THE TRANSCRIPTIONAL CONTROL OF SPX IN
RESPONSE TO OXIDATIVE STRESS

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ABSTRACT

The Transcriptional Control of *spx* in Response to Oxidative Stress

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The *Bacillus subtilis spx* gene encodes a global regulator that controls transcription initiation in response to oxidative stress by interaction with RNA polymerase (RNAP). It resides in the *yjbC-spx* operon and is transcribed from at least four promoters, three (P_1, P_2 and P_B) residing upstream of *yjbC* and one (P_M) located in the intergenic region between *yjbC* and *spx*. We uncovered a second intergenic promoter, P_3, from which transcription is elevated in cells treated with the thiol-specific oxidant diamide, by primer extension analysis. P_3 is recognized by the σ^A form of RNA polymerase (RNAP) in vitro without the involvement of a transcriptional activator. Deletion analysis together with point mutation analysis uncovered two negative *cis*-acting control elements within the P_3 promoter. Previously published studies and transcription factor/transformation array technology uncovered two transcriptional repressors, PerR and YodB that were potential candidates for the missing trans-acting factors affecting P_3 promoter utilization. PerR was previously characterized as the regulator of the inducible peroxide stress response in *B. subtilis*, while YodB is a novel DUF24/MarR type repressor that controls genes that are induced in response to phenolic compounds and

oxidative stress. The derepression of *spx* was detected in both *perR* and *yodB* mutants by examining the level of *spx* expression using the *spx-bgaB* fusion construct. The additive effect was observed in the *perR yodB* double mutant. The regions of *spx* P$_3$ DNA required for transcriptional repression by YodB and PerR were confirmed by DNase I footprinting analysis. PerR protects an area from approximately position -3 to +35. YodB binds a region from approximately positions -3 to -32. The binding of YodB and PerR proteins to *spx* P$_3$ promoter DNA was impaired by addition of diamide and H$_2$O$_2$ in vitro as determined by DNase I footprinting analysis. Besides *spx*, YodB also controls the divergently transcribed *yodC* gene which encodes a putative nitroreductase that is induced by disulfide stress. Microarray and proteome analyses were performed to identify other genes controlled by YodB. *yocJ (azoR1)*, encoding the putative FMN-dependent NADH-azoreductase, was the most strongly derepressed by *yodB* null mutation and was induced in response to diamide, catechol, MHQ and nitrofurantoin stress. *bsrB* encoding a small 6S RNA located downstream of *azoR1*, is co-transcribed with *azoR1* and increased in concentration in response to thiol-reactive compounds. The *yodB* mutant confers a catechol and MHQ resistance phenotype due to AzoR1 overproduction. In addition, the *yodBmhqR* double mutant, bearing the deletion of the *mhqR* gene encoding a MarR-like repressor, that overproduces AzoR1 and MhqR-regulated paralog AzoR2, exhibits hyper-resistance to thiol-reactive compounds. Thus, the detoxification of thiol-reactive substances in YodB and MhqR regulons show overlapping functions. DNase I footprinting analysis, together with promoter sequence alignments, uncovered YodB boxes which contain a common 15 bp consensus sequence for YodB-DNA interaction. The YodB protein contains three cysteine residues Cys6, Cys101 and Cys108. The conserved Cys6 contributes to the repression of *spx* and *azoR1* transcription by YodB. Moreover, mass spectrometry revealed YodB Cys modifications by catechol and MHQ.

CHAPTER 1
INTRODUCTION

Living microorganisms, especially bacteria, often encounter a variety of toxic substances and rapidly changing environmental conditions. Multiple environmental stresses such as low and high temperature, extremes of pH, high osmotic pressure, nutrient starvation and oxidizing substances can cause loss or reduction of bacterial viability and reproduction. For instance, the soil bacterium *Bacillus subtilis* encounters a variety of toxic and antimicrobial compounds produced by plants, fungi and other bacteria. Microbial pathogens such as the gastric pathogen *Helicobacter pylori* and the intestinal pathogen *Vibrio cholerae* become exposed to oxidative burst from host immune cells, gastric cells and phagocytes that are mobilized to combat infection. In order for bacterial cells to quickly adapt to the ever-changing external environment, rapid and accurate control of gene expression is essential. Regulation of gene expression operates at every single step of macromolecular biosynthesis, from DNA template utilization, to RNA synthesis and turnover, to post-translational modification and turnover of the protein product. In bacteria, the central step of the regulation of gene expression is transcription. Transcription is accomplished by an enzyme termed DNA-dependent RNA polymerase, or RNA polymerase (RNAP). The transcriptional control factors determine when and under what conditions transcription occurs, and how much RNA is transcribed. The control can be exerted at several steps, at the initial contact between promoter and RNAP, transcription initiation, elongation and termination. Transcription of a gene by RNA polymerase can be regulated by many different mechanisms involving specific factors (i.e., sigma factors, repressors, activators and enhancers).

A protein involved in regulating gene expression is called a regulatory protein or a regulator. In most cases, transcription initiation is regulated by proteins which are generally called transcription factors. They are usually bound to a regulatory cis-acting

site in or near a gene's promoter DNA. Its ability to bind to the DNA controls gene expression by switching the gene on (activator) or off (repressor).

This chapter provides a summary of the processes underlying the regulation of transcription and includes specific control mechanisms in prokaryotes. The chapter begins with the cellular components necessary for transcription in a bacterium. It also explains the role of transcriptional regulators and their special importance in controlling transcription initiation by serving as a link to the factors that sense changing environmental conditions. The regulation of gene expression in response to oxidative stress is subsequently described. The chapter also includes information on the sources, substances, and chemicals that induce oxidative stress. There follows a section describing redox-sensing mechanisms, where the activity of transcription factors is modulated through their redox-reactivity involving specific amino acid residues (i.e., cysteine). The redox-regulated transcription factors are also discussed. The roles of Spx-dependent transcriptional regulation against oxidative stress are introduced. The bacterial transcription repressors MarR-type family in response to ligands is subsequently discussed. The characterization of *spx* transcription which is induced in response to oxidative stress in *B. subtilis* is discussed in Chapter 2. In Chapter 3, the discovery of two negative regulators, which control *spx* transcription initiation, is reported. Two negative regulators, PerR and YodB, interact directly with the *spx* promoter DNA as examined in part by DNase I footprinting analysis. PerR is a previously well-characterized regulator of the inducible peroxide stress response in *B. subtilis*. YodB, the novel DUF24/MarR-type repressor, represses genes that are induced in response to phenolic compounds and oxidative stress. Our study on the role of YodB is documented in Chapter 4. The modification of YodB after exposure to thiol-reactive oxidants *in vitro* by mass spectrometry is reported in this chapter. Finally, conclusions and future directions are discussed in Chapter 5.

1.1 THE CELLULAR COMPONENTS NECESSARY FOR TRANSCRIPTION IN PROKARYOTES

The process of copying information stored in DNA to an RNA molecule is denoted transcription. In other words, RNA is the product of transcription and is the complement of the template strand (or non-coding strand). Transcription begins at the transcription start site near the promoter sequences of the gene's DNA that specifically bind RNA polymerase (RNAP), and ends at terminator sites after the transcription unit's coding sequences (Wagner, 2000).

Promoters are conserved DNA sequence sites that bind to RNAP to initiate the transcription reactions (Barnard *et al.*, 2004, Wagner, 2000). Bacterial RNAPs are isolated in two distinct forms: core RNAP ($\beta\beta'\alpha2$ ω or E) and RNAP holoenzyme ($\beta\beta'\alpha2$ ω σ) (Paget & Helmann, 2003, Young *et al.*, 2002, Young *et al.*, 2004). The σ subunit provides the specificity to the promoter DNA by reducing the non-specific DNA binding by core RNAP for the correct initiation of transcription of different kinds of genes under variety of growth conditions or the expression of various regulons (Burgess & Travers, 1970, Haldenwang, 1995). The RNAP core is capable of performing the transcription elongation reaction in an entirely processive way, after the σ subunit is discharged (Haldenwang, 1995, Helmann & Chamberlin, 1988, Wagner, 2000).

Numerous DNA-binding proteins, known as transcription factors interact at some point during initiation or elongation of transcription; some of these proteins function by making direct interactions with RNAP (Barnard et al., 2004, Fredrick & Helmann, 1997, Haldenwang, 1995). The function of transcription factors will be addressed in more detail in the latter sections.

1.1.1 The promoter and transcription start site

The majority of regulation of gene expression in bacteria occurs at the level of transcription. Although, a regulatory protein can significantly affect the efficiency of transcription, the specificity on interactions between RNAP and promoter of transcription reaction is equally important (Haldenwang, 1995). The RNAP holoenzyme starts

synthesizing an RNA polymer from only selected regions of a double-stranded DNA, rather than randomly from the whole molecule (Champness, 1997). The +1 nucleotide is the position on the DNA template where transcription of the gene into RNA begins. Transcription initiation involves at least three distinct intermediates: an initial "closed complex" where RNAP holoenzyme recognizes the two conserved hexamers in the promoter, located at -10 and -35 relative to transcription start site of +1; DNA melting occurs between roughly -11 to +2 to form the "open complex"; and then "initial transcribing" complexes in which short template RNA chains (i.e. abortive initiation) are synthesized and released prior to promoter clearance (Hsu, 2002, Young et al., 2002, Young et al., 2004). The formation of close complex (RPc) can be characterized by the association and dissociation rate constants k_1 and k_{-1}, respectively; K_B represents the binding equilibrium constant of this reaction (k_1/k_{-1}) and the isomerization to form the open complex (RPo) can be described by forward and reverse rate constants k_2 and k_{-2} (Wagner, 2000). Recently, abortive initiation has been described as involving a "scrunching" and releasing of DNA, in which DNA template is gathered into the transcription initiation complex as a short RNA oligonucleotide is synthesized, and then released to allow reinitiation (Kapanidis *et al.*, 2006, Revyakin *et al.*, 2006).

Promoter clearance or promoter escape refers to the last stage of transcription initiation, where RNAP loses contact with the promoter and starts moving along the DNA, prior to formation of the transcription elongation complex. It can be the rate-limiting step of transcription initiation besides the K_B and k_2 step (Hsu, 2002). Upon the transition from initiation to elongation or shortly thereafter, the σ subunit is released from the core RNAP (Burgess, 1971, Hansen & McClure, 1980, Shimamoto *et al.*, 1986, Stackhouse *et al.*, 1989). However, it had been reported that the σ^{70} subunit remains associated with RNAP in some transcription complexes of *E. coli* during transcription elongation (Bar-Nahum & Nudler, 2001, Kapanidis *et al.*, 2005). Thus, the promoter escape may or may not involve the releasing of σ subunit from core RNAP. At this point only general description of standard promoter will be given.

1.1.1.1 Core promoter elements. The minimal structural elements of the standard promoter are required to properly initiate transcription. In addition to the

transcription start site, the core promoter structure contains three elements: the -10 region, spacer region and -35 region (Fig. 1.1) (Wagner, 2000). The -10 region, a conserved recognition sequence for σ^{70}, is a short AT-rich region centered at about 10 base pairs upstream of the transcription start site (Champness, 1997). The conserved sequence exists among a certain class of promoters which define a sigma recognition site (Haldenwang, 1995).

The sequence between the -10 and -35 elements is called the spacer region (Wagner, 2000). Analysis of *E. coli* promoters showed that 92% of promoters had inter-region spacing of 17 ± 1 bp and 75% of defined start points were 7 ± 1 bases downstream of the -10 region (Harley & Reynolds, 1987). Although there is no consensus sequence for nucleotides in the spacer region, the variation in this region has been shown to affect promoter activity in *E. coli* (Hawley & McClure, 1983). In addition, mutation in the promoter spacing region also showed an effect on gene expression (Borst & Betley, 1993, Voskuil *et al.*, 1995). Oligonucleotide-directed mutagenesis identified the importance of bases for α-amylase promoter utilization in spacer sequence of *E. coli* and *B. subtilis*, which indicated that nucleotides in the spacer region are important for promoter utilization in the case of a weak promoter, but not that of strong promoters (Voskuil et al., 1995).

The conserved sequence located about 35 bp upstream of the first transcribed nucleotide is called -35 region (Wagner, 2000). This region is necessary for transcription initiation by interacting with RNAP σ subunit. The discriminator region, a G-C rich region downstream of -10 element , has potential to impede strand separation and also contacts the σ subunit (Haugen *et al.*, 2006). Further description of σ subunit-promoter interaction is presented in section 1.1.2.4. It is concluded that the sequences in the highly conserved -35 and -10 regions of the promoter as well as sequences and length of the spacer region contribute to the efficiency of transcription in bacteria.

1.1.1.2 Flanking elements. In addition to the -10 and -35 promoter recognition element, the upstream sequence region is frequently required for full promoter activity (Fig. 1.1) (Estrem *et al.*, 1999, Gourse *et al.*, 2000). The UP element resides upstream of

the -35 region of many promoters and contains a DNA sequence rich in A and T residues (Blatter *et al.*, 1994, Ross *et al.*, 1998). A specific interaction between UP elements and the C-terminal domain of the α subunit (αCTD) of RNAP stimulates transcription at some promoters in *E. coli* (Blatter et al., 1994, Ross et al., 1998, Tagami & Aiba, 1999). The best characterized UP element is that of the rRNA operon promoter *rrnB* P_1 in *E. coli*, the activity of which increases dramatically in the presence of the UP element (Ross *et al.*, 1993). The DNA sequence upstream of -35 of *lacUV5* promoter in *E. coli* does not match the UP element consensus sequence (Ross et al., 1998). Nevertheless, removal of DNA upstream of -35 reduced the *lacUV5* promoter activity (Ross & Gourse, 2005). Thus, the αCTD also interacts nonspecifically with upstream DNA in a promoter that lacks UP elements to elevate transcription initiation efficiency, in addition to interacting specifically with UP elements (Ross & Gourse, 2005). While the interaction between UP element and αCTD stimulates initiation of transcription, repressors exist that can compete with the αCTD for binding to this region by blocking αCTD/UP element contact (Quinones *et al.*, 2006). As it was shown in the study of P_{rstA}, the primary CTXΦ (*Vibrio cholerae* filamentous phage) promoter, the stimulatory effect of the UP element is not confined to the powerful *rrn* promoters. The activity of the P_{rstA} promoter is enhanced by the interaction of αCTD to an UP element (Quinones et al., 2006). Host repressor LexA represses P_{rstA} by binding to a specific sequence that overlaps with a promoter UP element resulting in inhibition of transcription initiation (Quinones *et al.*, 2005). Blocking the binding of LexA to promoter DNA by adding free α subunit indicates that LexA directly competes with the αCTD for their binding site. Thus, control of transcription initiation can be exerted by hindering αCTD contact with the upstream UP element. These examples, showing how transcription is enhanced by RNAP interaction with flanking promoter elements and how transcription is blocked when these interactions are prevented and illustrate the importance of RNAP-DNA contact outside of the core promoter.

1.1.2   RNA Polymerase (RNAP)

RNAP is a remarkable molecular machine that is essential for the first step of gene expression, transcription. Transcription initiation in prokaryotes is carried out by the holoenzyme, a complex of the five core subunits of RNAP ($\alpha_2\beta\beta'\omega$) and the σ factor (Young et al., 2002, Young et al., 2004). During initiation, RNAP must distinguish between promoter DNA and non-promoter DNA, a task accomplished in part by the initiation specific subunit σ. This is followed by the separation of the double helical DNA to expose the single template strand, and initiation of RNA synthesis by addition of nucleoside monophosphates to a growing polynucleotide chain designed according to base complementary (Burgess *et al.*, 1969). σ^{70}, the principle σ subunit of *E. coli*, when bound to core RNAP is capable of promoter specific recognition and enables transcription initiation of most genes during exponential growth.

This section will provide information about the function of RNAP and a description of the different subunits of RNAP based on the *E. coli* RNAP model, which is one of the best-studied RNAP complexes. The association of RNAP and transcriptional regulators in controlled gene expression will be addressed in more detail in section 1.3.1.

1.1.2.1 The a subunit.   The α subunit consists of 329 amino acids and is the subunit that initiates RNAP complex assembly (Blatter et al., 1994). It comprises two independently folding domains, the N terminal domain (αNTD; residues 8-231), and the C terminal domain (αCTD; residues 249-329), which are connected by a flexible 14 residues linker (Igarashi & Ishihama, 1991, Blatter et al., 1994). αCTD plays a role in transcription initiation by interacting with upstream promoter element (Blatter et al., 1994, Ross et al., 1993, Tagami & Aiba, 1999) and is the target for many transcription activators (Hirvonen *et al.*, 2001, Meng *et al.*, 2001). As mentioned above, the UP element-dependent promoter, *rrnB* P1, makes specific protein-DNA interaction with αCTD increasing the promoter activity by stimulating the open complex formation (Busby & Ebright, 1994, Rao *et al.*, 1994). In addition to the crucial interaction between UP element and αCTD, the natural length of the flexible linker between αCTD and

αNTD also allows for the optimal promoter activity by providing the ability of αCTD to access the UP element (Meng et al., 2001).

CAP, the catabolite gene activator protein in *E. coli*, is the well-characterized activator that interacts with αCTD. The CAP protein provides the link between the uptake and phosphorylation of glucose, and the transcriptional control of operons that function in the utilization of alternative carbon sources. Simple CAP-dependent promoters can be placed into two classes based on the DNA-binding sites for CAP and RNAP and the pattern of CAP-RNAP interaction during transcription activation (Busby & Ebright, 1994, Busby & Ebright, 1997). In class I CAP-dependent promoters, such as the *lac* promoter, the DNA site for CAP binding is located upstream of the DNA site for RNAP interaction (Busby & Ebright, 1994, Zhou *et al.*, 1993). CAP activates transcription at the *lac* promoter by recruiting αCTD to the DNA upstream of the -35 element resulting in increasing the affinity of RNAP to the promoter by positioning αCTD so as to make a potentially stimulatory interaction with σ^{70} (Busby & Ebright, 1994). The DNA-interaction site for CAP within the Class II CAP-dependent promoters, such as *gal* P1 promoter, overlaps the -35 region for RNAP binding site (Attey *et al.*, 1994, Belyaeva *et al.*, 1996, Busby & Ebright, 1997). The activation at the class II promoters involves more complex and multiple interactions between CAP and RNAP than activation by Class I CAP-RNAP contacts (Busby & Ebright, 1994, Busby & Ebright, 1997).

Interaction between a transcription regulator and the αCTD of RNAP also can have a negative effect on transcription initiation. For example, the GalR repressor protein interacts with αCTD to repress *gal* P1 promoter in *E.coli* (Choy *et al.*, 1997, Roy *et al.*, 2004). GalR represses the *gal* P1 promoter by inhibiting RNAP open complex formation, which is the rate-limiting intermediate of initiation, and this requires GalR interaction with αCTD (Roy et al., 2004).

1.1.2.2 The β and β' subunits. The *rpoBC* operon of *E. coli* encodes the β subunit, the second largest subunit of RNAP (1407 amino acids) and β′ subunit, the largest subunit of RNAP (1342 amino acids) respectively (Ishihama & Fukuda, 1980). In *E. coli*, β subunit contains nine conserved segments (A to I) and β′ subunit displays eight

conserved segments (A to H) all of which are separated by poorly conserved regions (Allison *et al.*, 1985, Severinov, 2000).

The RNAP-DNA interaction downstream of the transcription initiation start site is required for open promoter complex formation. Mutations in the β subunit of *E. coli* core RNAP affect promoter complex formation by Eσ[70] by impairing RNAP-DNA interaction. This results in a shortened transcription bubble (Nechaev *et al.*, 2000, Wigneshweraraj *et al.*, 2002). Many mutations in the β subunit confer resistance to rifampicin, an antibiotic that inhibits the function of RNAP in eubacteria (Jin & Gross, 1988). Moreover, several mutations in the β subunit of *E. coli* RNAP appear to weaken interactions with the promoters of the stringently controlled genes which respond to nutrition-limiting conditions (Zhou & Jin, 1998).

The N-terminus of the β′ subunit together with σ subunit are sufficient to induce promoter DNA melting at a rate similar to that catalyzed by authentic holoenzyme (Young et al., 2004). The residues 269-309 which are located at the conserved segment B of β′ subunit interact with σ[70] (Arthur & Burgess, 1998, Severinov, 2000).

The crystal structure of RNAP from *Thermus aquaticus* uncovered the organization of the multi-subunit RNAP. The β subunit included β lobes and β flap together with β′ subunit, which consists of the β′ lid, β′ clamp and the β′ jaw that forms part of the catalytic center with Mg^{2+} (Murakami *et al.*, 2002). The β flap domain interacts with σ subunit region 4 in initiation complex, however, the displacement of σ subunit region 4 from the β flap is required in elongation step (Nickels *et al.*, 2005). β flap is required for correct positioning of σ region 4.2 by seperating σ region 2 and region 4 which contributes to promoter recognition and allows -10/-35 promoter complex formation (Kuznedelov *et al.*, 2002). Although the β flap is important for transcription initiation, it is dispensable for transcription from extended -10 promoters, such as *gal P1* (Kuznedelov et al., 2002). The transcription activator, anti-σ factor AsiA, can compete with β flap for binding to σ region 4 by disrupting the σ region 4/β flap interaction resulting in inhibiting transcription initiation (Gregory *et al.*, 2004). In addition, β′ clamp and β′ jaw which form opposing walls of a trough are required to maintain stable DNA strand separation near the transcription start site (between -5 and -1) (Wigneshweraraj *et al.*, 2005,

Wigneshweraraj *et al.*, 2006). β′ lid protrudes from the clamp domain to seal the main channel and RNA exit channel (Murakami et al., 2002). Lid plays roles in RNA separation from RNA:DNA hybrid during RNA exit and in maintenance of the upstream end of the transcription bubble (Toulokhonov & Landick, 2006).

1.1.2.3 The ω subunit. The 90-amino acid ω subunit is encoded by *rpoZ*, which resides in the same operon as *spoT* gene (Gentry & Burgess, 1989). It is the smallest RNAP subunit, 10 kDa. *spoT* is involved for the maintenance of a stringent response under starvation conditions in *E. coli* (Gentry & Burgess, 1993). During RNAP assembly, the ω subunit acts through the largest subunit β′ and exhibits important structural and functional characteristics (Mathew & Chatterji, 2006). Initially, no characteristic phenotype was observed for the *rpoZ* null mutant of *E. coli* (Gentry & Burgess, 1989). However, recent studies showed that RNAP holoenzyme lacking ω has a defect in the normal response to ppGpp (Igarashi *et al.*, 1989, Vrentas *et al.*, 2005). The stringent factor ppGpp interacts with the active site of RNAP (Chatterji *et al.*, 1998) and inhibits the transcription of rRNA, tRNA and other stringently controlled genes (Barker *et al.*, 2001, Gralla, 2005, Paul *et al.*, 2004). In addition, ppGpp positively regulates the transcription of genes coding for enzymes that function in amino acid biosynthesis and transport (Barker et al., 2001, Paul et al., 2004). The model of the *T. thermophilus* ppGpp-RNAP structure reveals the segments of β′ connecting ω and the ppGpp binding region and suggests an allosteric effect of ppGpp binding or action that involves the ω subunit (Artsimovitch *et al.*, 2004, Vrentas et al., 2005). The segments of β′ connecting ω and the ppGpp binding region might alter the stability of kinetic intermediates on the pathway to open complex formation/dissociation, which amplify effects of ppGpp (Vrentas et al., 2005). DksA, a transcription regulator required for optimal ppGpp activity at the RNAP active site, along with ppGpp, bind directly to RNAP and combine to regulate gene transcription in response to amino acid starvation (Paul et al., 2004, Perederina *et al.*, 2004). Further studies demonstrated that DksA eliminates the requirement for ω with respect to ppGpp function to RNAP (Vrentas et al., 2005). DksA could potentially facilitate coordination of ppGpp bound Mg^{2+} ion, thereby stabilizing the ppGpp-RNAP complex (Perederina et al., 2004). Moreover, a decrease of *relA* mRNA

level is observed in an *rpoZ* mutant (Mathew & Chatterji, 2006). *relA* encodes ppGpp synthase I, which is responsible for the synthesis of the stringent factor ppGpp and many ribosomal proteins (Chatterji & Ojha, 2001). Thus, the product of *rpoZ* influences the expression of *relA* and is involved in stringent control (Mathew & Chatterji, 2006). The association between the ω subunit and the holoenzyme at the surface of the enzyme complex is observed, which suggests that it provides an accessible site for interaction with transcription factors (Dove & Hochschild, 1998). Therefore, the ω subunit of RNAP plays a role in RNAP function and control of its activity.

