNAP is also conceivable as interactions between αCTD and region 4.2 of σ have been suggested to potentially stabilize initiation complexes (Gourse *et al.*, 2000). Single amino acid substitutions in the σ^A region 4.2, K356A, H359A and R362A reduced *nasD* expression (Geng, 2007). The β-flap (flap domain of the β subunit of RNAP) is required for correct positioning of σ region 4.2 in the holoenzyme for interaction with -35 region DNA (Kuznedelov *et al.*, 2002). Recent findings by yeast two-hybrid system indicated that Spx and ResD, and not MgsR, interact with a β fragment carrying β-flap. $ResD_N$ and ResDc are unable to interact with β, suggesting either ResDc is unstable, or that the full-length protein is required for the interaction. We previously showed that ResDc is stable in *E. coli* but not produced in *B. subtilis*. We will screen if any ResD mutants available in our laboratory are unable to interact with β. The results will be confirmed by EMSA, pull-down, and far-western analysis with the subunits of RNAP to detect ResD interaction using anti-ResD antibody.

3.2.3 Are ResD mutants defective in signal transmission?

If we find that none of the ResD mutations generated in this study are RNAP-interaction mutations, we will test another possibility. As the regions A and B in ResDc are closely located to $ResD_N$, it might be possible that the conformation change induced by phosphorylation of D57 to activate ResDc is defective in these mutants. Transcription of the *cydABCD* operon requires ResD but not ResE at least in aerobic cultures (Puri-Taneja *et al.*, 2007), suggesting that transcription activation of *cydABCD* is phosphorylation independent. We will examine the effect of ResD mutations *on cydA* expression. If a mutant is defective in ResDc activation through phosphorylation, such a mutant likely has no significant effect on *cydA* transcription. In addition, investigating the effect of the ResD D57A mutant on *yjlC-lacZ* expression will provide insight on the effect of phosphorylation on ResD-dependent repression.

A deeper analysis of the ResD S146A mutant is also worthwhile as the surprisingly low level of *nasD* expression even compared to other severely affected ResD mutants, observed protein instability by Western blot analysis, and hindered ResD activity between aerobic and anaerobic conditions suggests a function for the residue apart from interaction with RNAP. Besides the structural role serine can play in hydrogen bond formation, the hydroxyl group of the side chain has been shown to be phosphorylated in regulatory proteins (Macek *et al.*, 2007). Thus, we will also investigate whether serine phosphorylation plays a role in protein stability.

3.2.4 Investigate interaction surface of the RNAP-ResD complex using structural studies

A structure has not been reported for RNAP from Gram-positive bacteria, nor for ResD. The OHSU Multiscale Microscopy Core houses Transmission Electron Microscope, optimized for high-resolution 3D imaging. Preliminary data obtained by negative staining using

conventional TEM showed that the RNAP sample is optimal for the high-resolution cryo-EM study both in homogeneity and concentration. Structural analysis of the RNAP-ResD complex using cryo-EM would allow us to elucidate the region of ResD participating in RNAP interaction, and the similarity and dissimilarity of interaction between RNAP-Spx and RNAP-ResD.

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