1.1.2.4 The σ subunit. Bacterial promoter-specific transcription initiation requires the RNAP σ factor which reversibly associates with the core RNAP complex and contributes to the initial promoter recognition, initiation of DNA melting and possibly promoter complex stability (Burgess et al., 1969, Dove *et al.*, 2003). The core RNAP ($\beta\beta\alpha_2\omega$) alone is capable for transcription elongation and termination but is insufficient to initiate transcription at promoter DNA. The primary σ factor, known as σ^{70} in *E. coli* and σ^{A} in *B. subtilis* is essential for general transcription in exponentially growing cells (Helmann & Chamberlin, 1988, Paget & Helmann, 2003).

Sequence alignments of the σ^{70} family members identify four conserved regions, of which regions 2, 3 and 4 contain DNA binding domains responsible for recognition of the promoter -10 element, the extended -10 element and -35 element respectively (Nickels et al., 2005, Dove et al., 2003, Paget & Helmann, 2003). Region 2 consists of four subregions, 2.1, 2.2, 2.3 and 2.4 (Lonetto *et al.*, 1992) involved in core binding and promoter recognition. Region 2.4 recognizes the -10 region of the promoter and region 2.3 involves in promoter melting (Lonetto et al., 1992, Paget & Helmann, 2003). Region 3 and 4 are separated by a flexible linker (region 3.2), which winds through the RNAP active site channel and out through the RNA exit channel (Murakami et al., 2002). Region 4 consists of two subregions, 4.1 and 4.2, in which region 4.2 participates in recognition of the -35 promoter sequence (Lonetto et al., 1992). In the initiation complex, σ^{70} region 4 is bound to the β-flap and region 3.2 is located within the RNA exit channel (Nickels et al., 2005). In the elongation complex, the extension of 16 nucleotides of

nascent RNA displaces σ^{70} region 3.2 from the RNA exit channel followed by displacement of σ^{70} region 4 from the β-flap domain (Nickels et al., 2005).

In vegetatively growing cells of *B. subtilis,* σ^A is the principal sigma factor and functions in a similar way as σ^{70} of *E. coli*. The common sequences of σ^A-dependent promoters at -10 (TATAAT) and -35 (TTGACA) recognition regions are identical to that of σ^{70}-dependent promoters (Haldenwang, 1995).

1.1.3 Alternative σ factors

In many bacterial species there are alternative σ factors that become active and abundant under specific environmental or metabolic conditions (Dove et al., 2003). The σ subunits bind reversibly to the core RNAP and can compete with different σ factors for core binding to promote transcription initiation of a specific collection of genes (Ishihama, 2000). Alternative σ factors confer distinct promoter selectivity to holoenzyme to change transcription patterns within the cell. This section includes a general description of alternative σ factors that have been well-studied in *E. coli* and *B. subtilis*.

E. coli contains at least six σ factors of the σ^{70} family, σ^{70} (RpoD), σ^S (RpoS), σ^{32} (RpoH), σ^F (FliA), σ^E (RpoE), and σ^{fecI} (FecI) (Gourse *et al.*, 2006, Gruber & Gross, 2003). The σ^{54} (RpoN) family, a second family of σ factors, functions in diverse processes including nitrogen metabolism, transport of dicarboxylic acids, pilus formation, formate dehydrogenase synthesis, metabolism of aromatic compounds and the expression of small heat shock proteins (Janaszak *et al.*, 2007).

The general stress-responsive alternative sigma factor, σ^S was first identified for its role in transcription during stationary phase. σ^S (RpoS) is very similar to the vegetative σ^{70} with respect to its structure, molecular function and promoter recognition properties (Gaal *et al.*, 2001, Gruber & Gross, 2003). Eσ^S and Eσ^{70} utilize the same core promoter sequences (Hengge-Aronis, 2002b). However, the selectivity of Eσ^S can be achieved with different sequence elements surrounding the promoter region and with tolerance for non-optimal -10 to -35 spacer lengths (Hengge-Aronis, 2002b). Eσ^S selectivity also can be attributed to the binding of *trans*-acting regulatory factors, such as cAMP-CRP, the leucine-responsive regulatory protein Lrp and the integration host factor IHF, to the

promoter region (Hengge-Aronis, 2002b, Colland *et al.*, 2000). σ^S is now recognized as the master regulator of the general stress response that is mobilized to cope with the consequences of many different stress conditions (Hengge-Aronis, 2002a).

The σ^{32} regulon plays a central role in the heat-shock response in *E. coli* (Gruber & Gross, 2003, Yura & Nakahigashi, 1999). The induction of heat shock proteins (HSPs) is positively controlled at the transcriptional level, in part, by the σ^{32} subunit of RNAP (Arsene *et al.*, 2000, Gruber & Gross, 2003). Major HSPs are molecular chaperones such as GroEL and DnaK, as well as proteases that control protein folding and degrade damaged proteins (Arsene et al., 2000). These are encoded by members of the σ^{32} regulon. GroEL and DnaK also function in the regulation of both σ^{32} activity and stability (Gruber & Gross, 2003, Guisbert *et al.*, 2004). DnaK negatively regulates σ^{32} activity by binding to σ^{32} and facilitating σ^{32} degradation (Guisbert et al., 2004). Damaged, unfolded proteins and σ^{32} compete for DnaK binding. The DnaK-σ^{32} interaction inhibits σ^{32}-dependent transcription (Guisbert et al., 2004). However, when the unfolded proteins are more abundant relative to DnaK, the level of free σ^{32} increases, resulting in the induction of heat shock genes (Guisbert et al., 2004). Thus, the tight regulation exerted by DnaK through its interaction with σ^{32} regulates heat shock gene expression ensuring that the cellular protein folding environment remains optimal.

The *fliA* or *rpoF* gene encodes the alternative sigma subunit of RNAP, σ^F (σ^{28}), which is involved in transcription of the flagellar and chemotaxis genes (Kundu *et al.*, 1997, Ohnishi *et al.*, 1990). Genes in the flagellar and chemotaxis regulon are coordinately regulated in three classes, early, middle and late (Chilcott & Hughes, 2000, Kundu et al., 1997). The FlhC and FlhD proteins encoded by the early genes *flhDC*, constitute the master regulator of the flagellar operon and are required for transcription from the class II promoters (Chilcott & Hughes, 2000). Two competing regulatory proteins, FlgM and FliA (σ^F) as well as the hook-basal body proteins are transcribed from class II promoters (Chilcott & Hughes, 2000). σ^F is required to activate the class III genes whose products function in flagellum assembly (Gruber & Gross, 2003). The negative regulator FlgM binds to σ^F to block class III gene transcription (Gillen & Hughes, 1991, Mytelka & Chamberlin, 1996). Once the hook-basal body is complete, FlgM is excreted

from the cell through the pore of the basal-body complex to yield free σ^F for the transcription of class III genes (Kutsukake, 1994).

The σ^E regulon, induced by extracytoplasmic stress, encodes periplasmic proteases and folding enzymes that sense misfolded proteins in the cell envelope (Gourse et al., 2006, Ruiz & Silhavy, 2005, Yura & Nakahigashi, 1999). σ^E activity is negatively regulated by RseB through RseA (Missiakas *et al.*, 1997). RseA, a membrane-bound anti-sigma-factor, with help of RseB inactivates σ^E by direct interaction with the sigma factor under non-stress conditions (Missiakas et al., 1997). When misfolded proteins accumulate in the periplasm as a result of damaging temperature or chemicals, the anti-sigma factor RseA is sequentially degraded by the proteases DegS (a periplasmic protease that cleaves the C-terminal part of RseA), YaeL (a intramembrane protease that cleaves the transmembrane domain of RseA, resulting in a cryptic proteolytic tag), and ClpXP (which completely degrades the residual RseA with the help of the SspB adapter protein) resulting in the activation of the σ^E-dependent extracytoplasmic stress response (Alba *et al.*, 2002, Flynn *et al.*, 2004).

FecI controls transcription of the *fecABCDE* operon containing the genes that function in the ferric citrate transport when the cell encounters iron depletion (Gruber & Gross, 2003). FecI expression is activated by iron starvation and the inducer, ferric citrate (Braun *et al.*, 2006). Under iron-limiting conditions, the inactive Fur protein (iron uptake repressor protein) dissociates from *fec* gene promoter resulting in induction of *fecI fecR* transcription (Braun et al., 2006). When ferric citrate is present, it binds to the outer membrane protein FecA, which serves as a signal receiver that activates *fecI fecR* transcription (Angerer *et al.*, 1995). FecR is required to activate FecI and FecI-RNAP interaction directs transcription from the *fecA* promoter (Angerer & Braun, 1998, Braun et al., 2006).

In summary, although most transcription in *E. coli* is initiated by RNAP holoenzyme containing σ^{70}, there are six other σ factors, and each recognizes a different set of promoters having a primary structure that is recognized by a specific sigma subunit. σ^S plays a crucial role in stationary phase or under stress conditions. σ^{32} controls heat shock promoters. σ^F is the master regulator of the flagellar genes. σ^E is induced by extracytoplasmic stresses and σ^{fecI} is used for promoters involved in iron transport.

There are at least 17 known alternative σ factors in *B. subtilis*, seven of which are members of the extracytoplasmic function (ECF) subfamily (Haldenwang, 1995, Kunst *et al.*, 1997). Vegetative cell σ factors are σ^A, σ^B, σ^C, σ^D, σ^H and σ^L whereas, alternative σ factors involved in the sporulation process are σ^E, σ^F, σ^G, σ^K (Haldenwang, 1995). The seven putative ECF sigma factors (SigV, SigW, SigX, SigY, SigZ, SigM and YlaC) contribute to genetic control in response to changes in the extracellular environment that lead to different kinds of stress. (Horsburgh & Moir, 1999, Kunst et al., 1997, Yoshimura *et al.*, 2004). In many cases, the gene encoding the ECF sigma factor is co-transcribed with one or more negative regulatory genes whose products function as anti-sigma factors by binding with the ECF sigma factor through its N-terminal region (Horsburgh & Moir, 1999, Yoshimura et al., 2004). In the remaining paragraphs of this section, only sigma factors related to the studies in this thesis will be described.

The σ^B regulon plays an important role in general stress response induced by heat, salt or ethanol stress (Petersohn *et al.*, 2001), and in some ways is a functional analogue of *E. coli* σ^S. Under non-stress conditions, σ^B is bound to anti-sigma factor RsbW, which prevents σ^B binding to core RNAP. (Benson & Haldenwang, 1993, Dufour & Haldenwang, 1994). The activation of σ^B requires the dephosphorylation of an antagonist protein, RsbV, which bind to RsbW, thereby forming an RsbV-RsbW complex, thus blocking anti-sigma- σ^B interaction. Free σ^B then can interact with RNAP leading to the transcription of σ^B- controlled genes under stress conditions (Voelker *et al.*, 1996, Haldenwang, 1995).

The ECF σ factor σ^M, encoded by the *sigM* (*yhdM*) gene, is activated in response to ethanol, heat, acid, superoxide stress and antibiotics that affect cell wall synthesis and essential for survival in media containing high salt concentration (Horsburgh & Moir, 1999, Cao *et al.*, 2005, Thackray & Moir, 2003). The expression of σ^M is maximal during early and mid exponential growth (Horsburgh & Moir, 1999). The *sigM* gene is cotranscribed with downstream genes *yhdL* and *yhdK,* which negatively regulate SigM activity (Horsburgh & Moir, 1999, Thackray & Moir, 2003). The N-terminal region of YhdL, the putative anti-sigma factor, interacts with SigM, whereas YhdK appears to interact with the trans-membrane domain of YhdL (Yoshimura et al., 2004). YhdK

binding to YhdL might provide the right conformation of YhdL for the anti-sigma function. The expression of *sigM* is positively autoregulated with transcription initiating from two promoters, P_M (σ^M-dependent promoter) and P_A (σ^A-dependent promoter) (Horsburgh & Moir, 1999). Sequence comparisons of the *sigM*, *sigW* and *sigX* promoter sites recognized by σ^M σ^W and σ^X respectively reveal similarities within their -35 elements, but these promoters contain different nucleotide sequences within the -10 elements (Horsburgh & Moir, 1999, Huang *et al.*, 1998a). The -10 element of P_M promoter contains the sequence CGTG whereas -10 element of CGTA and CGAC can be recognized by σ^W and σ^X respectively (Horsburgh & Moir, 1999, Huang et al., 1998a, Jervis *et al.*, 2007).

The σ^W, encoded by *sigW*, is modulated by a specific anti-sigma factor RsiW, encoded by *rsiW* (*ybbM*), which resides immediately downstream of *sigW* (Schobel *et al.*, 2004, Yoshimura et al., 2004). The σ^W-controlled genes encode products that function in detoxification and protection against antimicrobials (Cao *et al.*, 2002b, Butcher & Helmann, 2006). The activity of σ^W is controlled by three proteolytic steps of RsiW degradation resulting in the increase of free σ^W concentration and activation of the σ^W regulon (Schobel et al., 2004). The extracytoplasmic portion of RsiW undergoes proteolytic processing triggered by environmental stresses such as alkaline shock, and a truncated form of RsiW is further cleaved by second step catalyzed by the YluC intramembrane protease (Schobel et al., 2004). YluC cleaves RsiW and releases RsiW along with a cryptic proteolytic tag from the membrane, leading to the third proteolytic step by ClpXP, which results in the release of σ^W and transcription of σ^W-control genes (Frees *et al.*, 2007, Zellmeier *et al.*, 2006). The mechanism of the *B. subtilis* cell wall stress response mediated by σ^W/RsiW is similar to the mechanism of the *E. coli* extracytoplasmic stress response mediated by σ^E/RseA, although they are triggered by different stresses (Alba et al., 2002, Schobel et al., 2004). The role of the cytoplasmic part of RsiW as an inhibitory domain to prevent σ^W-controlled transcription in *B. subtilis* is in contrast to FecR, in which the cytoplasmic domain activates σ^{fecI}-mediated transcription in *E.* coli (Braun et al., 2006, Schobel et al., 2004).

1.2   REGULATION OF THE DEFENSES AGAINST OXIDATIVE STRESS

Bacteria possess defense systems against many threats, including oxidative stress, which is caused by exposure to reactive oxygen species (ROS) such as superoxide anion ($O_2\bullet^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO\bullet$) (Fig. 1.2) (Kiley & Storz, 2004, Storz & Imlay, 1999). Generation of reactive oxygen species can lead to damage of nucleic acids and cell membranes, as well as the inactivation of proteins (Imlay, 2002). In order to detect and respond to elevated levels of reactive oxygen species, cells employ redox-sensing transcription factors to regulate expression of antioxidant genes (Green & Paget, 2004, Zheng & Storz, 2000, Demple & Amabile-Cuevas, 1991).

Oxidative stress caused by encounters with reactive oxygen species is a common threat to all aerobic organisms. Cells respond to oxidative stress by expressing enzymes that detoxify the reactive oxygen species and repair the damage caused by those ROS. In many bacteria, superoxide dismutase (SOD) converts superoxide anions to H_2O_2 and O_2, whereas, H_2O_2 can be depleted by catalase or by glutathione peroxidases (Fig. 1.2) (Barford, 2004, Henle & Linn, 1997, Imlay, 2002). Oxidized thiols such as disulfide bonds within proteins can be restored to their thiol (SH) or thiolate ($-S^-$) state by thiol reductases, glutathione and thioredoxin (Paget & Buttner, 2003, Masip *et al.*, 2006). Oxidized glutathione and thioredoxin are products of substrate protein reduction, and are themselves reduced by glutathione reductase and thioredoxin reductase, respectively. These enzymes utilize the reducing power of NADPH to maintain the reduced state of glutathione and thioredoxin (Masip et al., 2006). Molecular chaperones, production of which is induced by the general stress response, are employed to mediate the refolding and degradation of unfolded and aggregated protein that accumulate after exposure to ROS (Barford, 2004, Graumann *et al.*, 2001).

1.2.1   Sources of substances causing oxidative stress

Protein cysteines are maintained in their thiol ($-SH$) or thiolate ($-S^-$) state in the highly reducing environment in cytoplasm. Therefore stable disulfide bonds rarely form (Ruddock & Klappa, 1999). However, the production of ROS that are capable of

modifying cysteine thiols are an inevitable by-product of aerobic metabolism (Henle & Linn, 1997, Paget & Buttner, 2003, Storz & Imlay, 1999). In addition to ROS formed by endogenous metabolism, exposure to exogenous oxidants, such as redox-active compounds and antibiotics secreted by plants and some microorganisms in order to suppress the growth of competitors, contribute to the intracellular formation of ROS (Imlay, 2002 , Kiley & Storz, 2004).

Superoxide anion ($O_2\bullet^-$) is a by-product of O_2 reduction in the electron transport chain (Fig. 1.2) (Green & Paget, 2004, Henle & Linn, 1997, Imlay, 2002). The superoxide anion attacks enzymes containing [4Fe-4S] clusters, which results in the release of iron and loss of enzyme activity. The reduced iron ions (Fe^{2+}) released from the enzymes react with H_2O_2 to yield the highly reactive hydroxyl radical (HO$\bullet$) by the Fenton reaction (Green & Paget, 2004, Henle & Linn, 1997, Imlay, 2002). Superoxide anion increases the free iron pool by releasing iron from [4Fe-4S] clusters resulting in hydroxyl radical-mediated DNA damage (Keyer *et al.*, 1995, Keyer & Imlay, 1996). Hydroxyl radical is a powerful oxidizing agent that targets all of the major macromolecules of cells such as RNA, DNA, protein and lipids, whereas superoxide and H_2O_2, while causing protein damage, are not strong enough oxidants to damage DNA (Imlay, 2002).

H_2O_2 is known to promote oxidative DNA damage by reacting with free iron (Fe^{2+}) to form HO$\bullet$ via Fenton reaction (Keyer et al., 1995, Keyer & Imlay, 1996). H_2O_2 is formed by spontaneous or enzyme-catalyzed reactions of HO$\bullet$ (Henle & Linn, 1997). H_2O_2 oxidizes the thiol side-chain of cysteine to form several different redox states such as disulfide and sulfenic acid to affect protein activity and structure (Graumann et al., 2001, Green & Paget, 2004, Paget & Buttner, 2003). DNA-binding activity can be regulated by the reaction of H_2O_2 to a metal center of the protein (Graumann et al., 2001, Herbig & Helmann, 2001). Most bacteria are killed by addition of 20 mM H_2O_2, addition of 50 μM H_2O_2 causes *E. coli* to suspend growth until the H_2O_2 is scavenged (Imlay, 2002).

There are many chemical compounds that induce oxidative stress in the cells. Paraquat, a $O_2\bullet^-$ generating agent, is an herbicide and has been used to analyze global gene expression of *B. subtilis* in response to oxidative stress (Helmann *et al.*, 2003a,

Mostertz, 2004 #1498). Treatment of the cells with the organic hydroperoxide, cumene hydroperoxide (CHP) results in the oxidation of a cysteine residue, to cysteine-sulfenic acid derivative that inactivates the protein (Fuangthong *et al.*, 2001).

Disulfide bonds play a major role in regulating protein function. Diamide [diazenedicarboxylic acid bis(N,N-dimethlylamide)], a specific oxidant of thiols, has been widely used to induce disulfide stress in the *B. subtilis* (Leichert *et al.*, 2003). Diamide changes the oxidative state of the thiols through a two-step mechanism. The first reaction occurs by adding thiolate anion (R-S⁻) to the diazene double bond to form sulfenylhydrazine (Kosower & Kosower, 1995, Leichert et al., 2003). The second thiolate reacts with sulfenylhydrazine to yield the end products disulfide bond and a hydrazine derivative in the second reaction (Kosower & Kosower, 1995, Leichert et al., 2003). The studies of the effects of diamide treatment on growth and survival in *B. subtilis* showed that diamide concentration up to 2 mM inhibits cell growth, a 10 mM concentration causes cell lysis and 1 mM concentration exhibits growth inhibition although does not affect viability (Leichert et al., 2003). Cells recover growth shortly after diamide has been consumed from the media (Leichert et al., 2003). Diamide treatment thus causes reversible oxidative thiol modifications, which also allow the study of cellular functions altered by a temporary perturbation of the thiol status in the normal cells.

Aromatic organic compounds are important industrial chemicals that have many applications and are widely present in the environment ranging in size from low-molecular-mass compounds, such as phenols, to biopolymers, such as lignin. (Oikawa *et al.*, 2001, Vardar & Wood, 2004, Vaillancourt *et al.*, 2006). Microorganisms are able to utilize aromatic compounds as the sole carbon and energy source by catabolic pathway to degrade each type of aromatic compound through anaerobic and aerobic strategies (Pessione *et al.*, 1996, Vaillancourt et al., 2006). Various intermediates including catechol (CAT) and hydroquinone occur during the degradation of monocyclic compounds which are the substrate for ring cleavage reactions by aerobic catabolism (Vaillancourt et al., 2006). Catechol is a major metabolite of benzene (Cavalieri *et al.*, 2002, Vaillancourt et al., 2006). It occurs in the degradation of benzene, phenol, benzoate, nitrobenzene and derivatives (Giuffrida *et al.*, 2001, Vardar & Wood, 2004, Tao *et al.*, 2004, Vaillancourt et al., 2006). Oxidation of catechol generates reactive oxygen species

(Cavalieri et al., 2002, Oikawa et al., 2001). It has been reported that oxidation of CAT generates electrophilic intermediates that form covalent adducts with cellular macromolecules, including DNA (Cavalieri *et al.*, 1997). The global gene expression profiles in response to CAT and antimicrobial compound 2-methylhydroquinone (2-MHQ) in *B. subtilis* using proteome and transcriptome analyses showed the induction of the thiol-specific oxidative stress response as well as the nitroreductase encoding *yodC* gene (Duy N.V., 2007, Tam *et al.*, 2006b). The phenolic compound 2-MHQ is ganomycin-related substance (farnesyl hydroquinones) which was isolated from the basidiomycete *Ganoderma pfeifferi* and exhibited antimicrobial activity against several Gram-positive and Gram-negative bacteria such as *B. subtilis*, *S. aureus*, *M. flavus* and *P. mirabilis* (Mothana *et al.*, 2000). 2-MHQ is derivative of hydroquinone which is metabolite of benzene (Vardar & Wood, 2004). It is also an intermediate in the degradation of nitroaromatic compound fenitrothion by *Burkholderia* sp. (Tago *et al.*, 2005). The bacterial metabolism of nitroaromatic compounds yields the common hydroquinone formation and its derivatives (Tago et al., 2005, Vaillancourt et al., 2006). In addition, *Pseudomonas* sp. and *E. coli* can oxidize toluene to cresol and cresol is hydroxylated to form 2-MHQ (Tao et al., 2004, Vardar & Wood, 2004). The oxidation of 2-MHQ also generates reactive oxygen species (Murata *et al.*, 1999).

Antibacterial compound nitrofurantoin is effective against most common Gram-positive and Gram-negative urinary tract pathogenic bacteria (Chamberlain, 1976). Its precise mode of action is unclear, however, it is known to inhibit the actions of a number of bacterial enzymes including inhibiting protein synthesis by nonspecific reactions with both ribosomal protein and rRNA (McOsker & Fitzpatrick, 1994). Proteomic analysis of *B. subtilis* in response to nitrofurantoin showed similar protein pattern to that of diamide (Bandow *et al.*, 2003). This suggests that nitrofurantoin mediates the *in vivo* formation of oxidized thiol groups in cytoplasmic proteins in *B. subtilis* as observed by diamide (Hochgrafe *et al.*, 2005). In addition, the isolation of nitrofurantoin-resistant mutants *Clostridium* sp strains from the human intestinal tract showed higher nitroreductase activities than did the corresponding parental wild-type strains (Rafii & Hansen, 1998). In rat liver mitochondria, nitroreductases convert nitrofurantoin to highly reactive electrophilic intermediates (McOsker & Fitzpatrick, 1994, Moreno *et al.*, 1984).

1.2.2 Redox-sensing and transcriptional control

The importance of thiol modifications of a protein's cysteine residues in redox sensing is illustrated in Fig. 1.2. The reaction of an ROS such as H_2O_2 with cysteinyl thiolates, which are major targets of H_2O_2, can lead to the formation of different modifications such as irreversible products sulfinic acid (-SO$_2$H), sulfonic acid (-SO$_3$H) (Fuangthong & Helmann, 2002). In addition, the oxidation by ROS promotes reversible redox state disulfide bond (-S-S-) formation, either intramolecular or intermolecular cysteine disulfide bond formation and mixed disulfides between cysteine-containing protein and a low molecular weight thiol (-S-S-R′). Disulfides can be reduced by a cellular reductant (R-SH$_2$) such as thioredoxin to generate the reduced form of the protein (Green & Paget, 2004, Kiley & Storz, 2004, Paget & Buttner, 2003). Cysteine residues can also be modified by reactive nitrogen species (RNS) such as nitric oxide (NO) to yield an S-nitothiol (R-SNO), and by peroxynitrite (NO$_3$⁻) to yield an S-nitrothiol (R-SNO$_2$) (Paget & Buttner, 2003).

The oxidation of cysteine or modifications of cysteine residues can serve as signals that induce specific cellular responses (Barford, 2004, Kiley & Storz, 2004). The thiols of a protein's cysteine residues can act as "redox switches" to sense changes in ROS concentration and to trigger signal transduction pathways leading to the induction of gene expression. There are two kinds of thiol-based regulatory proteins: those requiring metal cofactors and those with no metal involvement. In this chapter, oxidative stress by ROS will be mainly discussed.

1.2.2.1 Redox-sensing with no metal involvement. As mentioned above, the thiol of a cysteine residue is a primary target for oxidation that leads to a response to oxidative stress. The reversibility of most forms of thiol oxidations often plays a role as a mechanism for controlling protein activity. There are several proteins whose activities are modulated by thiol oxidation and reduction without metal involvement. The examples of these proteins: OxyR, a sensor for peroxide stress, OhrR, a sensor modulated by sulfenic acid formation and Spx, a sensor for disulfide stress, will be discussed in detail.

1.2.2.1.1 OxyR. Protection against oxidative stress in *E. coli* is mediated in part by the OxyR transcription factor that activates the expression of antioxidant defensive genes and genes involved in control of free iron concentration. Among members of the OxyR regulon are *katG* (hydroperoxidase I), *ahpCF* (an alkylhydroperoxide reductase), *oxyS* (a regulatory RNA involved in DNA repair), *gorA* (glutathione reductase), *grxA* (glutaredoxin 1), *fur* (ferric uptake regulator), *dps* (iron storage protein) and *yhjA* (predicted cytochrome c peroxidase) (Aslund *et al.*, 1999, Zheng & Storz, 2000, Partridge *et al.*, 2007). Ahp, an NAD(P)H-dependent peroxidase, scavenges a very low concentration of H_2O_2, whereas the catalase is more effective at higher concentrations (Seaver & Imlay, 2001). Grx1 (glutaredoxin 1) deactivates OxyR by disulfide bond reduction, providing a mechanism for autoregulation of OxyR activity (Zheng *et al.*, 1998). Free intracellular iron catalyses DNA damage via the Fenton reaction. Hence, the *fur* gene must be induced by OxyR to regulate the levels of intracellular iron (Varghese *et al.*, 2007).

OxyR is a LysR-type transcriptional regulator which positively controls its target genes in response to hydrogen peroxide and negatively regulates its own expression (Kim *et al.*, 2002, Kullik *et al.*, 1995). Mutational analysis suggested that the N-terminal helix-turn-helix motif serves as a DNA-binding domain in OxyR and the C-terminal region of OxyR functions in tetramerization (Kullik et al., 1995). The tetrameric form of oxidized OxyR binds to its target promoters where activated OxyR stimulates transcription by interacting with the carboxy-terminal domain of the RNAP α-subunit (Toledano *et al.*, 1994).

There are six cysteine residues in OxyR, but residues Cys119 and Cys208 are critical for optimal transcriptional activation (Aslund et al., 1999, Zheng et al., 1998). Cys199 and Cys208 form an intramolecular disulfide bond leading to the conformational change that activates the OxyR transcription factor upon oxidation by H_2O_2 (Zheng et al., 1998). The oxidation of Cys199 to –SOH is proposed to be the first step in OxyR activation (Zheng et al., 1998). Grx1 is responsible for the reduction of oxidized OxyR at the expense of glutathione (GSH) (Zheng et al., 1998). Moreover, thiol modifications can also occur, giving rise to different modified forms of OxyR, such as OxyR-SNO and OxyR-SSG when the reduced form of OxyR is treated with S-nitrosoglutathione and

glutathione disulfide respectively (Kim et al., 2002). All modified forms can be transformed back to reduced OxyR by DTT in vitro (Kim et al., 2002). Thus, OxyR can process different thiol modifications into distinct transcriptional responses (Kim et al., 2002).

In addition to oxidative stress by H_2O_2, diamide and nitrosative stress also activate OxyR (Kim et al., 2002, Zheng et al., 1998). Although OxyR mediates a primary response to oxidative stress, OxyR does not appear to mediate a major response to nitrosative and disulfide stress (Zheng et al., 1998).

1.2.2.1.2 OhrR. In *B. subtilis*, there are at least four transcription factors involved in response to oxidative stress: The alternative RNAP sigma factor σ^B, PerR, Spx and OhrR (Fuangthong et al., 2001, Mostertz *et al.*, 2004, Herbig & Helmann, 2001, Nakano *et al.*, 2003a). This section is concerned with OhrR, which regulates genes specifically in response to organic hydroperoxides such as tert-butyl hydroperoxide and cumene hydroperoxide (CHP) (Fuangthong et al., 2001).

ohrR encodes an organic peroxide sensing repressor (OhrR) which is a member of MarR family of transcription regulators (Fuangthong et al., 2001). OhrR binds to a perfect inverted repeat sequence, TACAATT-AATTGTA, upstream from *ohrA*, encoding thiol-dependent peroxidase that detoxifies organic hydroperoxides (Fuangthong et al., 2001). OhrR contains a single conserved cysteine residue (Cys15), which is required for the redox-sensing mechanism of OhrR but is not required for DNA binding (Fuangthong & Helmann, 2002). The structure of OhrR reveals that C15, positioned at the N terminus of helix 1, is hydrogen bonded to Tyr29 and Tyr40, which stabilizes the reduced form of OhrR (Hong *et al.*, 2005). OhrR senses oxidants through the formation of a reversible cysteine sulfenic acid-containing intermediate (C15-SOH) that retains DNA-binding activity and further generates either a mixed disulfide (S-thiolation) or a protein sulfenamide (sulfenyl-amide) derivative formed with a backbone amide, thereby leading to release from the DNA target, and derepression of *ohrA* transcription (Fuangthong & Helmann, 2002, Lee *et al.*, 2007). Since the intermediate form of OxyR (Cys-SOH) is further oxidized to disulfide bond formation, OhrR Cys-SOH , with only one Cys residue, might react with low molecular weight (LMW) intracellular thiols to form mixed

disulfides (Lee et al., 2007, Zheng et al., 1998, Fuangthong & Helmann, 2002). *B. subtilis* lacks glutathione but produces other LMW thiols such as cysteine (Cys) and reduced CoA (CoASH) (Newton *et al.*, 1996). In the presence of LMW thiols, the major sulfenic acid reactant is formed after exposure to CHP then rapidly converted to either the S-thiolated or sufenamide products (Lee et al., 2007). In the absence of Cys, the OhrR sulfenate is converted to cyclic sulfenamide derivative (Barford, 2004, Lee et al., 2007).

1.2.2.2 Redox-sensing proteins containing metals. The cysteine residue combines catalytic activity with an extensive redox chemistry and unique metal-binding ability (Barford, 2004). Several metal-bearing, redox-sensing proteins mediate gene regulation in response to oxidative stress. This section will focus on the molecular mechanisms of by three well-characterized metalloproteins that regulate gene expression or protein activity in response to oxidative stress. In *E. coli*, Hsp33 is a chaperone with redox-sensing capability that involves a zinc-binding domain. The RsrA anti-sigma factor contains zinc which plays a role in disulfide stress regulation in *Streptomyces coelicolor*. Finally, the peroxide sensing repressor PerR requires a metal ion to control the expression of peroxide resistance genes in *B. subtilis*.

1.2.2.2.1 Hsp33. Heat shock protein 33 (Hsp33) functions as either (i) a molecular chaperone, which assists protein folding by interacting with unfolded or partially folded proteins, thereby preventing non-productive folding or aggregation processes, or (ii) as a protease that degrades damaged proteins (Jakob *et al.*, 1999, Ruddock & Klappa, 1999). Exposure of the cell to stress conditions induces many molecular chaperones through increased transcription, however, heat shock protein Hsp33 activity is regulated at both the transcriptional and translational level in *E. coli* (Jakob et al., 1999).

Hsp33 is localized in the reducing environment of the cytoplasm of *E. coli*, in which the reduced Hsp33 is in an inactive form (Jakob et al., 1999, Winter & Jakob, 2004). The level of Hsp33 mRNA increases about 3 to 8 fold after heat treatment (Jakob et al., 1999), while its activity is also shown to be regulated by the redox switch (Jakob et al., 1999).

Hsp33 contains four conserved cysteines near the C terminus within the redox switch domain. The Cys residues are arranged in a $C_{232}XC_{234}$ and $C_{265}XXC_{268}$ motif (Ilbert *et al.*, 2007, Jakob et al., 1999). The Cys cluster is required for zinc binding (Jakob *et al.*, 2000), which stabilizes Hsp33 by protecting it from proteolytic digestion. Zinc coordination is also required for the activation process of Hsp33 by oxidative stress (Jakob et al., 2000). Unfolding of a linker region which connects the N-terminal substrate binding domain with the redox-sensitive zinc center is also important for protein activation (Ilbert et al., 2007).

Under reducing conditions, Hsp33 appears to act as a monomer in which the reactive cysteine residues coordinate a zinc atom (Ilbert et al., 2007, Jakob et al., 1999, Jakob et al., 2000). Severe oxidative stress causes zinc release and the nearby four conserved cysteines oxidize to form two intramolecular disulfide bonds connecting C_{232} with C_{234} and C_{265} with C_{268} (Graumann et al., 2001, Barbirz *et al.*, 2000, Jakob et al., 2000). This leads to the unfolding of the C-terminal redox switch domain and protein retains partial chaperone activity (Graumann et al., 2001). Heat stress promotes dimerization of oxidized Hsp33 monomers thereby accelerating Hsp33 activity (Graumann et al., 2001, Ilbert et al., 2007). The activation of Hsp33 involves conformational changes in two distinct regions, the zinc-redox center located at C-terminal redox switch domain that senses peroxide stress conditions and an adjacent linker region responding to heat stress (Ilbert et al., 2007). Thus, both oxidative stress and heat stress are required for full activation of the redox-regulated chaperone Hsp33 (Ilbert et al., 2007).

In summary, monomeric Hsp33 is reduced and inactive under non-stress conditions. Four conserved cysteine residues are present in the C-terminal redox switch domain that coordinates one zinc atom. Under oxidative stress, zinc is released and two intramolacular disulfide bonds are formed resulting in a conformational change. Heat stress stimulates oxidized Hsp33 to dimerize by unfolding the linker region to form a highly active chaperone. Thus, oxidation triggers Hsp33 dimerization and dimerization is stimulated by heat.

1.2.2.2.2 σ^R-RsrA. The thioredoxin system plays a major role in the response to changes in the cytoplasmic thiol-disulfide status in the Gram-positive antibiotic producing bacterium *Streptomyces coelicolor* (Paget *et al.*, 1998, Zeller & Klug, 2006). Transcription of *trxBA*, encoding thioredoxin reductase and thioredoxin, is initiated from two promoters, *trxBp1* and *trxBp2*, which are induced by diamide treatment (Paget et al., 1998). The level of *trxBp1* transcript is induced under disulfide stress in a σ^R-dependent manner (Paget et al., 1998). σ^R is encoded by *sigR* gene, which is transcribed from two promoters, *sigRp1* and *sigRp2* (Paget et al., 1998). σ^R itself contains no cysteines and so does not function alone in redox sensing during disulfide stress (Paget et al., 1998).

RsrA (regulator of *sigR*) encoded by the *rsrA* gene lying immediately downstream of *sigR*, regulates σ^R-directed transcription in response to disulfide stress (Kang *et al.*, 1999). RsrA is an anti-sigma factor that binds directly to σ^R only in the reduced form. RsrA activity is modulated by oxidation and reduction of cysteine residues in the RsrA protein (Kang et al., 1999). RsrA contains seven cysteine residues and C11, C41 and C44 are essential for anti-sigma activity (Paget *et al.*, 2001). Although C11 is not absolutely conserved, His37, Cys41 and Cys44 are conserved and form a HisXXXCysXXCys motif (Kang et al., 1999, Paget et al., 2001), which functions in zinc co-ordination. RsrA, containing this HisXXXCysXXCys motif, belongs to the ZAS (zinc-binding anti-sigma factor) family of proteins (Paget et al., 2001).

Zn(II) coordinated to three cysteine residues (C3, C41 and C44) and one histidine residue (H7) was proposed by Bae et al. in their model for the single zinc-binding site in RsrA (Bae *et al.*, 2004). However, the model proposed by Li et al., which was confirmed by Zdanowski et al., predicts the single zinc atom in RsrA is coordinated by C11, H37, C41 and C44 (Li *et al.*, 2003, Zdanowski *et al.*, 2006). During oxidation, zinc is released and a disulfide bond is formed, which results in RsrA dissociation from σ^R leading to activation of its regulon (Kang et al., 1999, Li et al., 2003, Paget et al., 2001). C11 preferentially forms a disulfide linkage with C44 , however, this disulfide likely oxidizes other cysteines in the protein through thiol-disulfide exchange as disulfide bond formation between C41 and C61 is formed in addition to the C11-C44 linkage (Kang et al., 1999, Li et al., 2003).

In conclusion, the thiol disulfide status of *S. coelicolor* is monitored by a regulatory system consisting of a σ^R and anti-sigma factor RsrA. In the reducing environment, RsrA binds a single zinc atom and forms a complex with σ^R that represses transcriptional activity of σ^R. The zinc atom in RsrA is coordinated by C11, H37, C41 and C44. During disulfide stress, intramolecular disulfide bond formation takes place and zinc is expelled resulting in the dramatic structural changes in RsrA, causing RsrA to dissociate from σ^R. The free σ^R then is free to interact with core RNAP and activate transcription of its target genes, including *trxBA*, in response to disulfide stress.

1.2.2.2.3 PerR. *Bacillus subtilis* PerR is a metal-dependent regulator that senses oxidative stress. PerR is a member of the ferric uptake repressor (Fur) family and requires either Fe or Mn as a cofactor for repression (Herbig & Helmann, 2001). *B. subtilis* contains three Fur homologs: Fur represses genes when the intracellular concentration of Fe(II) exceeds a threshold level, Zur represses Zn(II) uptake system and PerR regulates genes that function in alleviating oxidative stress (Helmann *et al.*, 2003b, Herbig & Helmann, 2001, Moore & Helmann, 2005). The PerR regulon includes KatA (the major vegetative catalase), AhpCF (alkyl hydroperoxide), ZosA (a zinc uptake P-type ATPase), HemAXCDBL (enzymes of heme biosynthesis), MrgA (a Dps-like DNA binding protein), Fur and PerR itself (Fuangthong *et al.*, 2002, Gaballa & Helmann, 2002, Helmann et al., 2003b).

The DNA-recognition sequence of Fur is bound by two dimers at the overlapping heptameric (7-1-7) core motifs (Baichoo & Helmann, 2002). Comparison of Per, Fur and Zur boxes in *B. subtilis* show a similarity in their consensus sequences with differences of only one or two bases at positions 5 and 6 in each half site (Fuangthong & Helmann, 2003, Moore & Helmann, 2005). Two nucleotide positions in the binding sites are critical for the ability of PerR, Fur and Zur to discriminate among the closely related operator sites (Fuangthong & Helmann, 2003).

Two overlapping Per box elements are present in the *perR* promoter region. The upstream Per box overlaps the -10 sequence of the *perR* promoter (Fuangthong et al., 2002). Primer extension analysis uncovered the *perR* transcription start site that initiates from a σ^A-type promoter (Fuangthong et al., 2002). PerR bound tightly to the *perR*

operator, with as little as 10 nM PerR protecting about a 25 bp region as shown by footprinting experiments (Fuangthong et al., 2002). In addition, the Per box is found in the *fur* regulatory region, which is the site of interaction between PerR and the *fur* operator sequence (Fuangthong et al., 2002).

PerR is a dimeric zinc-containing metalloprotein with a second regulatory metal-binding site that binds Fe^{2+} (PerR:Zn,Fe) or Mn^{2+} (PerR:Zn:Mn) (Herbig & Helmann, 2001). Under normal growth conditions, active PerR contains both zinc and iron (PerR:Zn,Fe) (Herbig & Helmann, 2001). PerR-regulated genes such as *mrgA*, *katA*, *zosA*, *ahpC*, *hemA*, *perR* and *fur*, are repressed in media supplemented with Mn(II) but lacking Fe(III) and the expression of those genes show little change in expression upon exposure to H_2O_2 (Fuangthong et al., 2002). In contrast, in media supplemented with Fe(III), the expression of *mrgA*, *katA* and *zosA* promoters are greatly induced by H_2O_2 whereas *ahpC* and *hemA* promoters are weakly induced by H_2O_2, while *perR* and *fur* promoter are rarely induced (Fuangthong et al., 2002). Thus, the iron-containing form of PerR is sensitive to as little as <10 μM H_2O_2, while the manganese form of PerR is relatively insensitive to H_2O_2 (Herbig & Helmann, 2001).

PerR contains four cysteine residues (C96, C99, C136 and C139) located in the C-terminal domain to form the high affinity Zn^{2+} binding site (Cys_4Zn), which is important to stabilize the dimeric form of the protein (Traore *et al.*, 2006, Herbig & Helmann, 2001). The Cys_4Zn site is not involved in sensing low levels of H_2O_2, however, intramolecular disulfide bonds between cysteine residues in the two CXXC motifs occur under severe oxidative stress conditions (10 mM H_2O_2) *in vitro*(Lee & Helmann, 2006a). PerR:Zn is inactive and does not bind a DNA fragment containing the Per box of the *mrgA* promoter both *in vivo* and *in vitro* (Lee & Helmann, 2006b). The low micromolar range of Mn^{2+} and even lower concentrations of Fe^{2+} activate DNA binding activity of PerR:Zn (Lee & Helmann, 2006a). Although the purified PerR:Zn can be reconstituted with Fe^{2+} or Mn^{2+} to form an active form which binds to *mrgA* Per box, Mn^{2+} is unable to restore DNA-binding activity to oxidized PerR (Lee & Helmann, 2006b).

Five residues (H37, D85, H91, H93 and D104), conserved among the PerR-like family of proteins are likely to be a binding site for the regulatory metal ion, Fe^{2+} or Mn^{2+} (Herbig & Helmann, 2001, Traore et al., 2006). Under aerobic conditions, addition of

Fe^{2+} to PerR:Zn results in inactivation of the protein; PerR is thus sensitive to metal-catalysed oxidation (MCO) by bound Fe^{2+} (Lee & Helmann, 2006b). Two residues H37 and H91 are further predicted to coordinate Fe^{2+} since oxidation of either H37 or H91 is sufficient for derepression (Lee & Helmann, 2006b). Oxidation of PerR is proposed to form 2-oxo-histidine residues, which are irreversible modifications (Lee & Helmann, 2006b).

In summary, the transcription repressor PerR regulates genes in response to oxidative stress and contains two metal centers. PerR contains a structural Zn^{2+}-binding site, which involves coordination of Zn by four conserved Cys residues (Cys_4Zn), and a regulatory metal binding site that contains either Fe^{2+} or Mn^{2+} (Herbig & Helmann, 2001, Lee & Helmann, 2006a, Traore et al., 2006). PerR:Zn is unable to recognize the Per box whereas PerR:Zn,Fe binds to the target genes and is sensitive to oxidation (Herbig & Helmann, 2001, Lee & Helmann, 2006b). Under harsh oxidative stress conditions, two intramolecular disulfide bonds are formed at the Cys_4Zn site leading to inactivation of PerR in its Zn,Mn-bound form (Lee & Helmann, 2006a, Lee & Helmann, 2006b). Oxidation of PerR:Zn,Fe is mediated by incorporation of an oxygen atom into a histidine residue (either H37 or H91), which is bound to Fe^{2+}, to form 2-oxo histidine (Lee & Helmann, 2006b). The free iron level is elevated under oxidative stress leading to exchange from the PerR:Zn,Mn form to peroxide sensitive PerR:Zn,Fe form (Keyer & Imlay, 1996, Lee & Helmann, 2006a). Thus, the PerR regulatory mechanism is mediated by metal-catalyzed oxidation in response to oxidative stress.

1.3   SPX-DEPENDENT TRANSCRIPTIONAL REGULATION AGAINST OXIDATIVE STRESS

The Spx protein of *B. subtilis* is a unique RNAP-binding protein that is highly conserved among low G-C-content gram-positive bacteria such as *Oceanobacillus*, *Listeria*, *Staphylococcus*, *Enterococcus*, *Lactococcus* and *Streptococcus* (Nakano *et al.*, 2003b, Zuber, 2004). The *spx* (suppressor of *clpP* and *clpX*) gene (formally *yjbD*) was first identified in *B. subtilis* to be the site of mutations that could suppress the defect in competence caused by *clpP* and *clpX* mutations (Nakano *et al.*, 2001). ClpXP, an ATP

dependent protease, is composed of a regulatory/substrate-binding subunit ClpX and a serine/threonine protease ClpP (Gottesman, 1999). ClpX and ClpP are required for regulated transcription in competence development and sporulation (Nakano et al., 2001, Nakano *et al.*, 2000b). Mutations in *clpX* and *clpP* have additional pleiotropic effects on developmentally regulated gene expression as well as causing severe growth impairment on certain media such as Luria-Bertani (LB) and competence media (CM) (Nakano et al., 2000b). Many phenotypes associated with mutations in *clpXP* can be alleviated by either elimination of Spx or by missense mutations in the *rpoA* gene that encodes the RNAP α C-terminal domain (Nakano et al., 2001, Nakano et al., 2000b).

ClpX and ClpP are also required for transcription of the *srf* operon, which encodes surfactin synthetase and the essential competence regulatory gene *comS* (D'Souza *et al.*, 1994, Hamoen *et al.*, 1995, Nakano et al., 2000b). The small protein ComS mediates environmental signaling by ComX pheromone and the two component regulatory system, ComPA, for competence development (Magnuson *et al.*, 1994, Weinrauch *et al.*, 1990). ComK, the competence transcription factor, forms a complex with the protease, ClpCP via the adapter protein MecA ; these interactions are eliminated by ComS, thereby freeing ComK, which then activates its own transcription as well as expression of late competence operons (Schlothauer *et al.*, 2003, Turgay *et al.*, 1998). Recent studies reported that PerR binds directly to a Per box upstream of the ComA boxes of the *srf* promoter and PerR exerts positive control over *srf* expression under non-stress conditions (Hayashi *et al.*, 2005).

A suppressor mutation of *clpX* , *cxs-1*, results in a change of Tyr263 to Cys in the C-terminal domain of RNAP α subunit (αCTD) which is able to suppress a *clpP* mutation with respect to *srf* transcription, but fails to suppress *clpP* with respect to competence development (Nakano et al., 2000b). The Spx protein accumulates to high concentration in *clpX* and *clpP* mutants but not in *clpC* mutant, thus Spx is a substrate for ClpXP catalyzed proteolysis (Fig. 1.3) and the accumulation of Spx has a negative effect on *srf* transcription (Nakano et al., 2001, Nakano *et al.*, 2002b). However, Spx is also a substrate for degradation by ClpCP, which requires the ClpC adapter proteins, MecA or YpbH in vitro (Nakano et al., 2002b). Spx forms a complex with ClpC, MecA and ComK and enhances ComK binding to ClpC-MecA in the presence of ComS (Nakano *et al.*,

2002a). When Spx and ClpP are absent, competence and sporulation are completely and partially restored, respectively (Nakano et al., 2002b).

In addition to *cxs-1*, *spx*[cxs-16], which confers an amino acid substitution at the conserved Gly52 residues (G52R) of the Spx protein, can suppress *clpX* mutation with respect to *srf* expression (Nakano et al., 2001, Nakano et al., 2003b). By western blot analysis, the Spx[cxs-16] protein is abundant in a *clpX* mutant, much like what is observed in the case of the Spx wild-type protein. Both mutant and wild-type Spx are substrates for the ClpXP protease (Nakano et al., 2003b, Nakano et al., 2002b). As mentioned above, transcription initiation of *srf* operon requires an activator ComA in addition to ClpXP protease which functions to degrade Spx protein (Gottesman, 1996, Nakano et al., 2000b). Either *spx* mutation (loss of function mutation or missense mutation *spx*[cxs-16]) or *cxs-1* which is a mutation in RNAP α-subunit can suppress the defect in competence development caused by *clpXP* mutations (Nakano et al., 2001, Nakano et al., 2000b). These data suggested that Spx exerts its negative effect on ComA-dependent transcription by interaction with αCTD of RNAP (Nakano et al., 2003b). The interaction between Spx and the α subunit of RNAP was confirmed by yeast two-hybrid analysis that showed interaction between RpoA and Spx, whereas RpoA was unable to interact with Spx[cxs-16]. Mutant RpoA[cxs-1], bearing the Y263C substitution in the αCTD, did not interact with WT Spx (Nakano et al., 2003b). The structure analysis also confirmed that the residue Y236 of the α subunit RNAP is part of the RNAP-Spx binding interface (Newberry *et al.*, 2005).These findings indicated that Spx is a negative transcriptional regulator in *B. subtilis* that disrupts activator-dependent transcription initiation by direct interaction with the α-CTD (Nakano et al., 2003b, Zuber, 2004).

The monomeric 15-kDa Spx protein belongs to the ArsC (arsenate reductase) family of proteins and contains a four stranded mixed β-sheet that is surrounded by α-helices (Newberry et al., 2005, Zuber, 2004). The structure of Spx and αCTD uncovered the residues of Spx that interact with helix α1of αCTD including R47, G52, T53, D54, E72, L76, Q77 and Y80 (Newberry et al., 2005). However, the CXXC motif of Spx is not essential for the repressor activity of Spx (Zhang *et al.*, 2006). Moreover, alanine-scanning mutagenesis of αCTD revealed residue positions required for Spx function and

ComA-dependent *srf* transcription activation (Zhang et al., 2006). Two residues C265
and K267 which lie in the region around α1 of RNAP were further examined (Newberry
et al., 2005, Zhang et al., 2006). Diamide sensitivity experiment together with SPPR
(Solid-phase promoter retention) demonstrated that the residue K267 is important for
ComA and Spx interaction, whereas the residue C265 likely interacts with only ComA
(Zhang et al., 2006). The results indicated that Spx and ComA use overlapping
interaction surfaces on αCTD, supporting the interference model of Spx-dependent
repression.

1.3.1   Spx-RNAP interaction functions in the disulfide stress response.

To better understand the role of Spx-RNAP interaction, microarray analysis was
performed to identify genes that were induced or repressed in the IPTG-treated *spx^{DD}*
cells, but not in the IPTG-treated *spx^{DD}rpoA^{cxs-1}* cells (Nakano et al., 2003a). *spx^{DD}* is a
mutant allele of *spx* in which the terminal Ala and Asn codons were replaced by two Asp
codons. It encodes a stable product that is resistant to the protease ClpXP (Nakano et al.,
2003a). Almost 200 genes were repressed by Spx^{DD}-RNAP interaction, which included
all the genes of the *srf* operon and several operons whose products function in purine,
pyrimidine and other genes that are associated with normal growth and metabolism (Fig.
1.3) (Nakano et al., 2003a). Among more than 100 genes that were induced by Spx^{DD}-
RNAP interaction, two of the most strongly expressed genes were *trxA* (encodes
thioredoxin) and *trxB* (encodes thioredoxin reductase), that function in thiol-redox
homeostasis (Fig. 1.3) (Nakano et al., 2003a). Induction of genes by Spx^{DD}-RNAP
overlaps with the genomic expression profile induced by disulfide stress caused by
treatment with the azo-thiol reactive compound, diamide, as shown by DNA macroarray
and proteome analysis (Leichert et al., 2003). This finding suggested that Spx functioned
in controlling the cell's response to disulfide stress (Zuber, 2004).

The *spx* gene resides in an operon with *yjbC* (Fig. 1.3) and the synthesis of the
yjbC-spx operon transcript is induced by diamide treatment (Leichert et al., 2003, Nakano
et al., 2001). The Cys-X-X-Cys (CXXC) motif at the N-terminal end of Spx suggests the
presence of a redox-sensing site, which could play a role in sensing ROS (Zuber, 2004).
As mentioned above, Spx is present in very low concentration in unperterbed cells but the

level of Spx increases after diamide treatment as shown by western blot analysis. The failure of Spx interaction with RNAP results in a diamide sensitive phenotype (Nakano *et al.*, 2005, Nakano et al., 2003a). Primer extension analysis indicated that transcriptional activation of *trxA* and *trxB* and transcriptional repression of *srf* require Spx-RNAP interaction during disulfide stress (Nakano et al., 2003a). In addition, intramolecular disulfide bond formation within the CXXC motif is required for the transcriptionally active form of Spx. Spx is unusual among transcriptional regulators as Spx does not bind DNA (Nakano et al., 2005). Thus, Spx acts as a global transcription factor through its interaction with αCTD and exerts both positive and negative control over transcription initiation of multiple genes in response to disulfide stress.

1.3.1.1   Spx-dependent positive control of transcription initiation. Many genes that function in maintenance of thiol homeostasis are induced by Spx-RNAP interaction and by the treatment with thiol-specific oxidant diamide (Leichert et al., 2003, Nakano et al., 2003a). The expression of *trxA* and *trxB* is highly induced under both conditions (Leichert et al., 2003, Nakano et al., 2003a). Spx-dependent transcriptional activation requires an intramolecular disulfide bond within the CXXC motif, although Spx alone does not bind to the target promoter DNA (Nakano et al., 2005). Spx concentration increases after diamide treatment as shown by western blot analysis. In addition, Spx protein level detected from cells expressing either C10A or C13A mutant forms of Spx, which are unable to sense disulfide stress upon diamide treatment, shows no difference as compared with those of wild-type cells (Nakano et al., 2005). The mechanism by which Spx activates transcription initiation is not clear. Since oxidized Spx is required for transcription activation, Spx-RNAP interaction might be weakened under reducing conditions (Nakano et al., 2005). However, the structure of Spx suggested that Spx might interact with another RNAP subunit, in addition to α, to position RNAP optimally for its transcriptional activation under oxidizing conditions (Newberry et al., 2005). Preliminary data suggests that Spx also interacts with the β subunit of RNA polymerase in the vicinity of the β-flap domain (M. M. Nakano and P. Zuber, unpublished).

1.3.1.2 Spx-dependent negative control of transcription initiation. Spx is termed anti-α factor because it disrupts RNAP αCTD-activator interaction to repress gene expression (Nakano et al., 2003b). The aforementioned *srf* operon, which requires the activator ComA for transcription initiation, is negatively controlled by Spx-RNAP interaction and Spx-dependent repression is also enhanced under disulfide stress (Nakano et al., 2003a). *srf* expression is highly repressed by Spx-RNAP interaction, diamide and H_2O_2 treatment (Mostertz et al., 2004, Leichert et al., 2003, Nakano et al., 2003a). Under normal conditions, the activator ComA (in phosphorylated form) activates *srf* transcription initiation while Spx is controlled by ClpXP proteolysis (Gottesman, 1996, Nakano et al., 2000b). Upon oxidative stress, expression of *spx* is induced and Spx degradation is reduced, leading to accumulation of Spx protein which binds to αCTD, thereby interfering with ComA-RNAP interaction (Nakano et al., 2003a).

Moreover, Spx-RNAP interaction also negatively controls expression of another activator-dependent promoter, the *hmp* gene encoding flavohemoglobin (Nakano et al., 2003b). *hmp* is a ResDE-controlled gene and is induced under anaerobic conditions (LaCelle *et al.*, 1996). A response regulator and transcriptional activator ResD, a homologue of ComA, is part of the ResDE two-component signal transduction system required for the transcription of genes that are induced in response in oxygen limitation (Nakano *et al.*, 2000a). Spx acts to disrupt the formation of the ResD (in phosphorylated form)-RNAP-DNA complex, resulting in the dissociation of RNAP, although no inhibitory effect on RNAP-promoter DNA interaction is observed by Spx (Nakano et al., 2003b). Thus, Spx negatively regulatd activator-stimulated transcription by destabilizing a complex of RNAP and transcriptional activators without interfering with the RNAP-promoter DNA interaction.

1.3.2 Roles of Spx in the control of gene expression.

The microarray transcriptome analysis and supporting data have uncovered genes that are under global transcriptional control mediated by the Spx-RNAP interaction. In addtion to *trxA* and *trxB*, Spx-controlled genes include those that function in organosulfur metabolism: 1) genes such as *ytmI*, *yxeI* and *ssu* operons that function in the utilization of organosulfur compounds; 2) genes involve in cysteine biosynthesis such as *yrrT* operon

and *cysK*; 3) genes required for methionine biosynthesis such as *metE* and *metK* (Nakano et al., 2003a).

Spx negatively controls gene transcription in response to the presence of sulfate but not cysteine. These genes include *ssu*, *yxeI*, *ytmI* and *yrrT* operons (Erwin *et al.*, 2005, Nakano et al., 2003a). The repressor CymR (formerly YrzC) functions in sulfate-dependent negative control of the *ytmI*, *yxeI* and *ssu* operons, whose products participate in cysteine biosynthesis from alternative sulfur sources(Even *et al.*, 2006). CymR protein level requires the activity of Spx although, *cymR* is indirectly transcriptionally controlled by Spx. Spx has no effect on in vitro transcription from *cymR* promoter DNA (Choi *et al.*, 2006). Spx negatively controls of *ytmI*, *yxeI* and *ssu* operons through stimulation of *cymR* expression in a sulfate-dependent manner (Choi et al., 2006, Erwin et al., 2005).

The transcript levels of the *yrrT* operon and *cysK*, required for converting methionine to cysteine, increase by Spx-RNAP interaction as determined by microarray transcriptome analysis (Nakano et al., 2003a). In the absence of cysteine, *yrrT* expression is positively controlled by Spx but negatively controlled by CymR (Choi et al., 2006). Unlike *trxA*, *trxB* expression that requires oxidized form of Spx to activate transcription initiation, high levels of oxidized Spx appear not to be essential to stimulate *yrrT* transcription (Choi et al., 2006, Nakano et al., 2005). Thus, *yrrT* is a member of *spx* regulon and under the direct positive control by Spx when methionine is the sole sulfur source.

Spx also plays important roles for growth, general stress protection and biofilm formation in the Gram-positive pathogen bacterium *Staphylococcus aureus* (Pamp *et al.*, 2006). The Ingmer group has demonstrated that: 1) Spx is essential for growth under both non-stress and stress conditions induced by diamide treatment, high salt, as well as high and low temperature conditions; 2) Spx is required for the expression of *trxB* (encoding thioredoxin reductase) under both non-stress conditions, after diamide treatment and upon exposure to high temperatures; 3) Spx positively and negatively controls the expression of genes encoding proteins involved in purine, pyrimidine and amino acid biosynthesis as determined by two-dimentional gel electrophoresis; 4) The enhancement of biofilm formation in the *spx* mutant strain is due to elevated transcription of the *icaABCD* operon, encoding proteins that function in biofilm formation, and a reduction in

the amount of *icaR* transcript which encodes a negative regulator of the *icaABCD* operon (Pamp et al., 2006).

1.4 BACTERIAL TRANSCRIPTION REPRESSORS ACTIVE IN RESPONSE TO LIGANDS: MEMBERS OF THE MARR FAMILY OF WINGED HELIX PROTEINS

Proteins belonging to the MarR (<u>m</u>ultiple <u>a</u>ntibiotic <u>r</u>esistance <u>r</u>egulator) family of transcriptional regulators control resistance to mutliple antibiotics, organic solvents, household disinfectants, detergents, oxidative stress agents and toxic chemicals as well as chemicals with aromatic rings (Alekshun & Levy, 1999). *E. coli* MarR represses marRAB operon transcription by binding to two binding sites: between the -35 and -10 (bp -31 to -9). MarR presumably interferes with RNAP binding, between transcription start site (bp 7-27) may interfere with either open complex formation or clearance (Alekshun *et al.*, 2000, Cohen *et al.*, 1993, Martin & Rosner, 2004). The crystal structure reveals MarR as a dimer with each subunit containing a winged-helix DNA binding motif (Alekshun *et al.*, 2001). The overall topology of MarR is α1, α2, β1, α3, α4, β2, W1 (wing region), β3, α5 and α6 (Alekshun et al., 2001, Hong et al., 2005). The region β1-α3-α4-β2-W1-β3 adopts the winged-helix fold which required for its DNA binding activity (Alekshun et al., 2001). Stabilization of the homodimer is mediated primarily by hydrophobic contacts involving 10 residues from each subunit between the N-terminal α-helix1 and the C-terminal helices 5 and 6 (Alekshun et al., 2001). Mutational analysis identified the N-terminus of MarR to be important for mediating protein-protein contacts between repressor subunits (Alekshun et al., 2000, Alekshun et al., 2001). Transcriptional regulation by MarR proteins is modulated by several anionic compounds (usually phenolic) including 2,4-dinitrophenol, plumbagin, menadione and salicylate (Alekshun & Levy, 1999, Schumacher & Brennan, 2002). Two salicylate binding sites per MarR subunit were revealed in the structure using crystals grown in the presence of high concentration of salicylate (250 mM) (Alekshun et al., 2001). Both salicylate molecules bind close to the DNA binding helix, which undergo significant

conformational changes to accommodate DNA binding (Alekshun & Levy, 1999, Schumacher & Brennan, 2002).

The heterotrophic, mesophilic bacterium *Deinococcus radiodurans* contains HucR (hypothetical uricase regulator) that belongs to the MarR family of transcriptional regulators. HucR is a repressor of an adjacent uricase enzyme that is involved in the aerobic purine degradation pathway (Wilkinson & Grove, 2004). The substrate of uricase, uric acid, is an efficient antagonist of HucR-DNA binding and uric acid is a scavenger of reactive oxygen species suggesting that HucR functions in the oxidative stress response (Wilkinson & Grove, 2004, Wilkinson & Grove, 2006). Transcription repression is also relieved when HucR binds to salicylic acid, which indicates that HucR has affinity for specific phenolic ligands (Wilkinson & Grove, 2004). The crytal structure of the HucR dimer uncovered the topology $\alpha 1$-$\alpha 2$-$\alpha 3$-$\beta 1$-$\alpha 4$-$\alpha 5$-$\beta 2$-$\beta 3$-$\alpha 6$-$\alpha 7$ (Bordelon *et al.*, 2006). $\alpha 2$, $\alpha 6$ and $\alpha 7$ form the dimerrization domain while the DNA-binding domain is composed of $\alpha 3$, $\beta 1$, $\alpha 4$, $\alpha 5$, $\beta 2$ and $\beta 3$ (Bordelon et al., 2006). Footprinting analysis identified the HucR dimer binding site containing an imperfect 8-bp incerted repeat with 2 bp separating each half of the palindrome (Wilkinson & Grove, 2004). The presence of a pair of similarly stacked histidine residues at a dimer interface can serve as a pH-sensor which could undergo a conformational change upon increasing proton concentration (Bordelon et al., 2006). Further decrease in pH results in significant loss of HucR-DNA complex formation as observed by EMSA (Bordelon et al., 2006). Thus, protonated uric acid could promote the conformational changes at the dimer interface resulting in repositioning of the DNA-binding domains.

B. subtilis OhrR belonging to the MarR family of transcription regulators binds the *ohrA* operator as a homodimer (Fuangthong et al., 2001). OhrR is the peroxide-sensing transcription factor that control expression of the organic hydroperoxide resistance (*ohr*) genes by oxidation of its unique and conserved cysteine (Cys15) resulting in the induction of *ohrR* and derepression of *ohrA* (Fuangthong et al., 2001, Fuangthong & Helmann, 2002). The structure of OhrR is similar to that of all other MarR family members which consist of six α helices and three β strands. The structure revealed two functional domains of each subunit of OhrR: the dimerization domain which involves the N and C termini, $\alpha 1$, $\alpha 2$, $\alpha 5$ and $\alpha 6$ and winged helix-turn-helix (WHTH) DNA

binding domain, which consists of β1, α3, α4, β2 and β3 (Hong et al., 2005). Cys15 is located at the N terminus of helix α1 involved in DNA binding and playing a key role in the peroxidation-mediated induction mechanism of OhrR (Hong et al., 2005). The ligand-induced oxidation of Cys to Cys-sulfenic acid likely causes a conformational change in the dimerization domain of OhrR resulting in an orientation of winged helix lobes of the dimer that alters the DNA binding activity of the protein (Hong et al., 2005, Wilkinson & Grove, 2006). The ligand-responsive form of OhrR prefers organic hydroperoxides over hydrogen peroxide due to the presence of a long strip of aromatic and nonpolar residues on helix α2 which provides the long hydrophobic landing pad for such lipophilic oxidants (Hong et al., 2005).

In summary, the physiological roles of MarR family of transcriptional regulators can be classified into three general categories: 1) regulation of response to environmental stress, 2) regulation of virulence factors, and 3) regulation of aromatic catabolic pathways (Wilkinson & Grove, 2006). Several members of the MarR family are capable of binding diverse anionic lipophilic (usually phenolic) compounds. MarR-DNA binding sites often overlap the -35 and/or -10 pormoter elements of their target genes suggesting that repression is achieved by inhibiting RNAP binding to the promoter. Ligand-binding at high-affinity sites causes a conformational change that antagonizes MarR-DNA interaction.
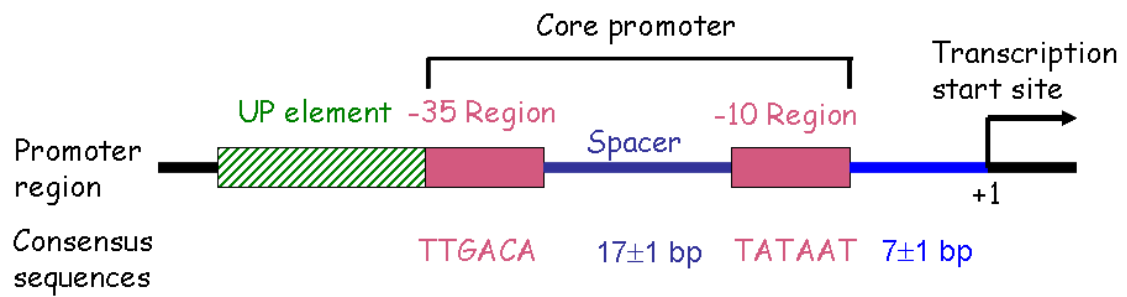
Figure 1.1 Typical *E. coli* consensus promoter structure. The core promoter consists of the -35 and -10 regions separated by a spacer sequence of 17±1 base pairs. Numbers indicate nucleotide positions relative to the transcription start site which is defined as +1. Upstream sequence region (UP element) usually contains AT-rich sequence upstream of the promoter core which affects promoter activity. Adapted from (Wagner, 2000).
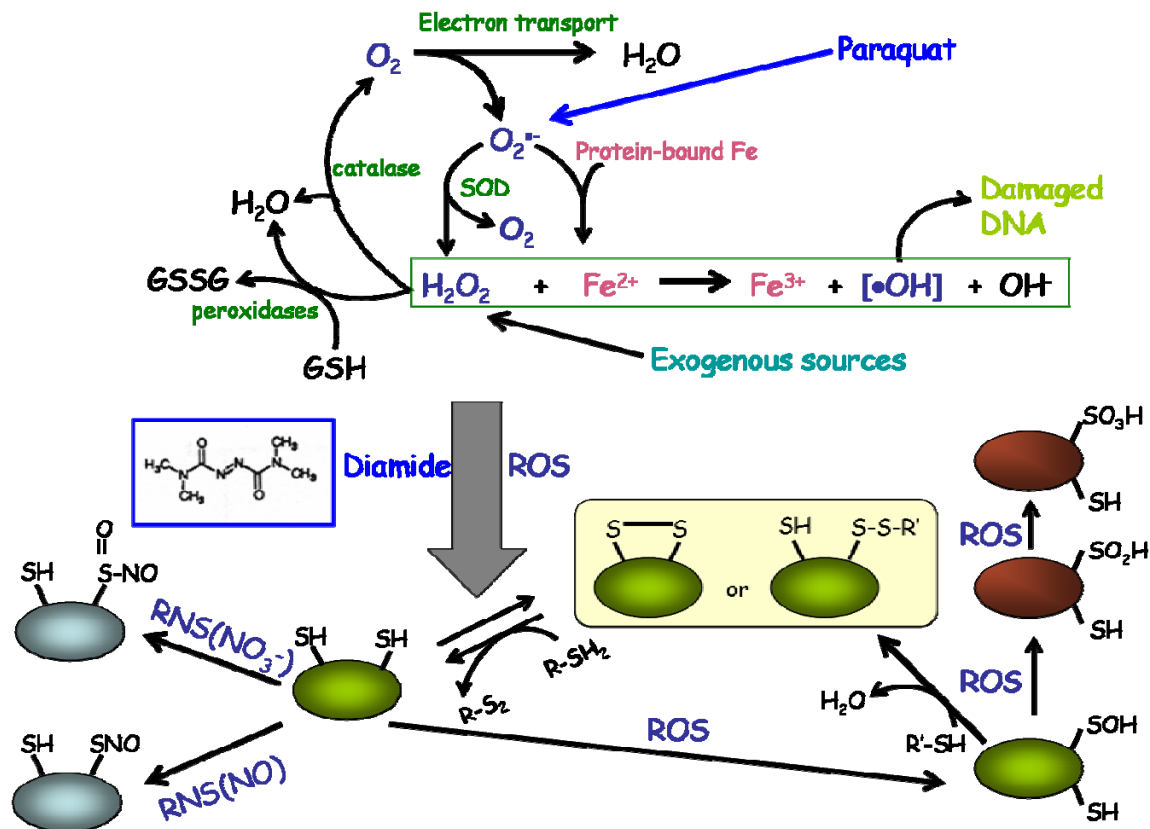
Figure. 1.2 Mechanism of oxidative cell damage leading to redox-sensing by thiol modification. Hydrogen peroxide (H_2O_2) and superoxide ($O2\bullet^-$) are generated endogenousely by autooxidation via electron transport chain reaction. Superoxide is rapidly converted to H_2O_2 by superoxide dismutase (SOD). H_2O_2 reacts with Fe^{2+} which is released from protein-bound Fe (Fe-S clusters) by superoxide to yield Fe^{3+} and the highly reactive hydroxyl redical ($\bullet OH$) in the Fenton reaction (indicated by green box) which cause DNA damage. H_2O_2 can be detoxified by catalase or by peroxidases. Reactive oxygen species (ROS), including superoxide, hydrogen peroxide and hydroxyl radical, are also generated exogenously. In the laboratory, superoxide generation can be induced by treatment of cells with paraquat. Diamide, thiol-specific oxidant, also is used to induce disulfide bond formation in the cell. The reaction of ROS with protein containing thiol side chain (-SH) leads to several different protein modifications such as reversible disulfide bond formation (S-S), mixed disulfide (S-S-R′), intermediate sulfenic acid (-SOH) and irreversible sulfinic acid (SO_2H) and sulfonic acid (-SO_3H) derivatives. The reduced form of the protein can be restored by a cellular reductant (R-SH_2). Oxidation of cysteine by reactive nitrogen species (RNO) such as nitric oxide (NO) and peroxynitrite (NO_3^-) give rise to S-nitrosothiol (R-SNO) and S-nitrothiol (R-SNO_2) respectively. Adapted from (Kiley & Storz, 2004, Paget & Buttner, 2003, Green & Paget, 2004, Henle & Linn, 1997, Storz & Imlay, 1999).
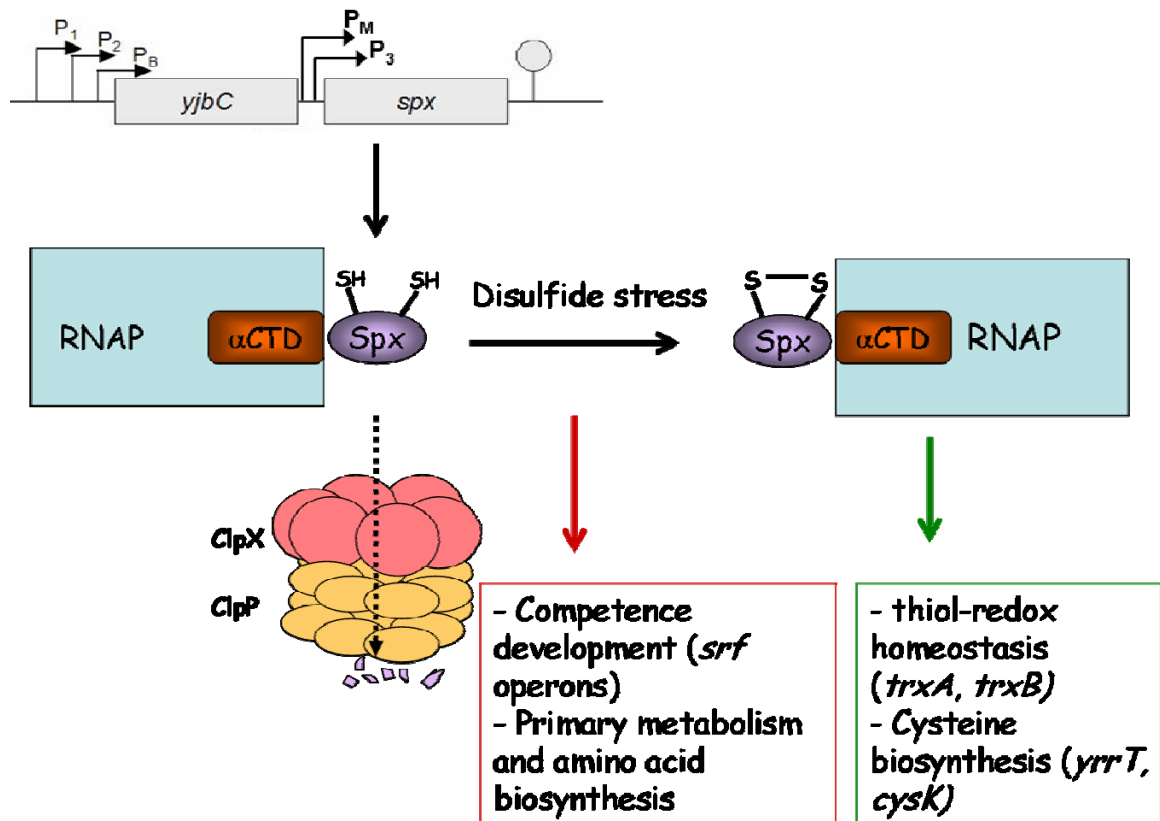
Figure 1.3 Roles of Spx during disulfide stress. The *spx* gene resides in a dicistronic operon with *yjbC* gene which contains three promoter including P$_1$, P$_2$ and P$_B$ and two promoters located within the intergenic region of *yjbC-spx*, P$_M$ and P$_3$.Under non-stress conditions, Spx is in the reduced form which is subject to proteolysis by ClpXP protease. When cells encounter disulfide stress, the interaction of oxidized Spx with αCTD of RNAP activates and represses gene expression as shown in green box and red box, respectively.

CHAPTER 2

TRANSCRIPTION FROM THE P₃ PROMOTER OF BACILLUS SUBTILIS SPX GENE IS INDUCED IN RESPONSE TO DISULFIDE STRESS*

2.1 INTRODUCTION

The *spx* gene of *B. subtilis* was discovered to be the site of mutations that suppressed null alleles of *clpX* and *clpP* with respect to competence development, sporulation gene expression and growth in minimal medium (Nakano et al., 2001, Zuber, 2004). It was() later characterized as an RNA polymerase (RNAP)-binding protein that repressed activator-stimulated transcription and activated transcription initiation at the *trxA* (thioredoxin) and *trxB* (thioredoxin reductase) promoters in response to oxidative stress (Nakano et al., 2005, Nakano et al., 2003a, Nakano et al., 2003b, Newberry et al., 2005). Recent reports indicate that Spx is an important regulatory factor in the stress response in *Staphylococcus aureus* (Pamp et al., 2006) and during *Listeria monocytogenes* infection (Chatterjee *et al.*, 2006). Spx concentration and activity increase when cells encounter a toxic oxidant that brings about disulfide stress (Nakano et al., 2005, Nakano et al., 2003a). Increases in Spx protein concentration are associated with a variety of stress conditions (Tam *et al.*, 2006a), including heat, salt, disulfide, and peroxide stress.

The *spx* gene resides in the *yjbC-spx* dicistronic operon located between the *opp* operon and the *mecA* gene (Fig. 2.1A). Previous studies had uncovered the complexity of the

yjbC-spx operon's transcriptional organization (Antelmann *et al.*, 2000). Four kinds of RNAP holoenzymes recognize promoter DNA sequences at the 5′ end of the operon and in the intergenic DNA between *yjbC* and *spx*. Upstream of the *yjbC* coding sequence are three putative transcription start sites uncovered by primer extension studies: P_1 recognized by the σ^A form of RNAP, P_2 and P_B utilized by the σ^W and σ^B RNAP holoenzymes, respectively. In the *yjbC-spx* intergenic region, the P_M promoter was identified (Thackray & Moir, 2003), which is utilized by the σ^M form of RNAP. The σ^B RNAP holoenzyme is a crucial component of the general energy and environmental stress responses (Hecker & Volker, 1998). The σ^W holoenzyme form is required for the transcription of genes that are induced by envelope stress, antimicrobial agents, and superoxide stress (Cao *et al.*, 2002a, Huang *et al.*, 1998b, Hoper *et al.*, 2006). Hence, part of the reason why Spx concentration increases in response to stress might be the activities of the RNAP holoenzyme forms that utilize promoters of the *yjbC-spx* operon.

In this report, the discovery of the fifth promoter in the *yjbC-spx* operon intergenic region is described. It is activated when *B. subtilis* cells are treated with the thiol-specific oxidant, diamide (Kosower & Kosower, 1995). The promoter, P_3, is utilized by the σ^A form of RNAP and is associated with two negative control elements. The accompanying paper describes the two repressors that target the *spx* P_3 promoter region.

2.2   RESULTS

2.2.1   Identification of P$_3$ promoter within the *yjbC-spx* operon

Fig. 2.1 shows genomic location and the organization of the *yjbC-spx* operon of *B. subtilis*. The *yjbC* operon resides between the *opp* (*spo0K*) operon and the *mecA* gene (Fig. 2.1A). Upstream of *yjbC* are three promoters, two of which are recognized by the σ^W (P$_2$) and σ^B (P$_B$) form of RNAP (Antelmann et al., 2000, Cao et al., 2002a, Petersohn *et al.*, 1999). The *yjbC* coding sequence is followed by an intergenic region of 184 bp that contains the previously described P$_M$ promoter of *spx* that is recognized by the σ^M form of RNAP (Jervis et al., 2007). The *spx* gene coding sequence begins 126 bp downstream of the start-point of the P$_M$ promoter (Fig. 2.1B) (Jervis et al., 2007). Because Spx activity and concentration are elevated upon oxidative stress, we felt that transcription of the *spx* gene might be controlled in response to the presence of a toxic oxidant (diamide). Primer extension analysis of total JH642 RNA using primers specific for *spx* and *yjbC* was conducted to determine if transcription from the various promoters of the *yjbC-spx* operon was induced by diamide treatment. No elevation of *yjbC* transcript levels was observed upon disulfide stress (data not shown but see Fig. 2.5D), but a transcript from a previously unidentified transcription start site was detected (Fig. 2.1C) and its concentration increased upon diamide treatment (see below). The start-site of transcription from the new P$_3$ promoter resides 79 bp from the start of the *spx* coding sequence. The same start-site was utilized by purified RNAP in vitro, as shown in the gel profile of the run-off transcription products in Fig 2.1D. No transcription from the P$_M$ promoter in vivo or in vitro was detected.

2.2.2   Diamide-dependent induction of transcription from P$_3$ does not require *spx*

Transcription of *spx* from P$_3$ was examined in cells of cultures that were subjected to disulfide stress. RNA was isolated from wild-type or *spx* mutant cultures before and after 10 and 30 min of diamide treatment. Denaturing gel analysis of primer extension reactions showed that transcript levels increased after 10 min of diamide treatment of wild-type and *spx* mutant cells (Fig. 2.2A). The Spx-dependent induction of *trxA*

(encoding thioredoxin) transcription by diamide (Nakano et al., 2003a) was included as a control (Fig. 2.2B). A similar pattern of transcriptional induction was observed in cells bearing the *rpoA*[cxs-1] mutation (Fig. 2.2B), which renders RNAP α C-terminal domain unable to interact with Spx. Thus, it was shown that the induction of *spx* transcription from the P_3 promoter by diamide treatment did not require the interaction of Spx with RNAP, which is required for *trxA* and *trxB* transcription.

Examination of the primer extension signal at 30 min following diamide treatment shows that there is more *spx* transcript in the *spx* and *rpoA*[cxs-1] cells than in those of the wild-type strain. This suggests that, while Spx-RNAP interaction might not be required for *spx* transcription, this interaction is necessary for the negative control required to restore expression to the pre-stress state. Aforementioned, the Spx-RNAP interaction is required for *trxA* and *trxB* transcription involving in thiol-homeostasis which help to restore the expression of *spx* to the pre-stress state.

2.2.3   Transcription from the *yjbC-spx* intergenic region contributes substantially to the total expression of *spx*

The *yjbC-spx* operon encodes a 1.2 kb RNA which specifies the YjbC and Spx proteins (Antelmann et al., 2000). Previous results (Nakano et al., 2001, Thackray & Moir, 2003) and the data presented in Fig. 2.1 and 2.2 show that the intergenic DNA contains at least two promoters that could drive *spx* expression. Two β-galactosidase (*bgaB*) fusions were constructed and their expression examined to determine to what extent the *yjbC-spx* intergenic region contributes to the expression of *spx* under non-stress conditions. The *yjbC-spx-bgaB* fusion of strain ORB4888 contained a fragment extending from the stop codon of *spx* at its 3′ end to 420 bp upstream of the *yjbC* coding sequence. The *spx-bgaB* fusion of strain ORB4889 contains a fragment bearing the same 3′ end as in the *yjbC-spx-bgaB* fusion, but its 5′ end is 538 bp upstream of the P_3 startsite, a point within the *yjbC* coding sequence. As shown in the time course experiment of Fig. 2.3, the promoters of the *spx-bgaB* fusion account for between 45 to 70% of the total *bgaB* activity directed by the *yjbC-spx* operon. Because the cells were grown in DSM medium at 37˚C, the stress conditions that would lead to maximal induction of σ^W and σ^B activity were likely not encountered. Such conditions might result in a much larger

contribution to *spx* expression by the *yjbC* promoter region. Nevertheless, the result of Fig. 2.3 indicates that *spx* gene contains transcriptional signals that direct expression independently of the *yjbC* transcription initiation region. This is in keeping with the observation that a fragment bearing the *spx* gene and upstream intergenic region is sufficient for the complementation of a *spx* null mutation (Nakano et al., 2003a).

2.2.4 Deletion analysis uncovers *cis*-acting negative control elements associated with promoter P$_3$

The result of Fig. 2.1D showed that the P$_3$ promoter can be utilized by purified σ^A RNAP in vitro without the presence of a positive control factor, suggesting that the diamide induction of P$_3$ transcription might be caused by a reversal of negative control. To identify cis-acting control sequences associated with the P$_3$ promoter, deletion and point mutational analysis was undertaken. Fig. 2.4 summarizes the results of deletion analysis of the *spx* promoter region. PCR-generated fragments in which sequences 5′ and 3′ of the P$_3$ promoter were omitted were introduced into the *bgaB* fusion vector. The levels of BgaB activity and the level of transcript determined by primer extension reactions were assessed. BgaB activity was used as a measure of uninduced expression, since diamide induction cannot be observed using the enzyme assay due to inactivation of BgaB. Therefore induction was observed by measuring the increase in the level of transcript from both the *spx-bgaB* fusion and from the endogenous *spx* gene using primer extension. The 5′ deletions had modest effects on basal expression of *spx*, but diamide induction was still observed. However deletions of the sequences 3′ of the P$_3$ transcriptional start-site resulted in higher basal level expression.

2.2.5 A negative control element resides downstream of the P$_3$ promoter sequence

As summarized in Fig. 2.4, deletions of the 5′ end had little effect on the diamide-dependent induction of P$_3$. This is shown in Fig. 2.5A, which is a gel profile of the primer extension reaction of RNA extracted from diamide-treated cells. A -40 deletion, which removes most of the DNA upstream of the P$_3$ promoter, does not change the induction pattern of P$_3$ transcription (lanes 1-3), which is also observed when the endogenous *spx* P$_3$ transcript is examined (lanes 4-6). Deletion of the DNA 3′ of the P$_3$ start site, with

endpoints at +30, +15, and +5, results in an increase in basal level transcription and loss of diamide induction as shown in primer extension experiments and in assays of *spx-bgaB* activity (Fig. 2.5B, lanes 1-9, Fig. 2.5C). The same RNA used in the primer extension analysis of *spx-bgaB* promoter mutants was used to examine the level of endogenous *spx* transcript, which increased within 10 min of diamide treatment (Fig. 2.5B, lanes 10-18). The data of Fig. 2.5B are strong evidence for a negative control element in the region 3′ to the P_3 promoter sequence.

Primer extension analysis also showed that the *yjbC* promoters do not contribute to the diamide induction of *spx* expression (Fig. 2.5D). The P_2 and P_B promoter transcripts can be detected in RNA from untreated cells, while diamide treatment has no effect on the level of P_2 transcript but results in loss of the P_B transcript. At present, we do not know if this repression of P_B is operon specific or due to reduction of σ^B activity upon disulfide stress.

2.2.6 Point mutations suggest the presence of a second negative control site within the P_3 promoter

Experiments were conducted to uncover mutations in *trans*-acting loci, with hopes of identifying the putative repressor controlling transcription from P_3. Cultures of *spx-bgaB* fusion-bearing cells were mutagenized by UV irradiation for 4 min and examined for elevated BgaB activity on agar medium containing X-gal as well as measured *spx*-directed BgaB activity (Fig. 2.S2). UV3 and UV5 variants isolated, all bore mutations in the intergenic region of *yjbC-spx* (-26 and 24 relative to transcription start site). In parallel experiments, *in vitro* mutagenesis by error-prone PCR was undertaken using a fragment of the P_3 promoter region as template. The pool of mutagenized fragments was inserted into the pDL *bgaB* fusion vector. Four variants isolated (ORB5434-1, 2, 3, 4) were examined the level of *spx-bgaB* expression (Fig. 2.S3). All of the mutants obtained by the two procedures bore multiple nucleotide substitutions (see Experimental Procedures). Therefore, site-directed oligonucleotide mutagenesis was carried out to create single substitutions, the locations of which were chosen from the mutant sequences obtained from the UV mutagenesis and error-prone PCR experiments. Seven single-site mutants obtained (Fig. 2.6A) were analyzed by

measuring *spx*-directed BgaB activity (Fig. 2.6B), and by primer extension analysis (Fig. 2.6C). Of the seven, five showed elevated basal *spx-bgaB* expression (Fig. 2.6B; T-26A, A-14T, A3G, T7C, T24C) and higher levels of transcripts as shown in primer extension reactions of RNA from untreated cells (Fig. 2.6C; T-26A, A-14T, A3G, T7C, T24C). While the A3G substitution results in a generally higher level of transcript in both treated and untreated cells, the other mutations showing higher basal expression and transcript levels show no increase in diamide induced expression, and hence, have reduced induction ratios compared wild-type cells, indicating that these mutations reduce transcriptional repression under non-stress conditions. Our results provide evidence for a second *cis*-acting, negative control region within the P_3 promoter region.

2.3   DISCUSSION

The transcription of *spx* is driven by five promoters of the *yjbC-spx* operon, although not all of the promoters are active under the culture growth conditions used in the study described herein. The promoters P_M and P_3 reside in the intergenic region of *yjbC-spx*, but only transcription from P_3 is observed in the primer extension analysis of RNA from cells grown in minimal TSS medium, before and after diamide treatment. The P_3 promoter contains a consensus -10 sequence that is utilized by the major, σ^A form of RNA polymerase, but has a -35 that shows a 3 of 6 nucleotide match for the consensus -35 region. Levels of transcript synthesized from P_3 increase 10 min after diamide treatment. Transcription from P_3 is catalyzed by σ^A holoenzyme in vitro without the requirement for an additional transcription factor, which suggested that transcription from P_3 is under negative control. The intergenic region contributes significantly to *spx* transcription as shown in experiments using *yjbC-spx*- and *spx-bgaB* fusions, and by complemention of a *spx* null mutation with respect to diamide sensitivity using a single copy of the *spx* gene with the accompanying *yjbC-spx* intergenic region. Spx concentration increases after diamide treatment, which was shown to be due in part to post-transcriptional control of *spx* expression (Nakano et al., 2003a), perhaps by down-regulation of ClpXP-dependent proteolysis of Spx. However, the observation that *spx*

transcription can be induced by disulfide stress suggests that transcriptional control can also contribute to elevated Spx levels during oxidative stress.

Mutational analysis of the regulatory region of the *spx* gene uncovered two negatively cis-acting elements. Deletion of sequences located downstream of the transcriptional start-site of P_3 resulted in higher basal level transcription and a reduced diamide induction ratio. We propose based on these data that the mutations define an operator to which a negative transcriptional regulator interacts. Attempts at identifying a regulator involved a mutant search for *trans*-acting loci that might confer constitutive transcription from P_3 and encode a repressor. No extragenic mutations conferring such a phenotype were isolated, but several *cis*-acting mutations were uncovered by screening mutants after UV mutagenesis. Five mutations, two downstream of the P_3 transcriptional start-site and three upstream and within the P_3 promoter sequence, were observed to cause elevated basal transcription. Of these, four T-26A, T-14T, T7C, and T24C reduced the diamide induction ratio and increased basal level transcription. The T7C and T24C mutations likely affect the downstream operator defined by deletion analysis. We propose that T-26 and A-14 positions define a second operator that is the interaction site of a second negative regulator. Our inability to uncover trans-acting loci that affect control of P_3 utilization suggested that there might be two mechanisms of negative control and both must be reversed to ensure proper induction of *spx* during disulfide stress; such a double mutant would likely be very rare.

Several transcriptome studies have been carried out to identify the genes that are induced by oxidative stress or that are controlled by the peroxide response regulator, PerR (Hayashi et al., 2005, Helmann et al., 2003a, Mostertz et al., 2004). The study of Hayashi et al. identified *spx* as being a member of the PerR regulon. The putative operator in the +5 to +30 region contains at least one sequence bearing a resemblance to a PerR box. Indeed, as we show in the accompanying paper, PerR protein binds to this region and in doing so represses *spx* transcription from P_3 in vitro. Furthermore, a second repressor encoded by the *yodB* gene was observed to bind to the second putative operator defined by the T-26 and A-14 mutations within the P_3 promoter sequence. The description of this dual negative control of *spx* transcription appears in the accompanying paper.

2.4   EXPERIMENTAL PROCEDURES

2.4.1   Bacterial strains and growth conditions

Bacillus subtilis strains used in this study are derivatives of JH642 and are listed in Table 2.1. Cells were cultivated in a shaking water bath at 37°C in Difco Sporulation medium (DSM) for β-galactosidase assays or TSS minimal medium (Fouet & Sonenshein, 1990) for diamide treatment experiment (Nakano et al., 2003a). Diamide was purchased from Sigma-Aldrich and used at a concentration of 1mM to induce disulfide stress.

2.4.2   Construction of the *yjbC* and *spx* translational fusions

The *yjbC-spx* promoter region was amplified by PCR using primer oML02-17 and oML02-6 (see table 2.2). The product was digested with BamHI and BclI then inserted into pUC19 digested with BamHI to generate pML11. The cloned *yjbC-spx* DNA was verified by DNA sequencing, then was digested with BamHI and SmaI to release the fragment containing 420 bp upstream of the *yjbC* start codon to 187 bp downstream of the start codon). The resulting fragment was then subcloned into plasmid pDL to construct pML18, a plasmid bearing a *bgaB* fusion, encoding thermostable β-galactosidase, (Yuan & Wong, 1995) and the *yjbC-spx* intergenic region. The fragment released from pML18 by EcoRI digestion and containing 538 bp upstream of the P_3 startsite to 187 bp downstream sequence of *spx* start codon, was inserted into pDL plasmid to generate pML19, the *spx-bgaB* fusion.

2.4.3   Constructions of promoter deletion mutations

The *spx* 3′ promoter region was amplified by PCR using primer oML02-7 in combination with oML02-22 (+50,wild-type), oML02-26 (+5), oML02-27 (+15), oML02-28 (+30) or oML02-29 (+40) (Table 2.2). The products were digested with BamHI and KpnI then inserted into pUC19 digested with the same enzymes to generate pML25, pML27, pML28, pML29 and pML33, respectively. The *spx* sequences were verified by DNA sequencing. The plasmids were digested with BamHI and EcoRI to

release a fragment extending from 330 bp upstream from the P_3 startsite to the 3′ deletion end-point. The fragments were then inserted into plasmid pDL that was digested with the same enzymes to generate pML26, pML30, pML31, pML32, and pML34 respectively. The *spx* 5′ promoter deletion region was amplified by PCR using primer oML02-22 in combination with oML02-23(-40), oML02-24(-60) or oML02-25(-100) (Table 2.2). The products were digested with BamHI and EcoRI, then inserted into pDL that was digested with the same enzymes to generate pML20, pML21 and pML22, respectively. The fragments extended from the 5′ deletion end-point to 50 bp downstream of the P_3 transcription start site. The *spx* sequences were verified by DNA sequencing. The strains bearing the wild-type *spx*, 5′ and 3′ *spx* deletion promoter-*bgaB* fusions were ORB5058 (wt), ORB4980 (-40), ORB4981 (-60), ORB4982 (-100), ORB5077 (+5), ORB5078 (+15), ORB5079 (+30) and ORB5115 (+40). Cells bearing the promoter-*bgaB* fusions were grown in DSM medium until the optical density $OD_{600} \sim 0.4$-0.5. After further incubation for 30, 60 and 120 min., samples of cells were harvested and prepared for β-galactosidase assays.

2.4.4　Site-directed mutagenesis

　　spx T-26A, T-20G, T-19G, A-14T, A+3G, T+7C and T+24C mutants allele were generated by PCR-based site-directed mutagenesis. First-round PCR was performed in two separate reactions with primer oMLbgaB in combination with oML02-38, oML02-40, oML02-42, oML02-44, oML02-46, oML02-48, oML02-50 (Table 2.2) using pML26 as template and primer oML02-37 in combination with oML02-39, oML02-41, oML02-43, oML02-45, oML02-47, oML02-49, or oML02-51 (Table 2.2) using pSN16 (Nakano et al., 2002b) as template. The PCR products were hybridized and subsequently amplified by a second round of PCR using oML02-37 and oML02-22. The second round of PCR products were then digested with EcoRI and BamHI restriction enzymes (to release the PCR fragment from -330 to +50 respectively to P_3 TSS) and inserted into pDL digested with the same enzymes to generate pML42, pML43, pML44, pML46, pML45, pML47 and pML48, respectively. The *spx* sequences in the plasmids were verified by DNA sequencing. The *spx* promoter point mutant strains used for β-galactosidase assays and

primer extension analyses were ORB6030 (T-26A), ORB6031 (T-20G), ORB6032 (T-19G), ORB6033 (A-14T), ORB6034 (A+3G), ORB6035 (T+7C), and ORB6036 (T+24C).

2.4.5 Assay of β-galactosidase activity

Assays of BgaB activity were performed according to previously published methods (Schrogel & Allmansberger, 1997). Activity was expressed as Miller Units (Miller, 1972). The data from β-galactosidase assays are presented with standard deviation for three to four independent experiments.

2.4.6 Primer extension analysis

Cultures of wild-type and P_3 promoter mutant strains were grown at 37°C in TSS medium. At mid-log phase, the culture was split into two cultures and to one, diamide was added to final concentration of 1 mM. Aliquots (20 ml) of cultures were withdrawn for RNA preparation at time 0 (mid-log phase) and after 10 min of with or without diamide (1mM) treatment. The culture was mixed with an equal volume of ice-cold methanol, after centrifugation the cell pellet was frozen at -80°C. The total RNA was prepared by RNeasy mini kit (Qiagen, Chatworth, CA). Primer corresponding to the *bgaB* sequence downstream of the inserted fragment (oMLbgaB) as well as the primer oML02-15 (specifying the endogenous *spx* sequence) were used to examine the transcript of *spx* fusions and endogenous *spx*. Primer oML02-10 was used to examine the transcript of *yjbC*. The same amount of RNA used in primer extension was applied to formaldehyde-agarose gel. 16S rRNA was visualized by staining with ethidium bromide to confirm that comparable amounts of total RNA were used for each reaction.

2.4.7 In vitro transcription assay

Linear DNA template for *spx* promoter was generated by PCR with primer oML02-7 and oML02-15 using plasmid pML18 as the template (encoding ~70-base transcript). The 0.1, 0.2 and 0.5 μM of DNA templates were mixed with 100 nM RNAP in 10 mM Tris-HCl pH 8.0, 50 mM NaCl, 5mM $MgCl_2$, 50 μg/ml BSA, 5 mM DTT at 37°C for 10 min. A nucleotide mixture (200 μM ATP, GTP and CTP, 10 μM UTP, 10 μCi α-^{32}P-UTP) was added to the reaction. The reaction mixtures (20 μl) were further

incubated at 37 °C for 20 min and the transcripts were precipitated by ethanol. Electrophoresis was performed as described (Liu & Zuber, 2000).

2.4.8 Ultraviolet (UV) irradiation mutagenesis

ORB5107 (*Pspx-bgaB, spx::neo*) was grown in DSM liquid media until OD_{600} = 0.7-1.0. Cells were harvested and washed with 0.1M $MgSO_4$. Cells were resuspended in 0.1M $MgSO_4$ then irradiated 500 μJ (UV stratalinker[TM] 1800) for 30 s, 1 min, 2 min, 3 min and 5 min. Irradiated cells were kept on ice and in the dark. Serial dilutions of untreated cells and UV-treated cells were plated on DSM plates for counting isolated colonies to determine survival rate (Fig. 2.S1). The frequency of auxotrophic mutants among the UV-treated cells was determined by replica plating on TSS agar containing 0.05% tryptophan and phenylalanine (Table 2.S1).

2.4.9 Error-prone PCR mutagenesis

The *spx* promoter region was amplified by PCR using primer oML02-7 and oML02-22. The error-prone PCR reaction contained error-prone PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 7 mM $MgCl_2$), 0.3 mM $MnCl_2$, 0.2 μM of each primers, 0.2 mM of dATP and dGTP, 1 mM of dCTP and dTTP. The product was digested with KpnI and BamHI then inserted into pUC19. The pooled plasmid was digested with EcoRI and BamHI followed by subcloning into plasmid pDL. The recombinant pDL pool (pML36) was used to transform JH642 to generate the strain ORB5434 bearing random nucleotide substitutions within the *spx* promoter region. The transformants (ORB5434) were screened for blue colonies on the DSM plates containing X-gal. The BgaB phenotype of the blue colony isolates was confirmed by β-galactosidase assays of samples collected from liquid DSM cultures (Fig. 2.S3). Confirmed BgaB[+] variants were analyzed by sequence analysis of the mutagenized *spx* promoter region. All of four mutants bore multiple nucleotide substitutions such as at position -14, 7 in ORB5434-1, -26, 24 in ORB5434-2, -19, -20, 3 in ORB5434-3 and -26, 24 in ORB5434-4.

Table 2.1 *Bacillus subtilis* strains and plasmide used

Strain or plasmid	Relevant phenotype or description[a]	Reference or source
Strains		
JH642	*trpC2 pheA1*	J. A. Hoch
ORB3621	*trpC2 pheA1 rpoA*(*Y263C*)	(Nakano et al., 2000b)
ORB3834	*trpC2 pheA1 spx*::*neo*	(Nakano et al., 2001)
ORB4888	*trpC2 pheA1 amyE*::*PyjbC-spx-bgaB cat*	This study
ORB4889	*trpC2 pheA1 amyE*::*Pspx-bgaB cat*	This study
ORB4980	*trpC2 pheA1 amyE*::*PspxΔ−40-bgaB cat*	This study
ORB4981	*trpC2 pheA1 amyE*::*PspxΔ−60-bgaB cat*	This study
ORB4982	*trpC2 pheA1 amyE*::*PspxΔ−100-bgaB cat*	This study
ORB5058	*trpC2 pheA1 amyE*::*PspxΔ+50(wt)-bgaB cat*	This study
ORB5077	*trpC2 pheA1 amyE*::*PspxΔ+5-bgaB cat*	This study
ORB5078	*trpC2 pheA1 amyE*::*PspxΔ+15-bgaB cat*	This study
ORB5079	*trpC2 pheA1 amyE*::*PspxΔ+30-bgaB cat*	This study
ORB5107	*trpC2 pheA1 amyE*::*PspxΔ+50(wild-type)-bgaB cat spx*::*neo*	This study
ORB5115	*trpC2 pheA1 amyE*::*PspxΔ+40-bgaB cat*	This study
UV3, UV5	Irradiated ORB5107	This study
ORB5434	*trpC2 pheA1 amyE*::*PML36*	This study
ORB6030	*trpC2 pheA1 amyE*::*Pspx*(T−26A)-*bgaB cat*	This study
ORB6031	*trpC2 pheA1 amyE*::*Pspx*(T−20G)-*bgaB cat*	This study
ORB6032	*trpC2 pheA1 amyE*::*Pspx*(T−19G)-*bgaB cat*	This study
ORB6033	*trpC2 pheA1 amyE*::*Pspx*(A−14T)-*bgaB cat*	This study
ORB6034	*trpC2 pheA1 amyE*::*Pspx*(A+3G)-*bgaB cat*	This study
ORB6035	*trpC2 pheA1 amyE*::*Pspx*(T+7C)-*bgaB cat*	This study
ORB6036	*trpC2 pheA1 amyE*::*Pspx*(T+24C)-*bgaB cat*	This study

Strain or plasmid	Relevant phenotype or description[a]	Reference or source
Plasmids		
pSN16	*yjbC-spx-lacZ* fusion plasmid	(Nakano et al., 2002b)
pML11	*yjbC-spx* transcriptional fusion in pUC19	This study
pML18	*yjbC-spx* transcriptional fusion in pDL	This study
pML19	*spx* transcriptional fusion in pDL	This study
pML20	*spx* promoter fragment (-40) in pDL	This study
pML21	*spx* promoter fragment (-60) in pDL	This study
pML22	*spx* promoter fragment (-100) in pDL	This study
pML23	*yjbC* promoter fragment in pDL	This study
pML25	*spx* promoter fragment (wild-type +50) in pUC19	This study
pML26	*spx* promoter fragment (wild-type +50) in pDL	This study
pML27	*spx* promoter fragment (+5) in pUC19	This study
pML28	*spx* promoter fragment (+15) in pUC19	This study
pML29	*spx* promoter fragment (+30) in pUC19	This study
pML30	*spx* promoter fragment (+5) in pDL	This study
pML31	*spx* promoter fragment (+15) in pDL	This study
pML32	*spx* promoter fragment (+30) in pDL	This study
pML33	*spx* promoter fragment (+40) in pUC19	This study
pML34	*spx* promoter fragment (+40) in pDL	This study
pML35	Random mutagenesis of *spx* promoter in pUC19	This study
pML36	Random mutagenesis of *spx* promoter in pDL	This study
pML42	*spx(T−26A)* in pDL	This study
pML43	*spx(T−20G)* in pDL	This study
pML44	*spx(T−19G)* in pDL	This study
pML45	*spx(A3G)* in pDL	This study
pML46	*spx(A−14T)* in pDL	This study
pML47	*spx(T7C)* in pDL	This study
pML48	*spx(T24C)* in pDL	This study

[a]wt, wild-type.

Table 2.2 Oligonucleotides used

Oligonucleotide	Sequence
oMLbgaB	5′-CCCCCTAGCTAATTTTCGTTTAATTA-3′
oML02-6	5′-GATGATACGATCTATTAGTTTGCCAAAC-3′
oML02-7	5′-CCCAAGCTTGCATTTCAAGCATGCTCAG-3′
oML02-10	5′-TCAATTGGAAAGTACTCGCTAAGC-3′
oML02-15	5′-TCCTCTAATTAGTAGGATGAACAT-3′
oML02-22	5′-CGGGATCCCGAACATCTATTTTATTC-3′
oML02-23	5′-GGAATTCCGATACTGTTCACACTT-3′
oML02-24	5′-GGAATTCCTTGACACATTTTTTTC-3′
oML02-25	5′-GGAATTCCAGAATAAGAACATATC-3′
oML02-26	5′-CGGGATCCTCTTTACAGGTATTA-3′
oML02-27	5′-CGCGGATCCAAAAAAGGAATCTTT-3′
oML02-28	5′-CGCGGATCCAATTGAAATTACTC-3′
oML02-29	5′-CGCGGATCCTTTATTCTTAAATTG-3′
oML02-37	5′-GGAATTCCGGTACCGGCGCCGGTCATT-3′
oML02-38	5′-GTTCACACaTACTTTTTTATAG-3′
oML02-39	5′-AAAAGTAtGTGTGAACAGTATCC-3′
oML02-40	5′-CACTTACTTgTTTATAGTATAATACC-3′
oML02-41	5′-CTATAAAcAAGTAAGTGTGAAC-3′
oML02-42	5′-CACTTACTTTgTTATAGTATAATACC-3′
oML02-43	5′-CTATAAcAAAGTAAGTGTGAAC-3′
oML02-44	5′-ACTTTTTTATtGTATAATACCTG-3′
oML02-45	5′-CTATAAcAAAGTAAGTGTGAAC-3′
oML02-46	5′-ACCTGTAAgGATTCCTTTTTTAGAG-3′
oML02-47	5′-AGGAATCcTTACAGGTATTATACT-3′
oML02-48	5′-GTAAAGATcCCTTTTTTAGAGTAA-3′
oML02-49	5′-CTAAAAAAGGgATCTTTACAGGT-3′
oML02-50	5′-GAGTAATcTCAATTTAAGAATAA-3′
oML02-51	5′-TAAATTGAgATTACTCTAAAAAAGG-3′

[a]Bases in lowercase type are mutagenic

Table 2.S1 Percentage of auxotrophic by UV mutagenesis

Irradiation time (min)	Auxotrophic (%)
0.5	1
1	2
2	2
3	7
5	25 (2 auxotrophic out of 8 colonies)
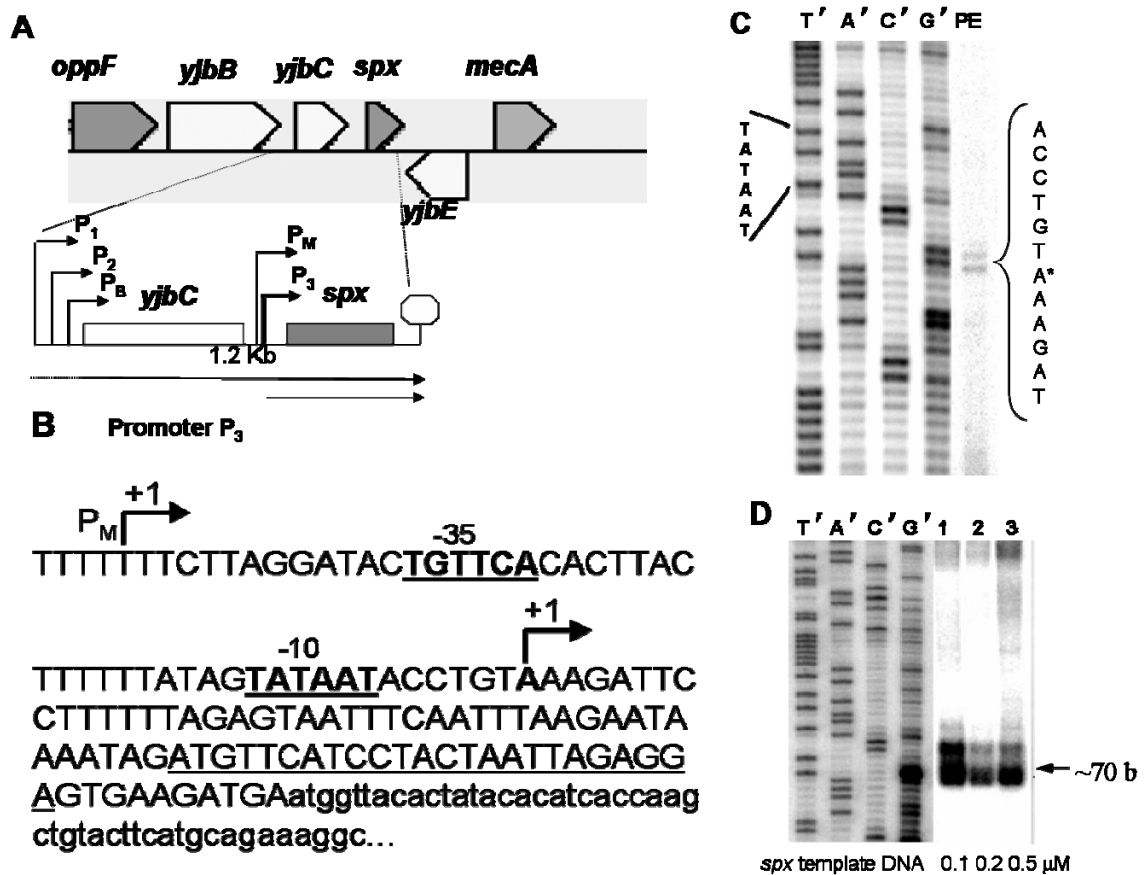
Figure 2.1 (A) Organization of *yjbC-spx* operon. Promoters (P$_1$, P$_2$ and P$_B$) of *yjbC* are indicated by bent arrows as is the P$_M$ promoter of the *spx* gene recognized by σ^M RNAP (Jervis et al., 2007). The arrow beneath the operon diagram indicates 1.2 kb mRNA identified by northern blot analysis [hybridization labeled-probe specific for *yjbC* (Antelmann et al., 2000)]. The *spx* promoter P$_3$ is indicated by the bold, bent arrow. (B) Sequence of the *spx* P$_3$ promoter region. The -10 and -35 promoter sequences are indicated by bold, underlined letters. The P$_3$ transcription start site is marked by +1 and an arrow. The complemented nucleotide sequence of the labeled primer used in the primer extension experiments is underlined. The nucleotide sequence of the *spx* coding sequence is shown in lower case letters. (C) Denaturing polyacrylamide gel profile of primer extension reaction using RNA extracted from JH642 cells. Sequencing reaction and primer extension utilized the primer described in B. (D) Denaturing polyacrylamide gel profile of in vitro run-off transcription products of reactions containing purified RNAP (100 nM), *spx* promoter DNA (0.1,0.2 and 0.5 μM as indicated), and radiolabeled nucleotide triphosphate mix. Reactions were incubated at 37°C for 20 min.
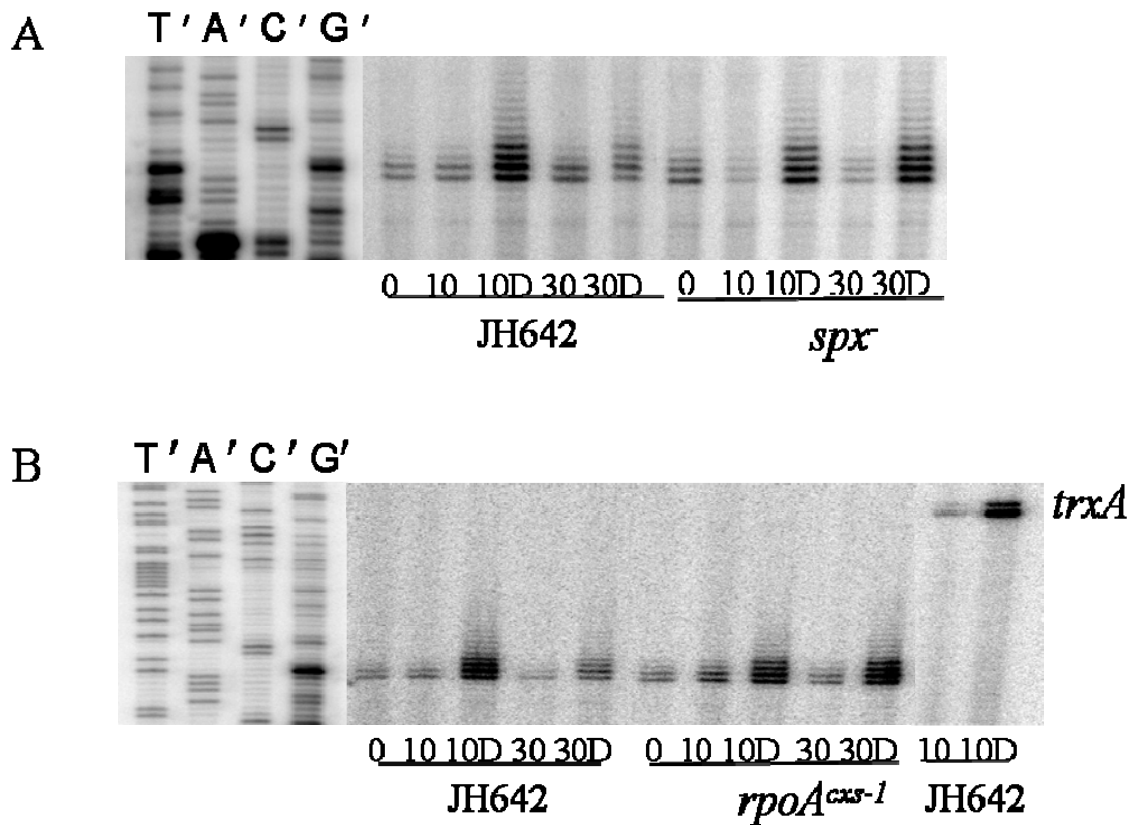
Figure 2.2 Primer extension analysis shows the increase in *spx* transcript levels after diamide treatment. Total RNA was extracted from cells grown in TSS medium and harvested at mid-log phase (0) and then after cells were treated for 10 and 30 min with 1 mM diamide (10D and 30D, respectively) and without diamide (10 and 30, respectively). The labeled primer shown in Fig. 2.1B was used for primer extension reactions. The gel profile of the primer extension reaction using a *trxA*-specific primer (Nakano et al., 2003a) was a control for diamide-induced transcript accumulation. The dideoxy sequencing ladders are shown on the left. For dideoxynucleotide sequencing, the nucleotide complementary to the dideoxynucleotide added in each reaction mixture is indicated above the corresponding lane (T′, A′, C′ and G′). (A) Total RNA was extracted from wild-type JH642 and ORB3834 (*spx* null) cells. (B) Total RNA extracted from wild-type JH642 and ORB3621 (*rpoA*$^{cxs-1}$) cells.
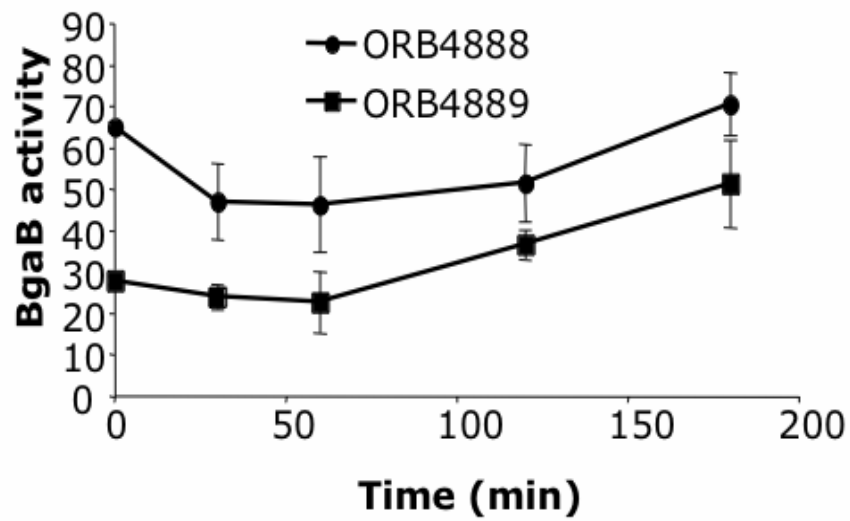
Figure 2.3 Assay of *yjbC-spx-* and *spx*-directed β-galactosidase (BgaB) activity. Expression of *yjbC-spx-bgaB* and *spx-bgaB* was determined as BgaB activity in Milller units. Cells were grown in DSM. Time 0 indicates the mid-log phase. ●, ORB4888 (*yjbC-spx-bgaB*); ■ , ORB4889 (*spx-bgaB*).
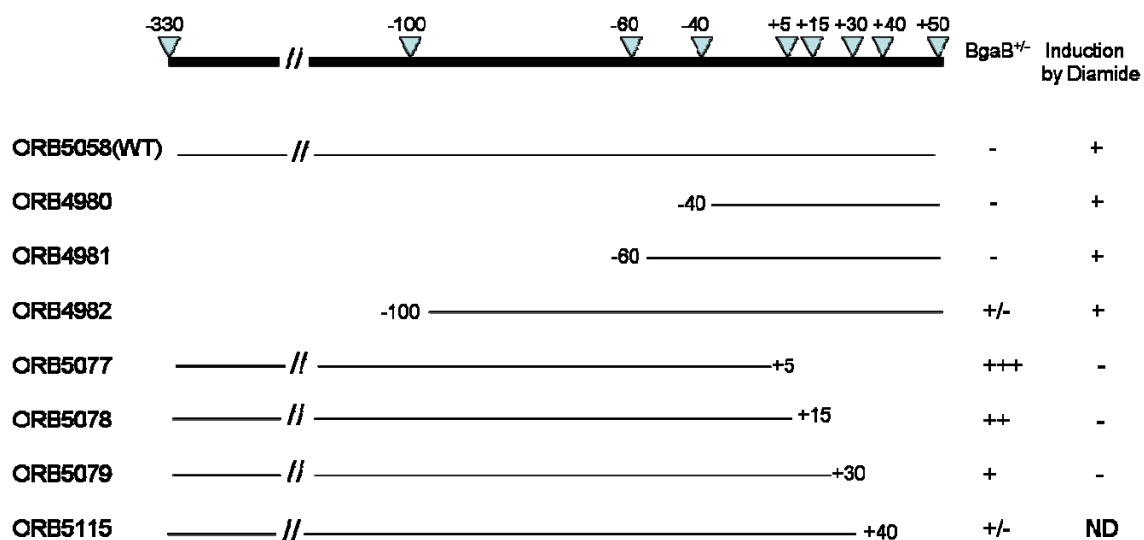
Figure 2.4 Summary of deletion analysis of *spx* P$_3$ promoter region. The top bar shows the intact *spx* promoter and the positions of deletion endpoints relative to the transcription start site. All constructs generated by PCR were fused with the promoterless *bgaB* gene as the reporter. The *bgaB* expression from the respective fusions after integration into the chromosome was examined by screening on DSM agar plates containing 40 μg/ml X-gal (blue +, white – and pale blue +/-). Transcriptional induction determined by extraction of RNA from diamide treated and untreated cells bearing *spx-bgaB* fusions followed by primer extension analysis. + indicates the transcriptional induction after diamide treatment was detected. – indicates loss of induction was detected after diamide treatment. ND, no data.
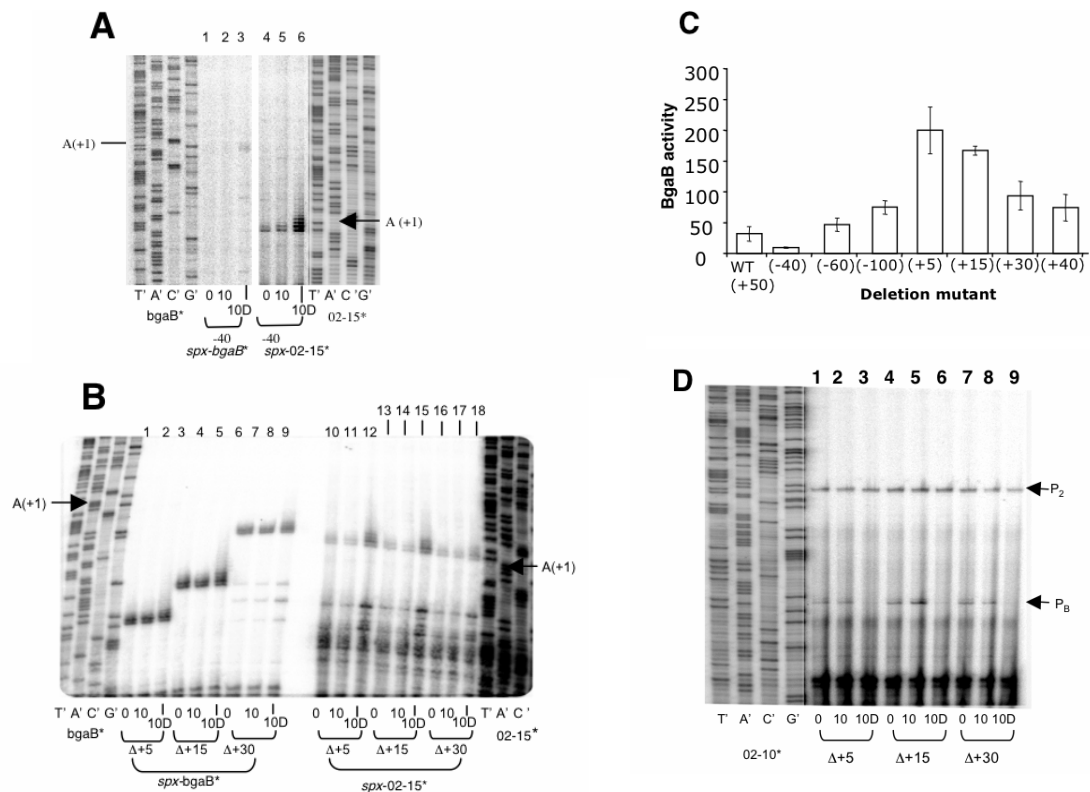
Figure 2.5 Primer extension analysis of RNA extracted from -40 deletion ORB4980 (A), 3′ deletion ORB5077 (Δ+5), ORB5078 (Δ+15) and ORB5079 (Δ+30) (B,C) cells in TSS cultures subjected to diamide treatment. Cells were treated with 1 mM diamide (10D) and without diamide (0 and 10 min) after OD_{600} reached 0.4-0.5. Labeled primers specific to the *bgaB* fusion (bgaB*) and endogenous *spx* (02-15*) were used to detect *spx* transcripts. Marker lanes of sequencing reactions show the nucleotide positions corresponding to the *spx-bgaB* (A, B), endogenous *spx* (A, B) start points. (A) Primer extension analysis of *spx-bgaB* and endogenous *spx* transcripts in total RNA extracted from -40 deletion strain (ORB4980). (B) Primer extension analysis of *spx-bgaB* and endogenous *spx* transcripts in total RNA extracted from 3′ deletion strains (ORB5077 (Δ+5), ORB5078 (Δ+15), and ORB5079 (Δ+30). (C) Expression of *spx-bgaB* fusion derivatives bearing promoter deletions as determined by assays of *spx*-directed β-galactosidase activity. Cells were grown in DSM. The expression was determined as BgaB activity in Miller units at time 30 min after cultures reached mid-log phase. (D) Primer extension analysis of *yjbC* transcripts in total RNA extracted from 3′ deletion strains.
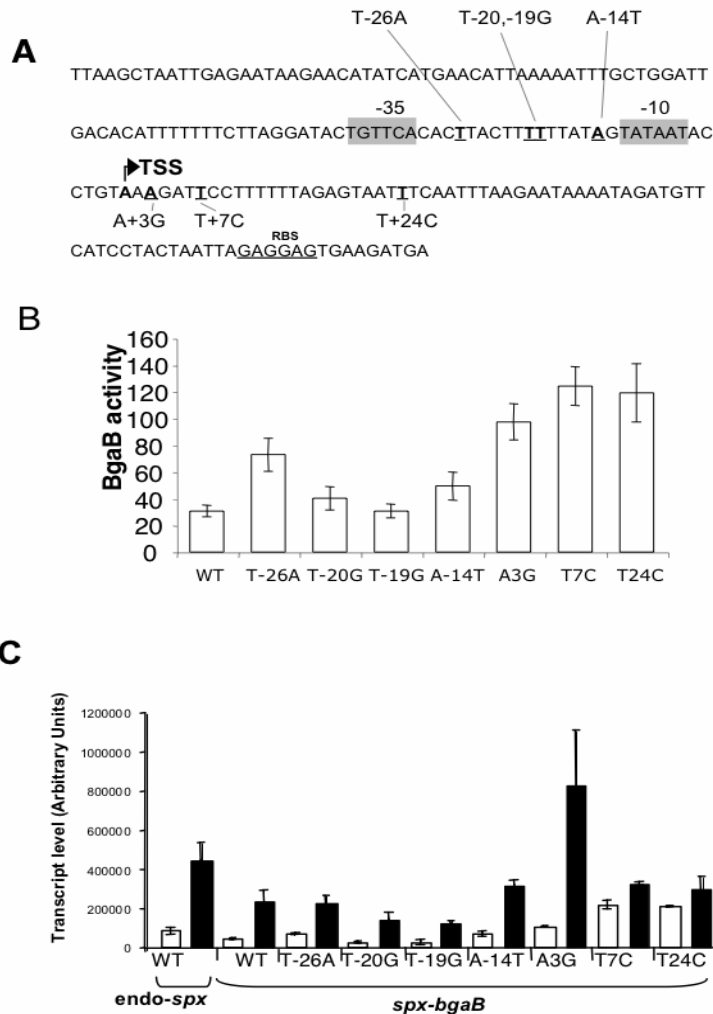
Figure 2.6 Analysis of *spx* promoter P$_3$ point mutations. (A) *spx* promoter region. *spx* sequence shown along with the locations of seven nucleotide substitutions as indicated by underline (T-26A, T-20G, T-19G, A-14T, A+3G, T+7C and T+24C). The bent arrow indicates transcription start site, TSS. RBS, ribosome binding site. -10 and -35 promoter sequences are indicated by shaded boxes. (B) The PCR fragments of each point mutation bearing *spx* sequence from -330 to +50 were inserted into plasmid pDL (the *bgaB* fusion vector). The constructed plasmids were integrated into the *amyE* locus of *B. subtilis*. Cells were grown in DSM. The expression was determined as BgaB activity in Miller units at time 30 min after cultures reached mid-log phase. (C) Primer extension analysis of point mutations in the P$_3$ sequence of the *spx-bgaB* fusion after diamide treatment. The transcripts of the *spx* fusions and endogenous *spx* (endo-*spx*) of each point mutant without (white bar) and with (black bar) diamide treatment for 10 min. RNA was extracted from cells harvested from cultures that reached OD$_{600}$ of 0.4-0.5. *spx-bgaB* transcripts were detected by primer extension analysis. Results are presented, with standard deviation, for radiolabeled primer extension product from two independent RNA extracts and duplicate primer extension reactions for each extract. endo-*spx* denotes endogenous *spx* gene transcript level.
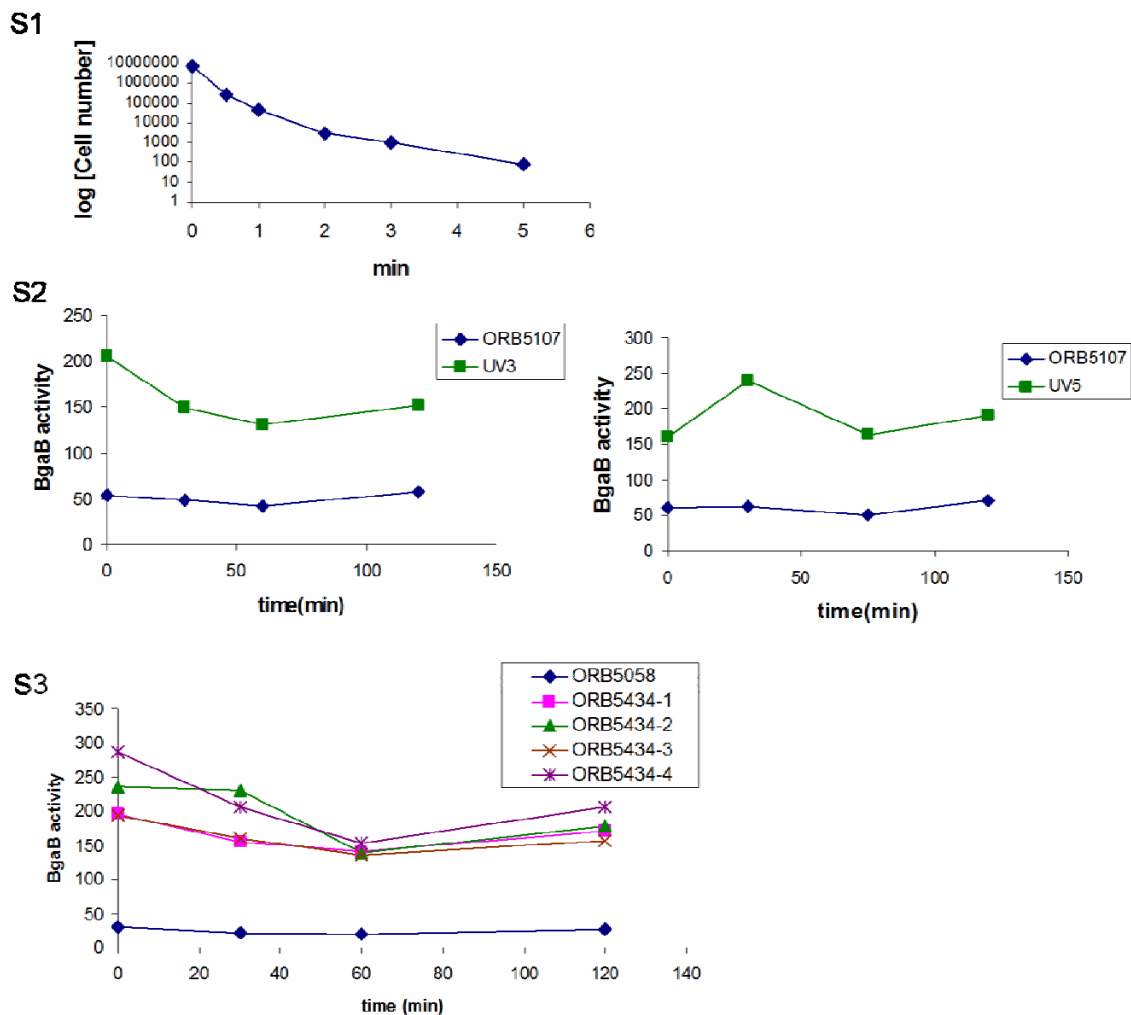
Figure 2.S1 Survival rate of UV-treated cells. Serial dilution of untreated cells (0 min) and UV-treated cells for 0.5, 1, 2, 3 and 5 min were plated for counting isolated colonies to determine survival rate. **S2** The expression of UV-treated *spx-bgaB* fusion cells. Untreated cells (ORB5107) and UV-treated cells (UV3 and UV5) were grown in DSM, and their β-galactosidase (BgaB) activities were determined. Time zero indicates the mid-log phase. **S3** The expression of BgaB fusion cells containing random nucleotide substitutions within the *spx* promoter region. The wild-type *spx-bgaB* fusion cells (ORB5058) and the pool of mutagenized *spx* fragments in BgaB fusion cells (ORB5434) were grown in DSM and samples were collected for β-galactosidase assay.

CHAPTER 3

DUAL NEGATIVE CONTROL OF SPX TRANSCRIPTION INITIATION FROM THE P₃ PROMOTER BY REPRESSORS PERR AND YODB IN BACILLUS SUBTILIS[*]

3.1   INTRODUCTION

Spx is a highly conserved transcriptional regulatory protein of low GC content Gram- positive bacteria (Chatterjee et al., 2006, Duwat *et al.*, 1999, Frees *et al.*, 2001, Pamp et al., 2006, van de Guchte *et al.*, 2006, Zuber, 2004). It does not possess sequence-specific DNA-binding activity, but instead directly targets RNA polymerase (RNAP) (Nakano et al., 2003b, Newberry et al., 2005), an interaction that interferes with contact between transcriptional activators (Nakano et al., 2003b, Zhang et al., 2006) and RNAP, while activating transcription at promoters of genes whose products function in intracellular thiol homeostasis (Nakano et al., 2005, Nakano et al., 2003a) and responses to encounters with toxic oxidants (Nakano et al., 2003a, Pamp et al., 2006, Tam et al., 2006a, Tam et al., 2006b). Recent studies suggest that Spx plays a role in cellular invasion by a microbial pathogen (Chatterjee et al., 2006). Spx does not belong to any other known family of transcriptional regulatory proteins, but resembles the arsenate reductase ArsC of plasmid R773, in terms of its primary and higher order structure (Martin *et al.*, 2001, Newberry et al., 2005, Zuber, 2004).

The *spx* gene resides in the *yjbC-spx* operon of the *B. subtilis* genome and is transcribed from at least five promoters by four forms of RNAP holoenzyme (σ^A, σ^B, σ^M,

and σ^W). Induction of *spx* expression has been associated with phosphate starvation, ethanol and oxidative stress (Antelmann et al., 2000, Nakano et al., 2003a, Thackray & Moir, 2003), while induction of the *spx* regulon is activated by a variety of stress conditions that include heat shock, salt stress, oxidative stress, and toxic phenolic compounds (Nakano et al., 2003a, Tam et al., 2006b, Tam et al., 2006a). Control of *spx* expression in response to oxidative stress operates at three levels. Spx protein levels increase independently of *spx* transcriptional control, perhaps by down-regulation of ClpXP-catalyzed proteolysis (Nakano et al., 2003a, Nakano et al., 2002b). Spx activity is under redox control through its CxxC disulfide center (Nakano et al., 2005). And, finally, transcription from the newly discovered P_3 promoter of *spx* is induced by disulfide stress, as shown in the accompanying paper. The tight control of *spx* expression and the activity of its product likely provides assurance that Spx is not overproduced inappropriately, since high concentrations of Spx have a severe effect on growth and a variety of transition state developmental processes (Nakano et al., 2001, Nakano et al., 2000b, Nakano et al., 2003a, Nakano et al., 2003b).

The analysis of the P_3 promoter region of *spx* uncovered two negatively *cis*-acting elements, mutations in which cause derepression of *spx* transcription under normal growth conditions. In this report, we show that the two sites participate in repression of *spx* transcription that is exerted by the direct interaction of two negative transcriptional regulators, PerR and YodB. PerR is the previously characterized peroxide stimulon control factor (Herbig & Helmann, 2001, Lee & Helmann, 2006b), while YodB is a novel DUF24 family member that exerts repression that is sensitive to treatment with oxidants.

3.2   RESULTS

3.2.1   Two genes identified that negatively control *spx* transcription

In the accompanying paper, the discovery of the *spx* P_3 promoter and its activation during disulfide stress are described. P_3 resides in the intergenic region between *yjbC* and *spx*, 79 bp upstream from the *spx* ATG start codon. It is utilized by the σ^A form of RNA polymerase and is the only transcriptional start site detected in the *yjbC-spx* intergenic region when cells are grown under the conditions (non stress and disulfide stress) used in

our studies. Mutational analysis has uncovered two potential *cis*-acting negative control elements for P_3-directed transcription initiation, one downstream of the transcriptional start site and the other within the P_3 promoter itself.

Four different forms of RNA polymerase (σ^A, σ^B, σ^W, and σ^M) contribute to *spx* transcription, and there has been one published report of a negative regulatory factor, PerR, that controls *spx* expression (Hayashi et al., 2005). We sought to uncover other transcription factors that exert negative control on transcription from P_3 using transcription factor/transformation array technology (Hayashi *et al.*, 2006). A *lacZ* fusion of the *yjbC-spx* intergenic region extending from -538 to the *spx* stop codon was constructed and screened for increased β-galactosidase activity in the transcription factor mutant backgrounds.   Figure 3.1 shows a portion of the array, in which a colony of the fusion bearing strain transformed with *yodB*::*cat* DNA appears slightly more blue on the X-gal DSM agar plate than neighboring transformant colonies.

To validate the transformation array results and the previously reported microarray data, the *spx-bgaB* fusion was introduced into a *perR* and a *yodB* drug resistance-insertion mutant and fusion expression was examined. Expression of *spx-bgaB* was constant throughout growth and into stationary phase in wild-type *B. subtilis* cells (Fig. 3.2A). In the *perR* mutant (ORB6268), *spx-bgaB* activity increased significantly over that of wild-type cells. A lower level of derepression was observed in the *yodB* mutant and an additive effect of *yodB* and *perR* absence was observed in the double mutant. Fig. 3.2B shows the activity of *spx-bgaB* in a *yodB* mutant (ORB6288) that bears an ectopically expressed wild-type copy of *yodB*, which confirms that the absence of YodB in the *yodB* insertion mutant is the cause of *spx-bgaB* derepression.

Primer extension analysis was performed to determine the level of P_3 transcript in JH642 cells as well as cells of the *yodB* mutant derivative (ORB6208), *perR* mutant (ORB6267), and the *yodB perR* double mutant (ORB6324). As had been shown previously, diamide treatment increased *spx* P_3 transcript levels (Fig 3.3, lanes 1-3). An increase in P_3 transcript was also observed in the diamide-treated *yodB* (lanes 4-6) and *perR* (lanes 7-9) mutant cells. Importantly, P_3 transcript concentration was high in untreated *yodB* and *perR* mutants when compared to untreated wild-type cells (compare lanes 1 and 2 to 4-5 and 7-8, which is indicative of the roles the two regulators play in the

negative control. A further increase was observed in the untreated cells of the *yodB perR* double mutant (lanes 10-11), as was predicted from the BgaB assay data of Figure 3.2 which showed a level of transcriptional activity that was higher than the activity of either the *yodB* or *perR* single mutant. Unexpectedly, the double mutant, when treated with diamide, reproducibly showed sharply reduced *spx* P_3 transcript (lane 12).

3.2.2 Purified PerR and YodB proteins repress transcription from *spx* P_3 *in vitro*

YodB and PerR protein was obtained from *E. coli* expression systems in a His-6 tagged form for use in transcription reactions and protein-DNA binding experiments. Both proteins were purified from cleared lysates by Ni-chelate and ion exchange chromatography. The PerR protein was used in His-tagged form (Hayashi et al., 2005), while His-6 YodB was proteolytically cleaved to release the N-terminal His-tag sequence (see Experimental Procedures).

The purified YodB and PerR proteins were added to in vitro run-off transcription reactions containing purified RNAP and σ^A from *B. subtilis* and linear *spx* P_3 DNA fragments generated by PCR. Template DNA was also obtained by PCR amplification of mutant P_3 promoter DNA that was isolated as described in the accompanying paper. The *spx* mutations T24C, T-26A, T-20G, T-19G, and A-14T caused *spx* transcription from P_3 to be higher than that observed in wild-type cells (Leelakriangsak & Zuber, 2007). Deletions of the region 3′ to the P_3 transcription start site also resulted in derepression of transcription from P_3. Addition of YodB to the run-off transcription reactions showed that YodB could repress transcription from P_3 in vitro (Fig. 3.4), but not when template DNA was made from T-26A, T-20G, or T-19G DNA. The A-14T template showed reduced YodB-dependent repression compared to +40 deletion P_3 DNA. YodB could repress transcription of a template made from T24C mutant DNA or from DNA of the deletion mutant Δ+5 (lacking DNA 3′ from position +5). In contrast, PerR could repress transcription of DNA made from +40 deletion, T-26A, T-20G, T-19G, or A-14T *spx* P_3 DNA, but not from Δ+5 or from T24C. Neither repressor significantly affected transcription from the control *rpsD* (ribosomal protein S4) promoter DNA. These results suggested that sequences in the *spx* P_3 promoter were required for YodB-dependent repression, whereas sequences 3′ of the *spx* P_3 start site were necessary for PerR-

dependent repression. As predicted from work described in the accompanying paper, the *in vitro* transcription analysis uncovered two *cis*-acting elements, one being the site of YodB interaction, and one required for PerR-dependent control (Leelakriangsak & Zuber, 2007).

Further verification of the roles of YodB and PerR in the control of *spx* transcription initiation and the respective *cis*-acting elements was obtained by examining the *in vivo* basal level of *spx* expression using the *spx-bgaB* fusion construct. Fig. 3.5 shows that expression of mutant *spx-bgaB* fusions bearing mutations in the regulatory *cis*-acting sites in wild-type cells and *perR* (Fig. 3.5A) or *yodB* (Fig. 3.5B) mutant cells. Only the two mutations downstream of the transcription start site elevated significantly lowered the ratio of basal BgaB activity in *perR* vs. wild-type cells (Fig. 3.5A), while the upstream mutations residing in the P_3 promoter affected the ratio of *yodB* vs. wild-type expression. These data confirm that YodB acts on the upstream *cis* regulatory sequence, while PerR acts on the negative control element located 3′ to the transcription start site.

3.2.3   YodB and PerR proteins interact with two distinct regions of the *spx* P_3 promoter DNA

DNase I footprinting analysis was performed to confirm that the regions of *spx* P_3 DNA required for transcriptional repression *in vitro* were sites of YodB and PerR interaction. End-labeled P_3 promoter DNA was combined with YodB protein, PerR protein or both, followed by digestion with DNase I, denaturing gel electrophoresis and phosphorimaging. As predicted from mutational and in vitro transcriptional analysis, PerR interacted with DNA 3′ to the start site of P_3 transcript synthesis, protecting an area from approximately -3 to +35 (Fig. 3.6A). YodB protected a region from -3 to -32 (Fig. 3.6A, lanes 8-10), which encompasses the nucleotide positions that are the sites of mutations that affect YodB-dependent repression.

Reduced binding of YodB was observed in footprinting reactions that contained *spx* promoter DNA with either the T-26A (Fig. 3.6B) or T-19G (Fig. 3.6C, and see Fig. 3.8) mutations, both of which give rise to elevated *spx* transcription in vivo. Neither mutation affected binding of PerR (Fig. 3.6B and C). The mutations T24C residing within the envelope of PerR contact (see Fig, 3.8), caused an altered DNaseI digestion pattern

(Fig. 3.6D, compare lanes 1 and 4) and reduced the protection afforded by PerR interaction (Fig. 3.6D, lanes 2-3 and 5-6). These findings are consistant with the *in vivo* and *in vitro* phenotype of the *spx* P$_3$ mutations and the *yodB* and *perR* insertion mutations.

3.2.4 Diamide and hydrogen peroxide reduce binding of YodB and PerR proteins to *spx* P$_3$ promoter DNA

The *spx* gene is a member of the PerR regulon (Hayashi et al., 2005) and we reasoned that binding of PerR to *spx* promoter DNA might be sensitive to toxic oxidants such as diamide and H$_2$O$_2$. Using the DNase I protection assay, we determined if binding of PerR and YodB to P$_3$ was reduced if diamide or H$_2$O$_2$ was added to the footprinting reactions. As shown in lanes 1-5 of Fig. 3.7A, PerR bound poorly to the downstream operator sequence when diamide was present. The same result was observed when diamide was added to a binding reaction containing YodB and *spx* P$_3$ promoter DNA (Fig. 3.7A, lanes 6-11). Binding of both PerR and YodB in combination is also impaired by diamide (lane 12). Diamide did not affect the repressor CymR (Choi et al., 2006, Even et al., 2006) interaction with one of its target, the *yrrT* operon promoter (Fig. 3.7B), nor did it affect the activity of DNase I (lanes 1 and 2). Addition of hydrogen peroxide to the DNase I footprinting reactions (Fig, 3.7C) also impaired the binding of PerR (lanes 3-6), YodB (lanes 7-10) and YodB plus PerR (lanes 11 and 12) to the *spx* P$_3$ promoter DNA. The concentrations used are slightly higher than the range of concentrations used in previous studies (10 mM to 75 mM) to inactivate PerR repressor *in vitro* (Herbig & Helmann, 2001). Again, DNase I activity was not impaired by H$_2$O$_2$ at the higher concentration tested in the DNA-protein binding experiments (Fig. 3.7C, lanes 1 and 2).

The above results suggest that hydrogen peroxide treatment would lead to impairment of YodB/PerR-dependent negative control. Indeed, we observed increased *spx*P$_3$-directed β-galactosidase activity in *spx-lacZ* fusion bearing cells after treatment with 100 μM H$_2$O$_2$. Untreated cells showed activity of 68.4±1.8 and treated cells had activity of 98.7±0.8 after 30 min. H$_2$O$_2$ treatment.

3.3 DISCUSSION

The *yjbC-spx* operon is under complex transcriptional control involving the activities of four RNAP holoenzyme forms utilizing promoters in the *yjbC* promoter regions and in the intergenic region of *yjbC* and *spx*. Under the growth conditions used in the study reported here and the accompanying paper (Leelakriangsak & Zuber, 2007), transcription of *spx* was observed to be initiated from an intergenic promoter, P$_3$, that is recognized by the major σ^A form of RNAP. Although a promoter recognized by σ^M resides in the intergenic region, transcription from its start site was not detected in our experiments. The only promoter that was utilized in response to oxidative stress in our experiments was the P$_3$ promoter.

Studies described in the accompanying paper uncovered the P$_3$ promoter and two putative *cis*-acting negative control elements, mutations in which caused derepression of *spx* transcription *in vivo* (Leelakriangsak & Zuber, 2007). As shown herein, the two cis-acting sites are operators for two repressor proteins PerR and YodB. The *perR* and *yodB* null mutations also lead to derepression of *spx* transcription from P$_3$ and the products of the two genes interact directly with *spx* promoter DNA as illustrated in Fig. 3.8. No cooperative interaction between YodB and PerR was detected by DNase I footprinting analysis, (M. L. and P. Z., data not shown). Binding of PerR and YodB to their operators can be reversed by introduction of an oxidant, either hydrogen peroxide or diamide to Protein-DNA binding reactions. We propose that *spx* is regulated at the level of gene transcription by the two repressors, which are inactivated by toxic oxidants.

Previous studies involving genome-wide analysis of gene expression have uncovered *spx* as a stress-induced gene. Proteomic and transcriptomic studies have shown that *spx* transcript levels increase in response to ethanol stress and phosphate limitation (Antelmann et al., 2000, Thackray & Moir, 2003). Mutations of *yjbC* and *spx* have been reported to confer a salt-stress-sensitive phenotype (Petersohn et al., 2001), although our attempts at repeating this result have not succeeded. Transcriptomic analysis of the oxidative stress response have not uncovered the *spx* gene as being induced by reactive oxygen species, but microarray analysis of the PerR regulon showed that *spx* transcript levels increased in a *perR* mutant (Hayashi et al., 2005). Additionally, treatment of cells with the thiol-specific oxidant, diamide was reported to increase *spx*

transcript levels (Leichert et al., 2003). The accompanying paper provides data that diamide induction involves accelerated transcription from the P_3 promoter of *spx* (Leelakriangsak & Zuber, 2007). This is likely due in part to the inactivation of PerR, which interacts with its operator located downstream of the P_3 transcriptional start site.

PerR possesses a zinc binding domain in which a zinc atom is coordinated by four cysteines in a CxxC…CxxC arrangement. The cysteines are quite resistant to oxidation due to the interaction with zinc, and only high concentrations of hydrogen peroxide can oxidize them in vitro with the release of zinc (Lee & Helmann, 2006a). No oxidation of the cysteines was observed *in vivo* (Lee & Helmann, 2006a), indicating that the major oxidant-sensing mechanism of PerR involves the histidines that coordinate ferrous ion (Lee & Helmann, 2006b). PerR is resistant to oxidation by diamide, and releases zinc only when treated with the oxidant under harsh conditions (elevated temperature or in the presence of a denaturant) (Lee & Helmann, 2006a). Thus, the zinc binding domain is believed to serve a structural function, rather than being a major oxidant-sensing domain of PerR. However, diamide treatment results in loss of PerR binding to its operator in the *spx* P_3 promoter, which would implicate the zinc-coordinating cysteine residues as a redox target of the oxidant. Perhaps partial oxidation of the cysteines reduces the affinity of PerR for the operator in *spx* P_3.

The PerR protein used in the experiments reported here was obtained by purification under aerobic conditions that results in loss of the Fe^{++} cofactor by oxidation. This cofactor is required for optimal reactivity of the His residue with peroxide (13, 15). The PerR protein of our studies is mostly devoid of Fe as determined by inductively coupled plasma optical emission spectrometry (ICP-OES, 2-3% of total PerR protein is of the holo-form, data not shown). PerR is responsive to the presence of H_2O_2 in vitro (Fig. 3.7), but likely does not possess optimal reactivity.

YodB is a member of the DUF24 family of winged-helix transcription factors, of which the *B. subtilis* HxlR (Yurimoto *et al.*, 2005) is the only member of known regulatory function. HxlR activates the *hxlAB* operon encoding the ribulose monophosphate pathway of formaldehyde fixation. HxlR is activated when cells encounter formaldehyde, but the mechanism of activation is not known. YodB is also similar to members of the ArsR family of transcriptional repressors, but it lacks the

conserved cysteine residues in the H-T-H motif that function in metalloid coordination via trigonal geometry (Harvie *et al.*, 2006). Most close orthologs of YodB are found in Bacilli, Listeria, Staphylococcus, and Clostridia. These orthologs, along with HxlR, have a conserved cysteine residue at or near position 6 at the N-termini, but YodB is unique among them due to the presence of two cysteine residues near its C-terminus. Like PerR, addition of diamide or hydrogen peroxide to DNase I footprinting reactions resulted in loss of DNA binding activity. Current efforts are focused on the possible role of the cysteine residues in redox control of YodB activity. The *yodB* gene resides adjacent to *yodC*, which encodes a putative nitroreductase. *yodC* and *yodB* are in divergent orientation with respect to one another and preliminary analysis of *yodC-lacZ* expression has suggested that YodB is a repressor of *yodC* transcription (M.L. and P. Z., unpublished). The expression of the *yodB* and *yodC* genes are induced by catechol treatment (Tam et al., 2006b), conditions that also induce the *spx* regulon. Paralogs of YodC include NfrA, an NAD(P)H-linked flavin binding nitroreductase that is encoded by a gene controlled by Spx and induced by heat shock and oxidative stress (Moch *et al.*, 2000, Tam et al., 2006b). YodB might serve as another regulator that participates in the oxidative stress response of *B. subtilis* through its control of *yodC* and *spx*.

A curious observation is the reduction of *spx* transcript in the *perR yodB* double mutant when cells are treated with diamide. The total pleiotropic effects of the two mutations combined are unknown at this time as are the effects on transcription of the *yjbC-spx* operon. These questions are the subject of current investigations.

The study described herein has uncovered yet another level of control that governs the expression of *spx*. Previous reports detailed the redox control of Spx activity mediated by the N-terminal CxxC disulfide motif (Nakano et al., 2005), and post-transcriptional control of Spx protein levels that includes proteolytic turnover of Spx by the ATP-dependent protease ClpXP (Nakano et al., 2003a, Nakano et al., 2002b). The transcriptional control targeting the P_3 promoter represents another layer of regulation involving dual control by PerR and a previously unknown negative control factor, YodB. The conservation of Spx, PerR, and YodB among low %GC Gram positive species suggests an important network of control governing the bacterium's response to toxic oxidants.

3.4   EXPERIMENTAL PROCEDURES

3.4.1   Bacterial strains and growth conditions

Bacillus subtilis strains used in this study are derivatives of JH642 and are listed in Table 3.1. Cells were cultivated in a shaking water bath at 37°C in Difco Sporulation medium (DSM) (Schaeffer *et al.*, 1965) for β-galactosidase assays or TSS minimal medium (Fouet *et al.*, 1990) for diamide treatment experiment. Diamide was purchased from SIGMA-Aldrich. To create ORB6118, the strain used in the transformation array experiment, the following constructions were carried out. Plasmid pSN16 (Nakano et al., 2002a) was digested with EcoRI to release the fragment containing only *spx* promoter (*spx* coding sequencing including 538 bp upstream of P_3) to generate pML8 (*spx-lacZ* fusion). pML8 was transformed into ZB307A (Zuber & Losick, 1987) with selection of chloramphenicol resistance. SPβ-transducing lysate was produced by heat induction then was transduced into ZB278 (Zuber & Losick, 1987). Phage generated from this strain was used to transfer the *spx-lacZ* fusion into wild-type background by transduction into JH642 with selection for chloramphenicol resistance to generate strain ORB4563. Plasmid pCm::Sp (Steinmetz & Richter, 1994) was used to transform to ORB4563 replacing Cm^r cassette to Spc^r cassette to generate strain ORB6118.

The isogenic *yodB* and *perR* mutants were constructed by transformation of JH642 with chromosomal DNA of TF277 (this study) and HB2078 (Fuangthong et al., 2002), respectively. The resulting strains were designated ORB6208 (*yodB*::*cat*) and ORB6267 (*perR*::*kan*).

The *yodB* mutant (TF277) was constructed as follows. Upstream and downstream regions of *yodB* were amplified by PCR with the primer sets yodB-F1/yodB-R1 and yodB-F2/yodB-R2 (Table 3.2), respectively. The *cat* (chloramphenicol acetyltransferase) gene was amplified by PCR from plasmid pCBB31 using primers PUC-F and PUC-R. The 5′ ends of yodB-F2 and yodB-R1 are complementary to the sequence of PUC-F and PUC-R, respectively. Three PCR products were mixed and used as templates for the second PCR with primers yodB-F1 and yodB-R2 (Table 3.2). The resultant PCR fragment amplified via overlap extension was used for transformation of *B. subtilis* 168.

The fragment containing *spx* P$_3$ promoter region (-330 to +50 relative to the P$_3$ transcription start site) was fused with the promoterless *bgaB* gene (plasmid pDL, (Yuan & Wong, 1995)) as the reporter that was integrated into the *amyE* locus of JH642 to generate strain ORB5058 (spxP$_3$Δ+50(wt)-*bgaB*) (Leelakriangsak & Zuber, 2007). The plasmid pCm::Tc (Steinmetz & Richter, 1994) was used to transform ORB5058 competent cells, thereby replacing *cat* cassette with a *tet* (tetracycline resistance) cassette to generate strain ORB6284. For *perR*, *yodB* and *perR yodB* disruption strains bearing the *spx-bgaB* fusion, chromosomal DNA of ORB6208 (*yodB*::*cat*) was transformed into ORB6284 to generate ORB6288 (*spx*P$_3$Δ+50(wt)-*bgaB*, *yodB*::*cat*). Chromosomal DNA of ORB6267 (*perR*::*kan*) was used to transform cells of strain ORB5058 to generate ORB6268 (*spx*P$_3$Δ+50(wt)-*bgaB*, *perR*::*kan*). The *yodB perR* double mutant was constructed by transformation of ORB6288 with chromosomal DNA from ORB6267 to generate ORB6594 (*spx*P$_3$Δ+50(wt)-*bgaB*, *yodB*::*cat*, *perR*::*kan*).
For complementation experiment of *yodB*, Primers oyodB-EcoRI and oyodB-HisB were used to amplify the *yodB* gene from *B. subtilis* strain JH642 chromosomal DNA. The PCR fragment (about 550 bp, including the coding region of *yodB* as well as 200 bp of upstream sequence and 15 bp of downstream sequence) was digested with EcoRI and BamHI and ligated with pUC19 digested with the same enzymes to generate pML63. The *yodB* sequence in plasmid pML63 was verified by DNA sequencing. pML63 was cleaved with EcoRI and BamHI and the released *yodB* fragment was inserted into pDG795 (Guerout-Fleury *et al.*, 1996) that was digested with the same enzymes, to generate pML64. pML64 was introduced by transformation, with selection for erythromycin/lincomycin, into *B. subtilis* strain JH642, where the *yodB* fragment integrated into the *thrC* locus. The resulting strain was designated ORB6606 (*thrC*::*yodB*). Chromosomal DNA of ORB6606 was used to transform ORB6288 to generate ORB6616 (*spx*P$_3$Δ+50(wt)-*bgaB*, *yodB*::*cat*, *thrC*::*yodB*). Cells were grown in DSM medium until OD600 ~ 0.4-0.5. The cells were harvested and prepared for β-galactosidase assays after further incubation for 30, 60 and 120 min.

Double mutants bearing *spx-bgaB* fusions with *cis*-acting mutations and *yodB* or *perR* mutations were constructed as follows. The plasmid pCm:Sp was used to transform ORB6208 competent cells, thereby replacing *cat* cassette with a *spc* cassette to generate

strain ORB6299. Chromosomal DNA of ORB6267 (*perR::kan*) or ORB6299 (*yodB::spc*) was used to transform ORB6030 (T-26A), ORB6031 (T-20G), ORB6032 (T-19G), ORB6033 (A-14T), ORB6034 (A3G), ORB6035 (T7C) and ORB6036 (T24C) to generate ORB6684 (T-26A, *yodB::spc*), ORB6685 (T-26A, *perR::kan*), ORB6686 (T-20G, *yodB::spc*), ORB6687 (T-20G, *perR::kan*), ORB6688 (T-19G, *yodB::spc*), ORB6689 (T-19G, *perR::kan*), ORB6690 (A-14T, *yodB::spc*), ORB6691 (A-14T, *perR::kan*), ORB6692 (A3G, *yodB::spc*), ORB6693 (A3G, *perR::kan*), ORB6694 (T7C, *yodB::spc*), ORB6695 (T7C, *perR::kan*), ORB6696 (T24C, *yodB::spc*) and ORB6697 (T24C, *perR::kan*).

3.4.2 Transcription factor array analysis

The transcription factor/transformation array analysis was performed as previously described (Hayashi et al., 2006). Competent cells of strain ORB6118 bearing the prophage SPβ*c2del*::Tn*917*::pML8-15 (*spx*P$_3$-*lacZ*) were used for the transformation array.

3.4.3 Protein purification

The *yodB* coding sequences was amplified by PCR using primer oYodB-HisN and oYodB-HisB (Table 3.2). The PCR product were digested with NdeI and BamHI restriction enzymes and inserted into pPROEX-1 (Life Technologies) digested with the same enzymes to generate pML54. *E.coli* M15 cells carrying pRep4 and pHis-PerR (Hayashi et al., 2005) was cultured in 100 ml LB medium or BL21 (pML54) was cultured in 500 ml LB medium and IPTG (final concentration; 0.5 mM) was added at the mid-log phase (OD600= 0.6). After 5 hr, the cells were harvested, collected by centrifugation and resuspended in lysis buffer A (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole) with 1 mM PMSF. The cells were disrupted using a French pressure cell at 1,000 psi and centrifuged. An equal volume of 50% Ni-NTA resin (Qiagen) was added to the lysate, mixed into the column and shaken at 4°C for 3 hr. The column was washed with wash buffer B (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 20 mM imidazole). His6-PerR or His6-YodB was eluted with elution buffer C (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 200 mM imidazole). PerR or YodB eluted from Ni-NTA column was further purified by High Q column

chromatography (Bio-Rad) with 50-500 mM NaCl gradient. 1 mg of His-tagged YodB was incubated with 500 U AcTEV^{TM} (Invitrogen) at 30° C for 4 hr to remove the N-terminal His6 tag. The AcTEV^{TM} protease was removed from the cleavage reaction by Ni-NTA resin followed by elution the YodB with elution buffer containing 20-40 mM imidazole. The purified PerR and cleaved-YodB were extensively dialysed against TEDG buffer (50 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 2 mM DTT and 10% glycerol) and stored at -80°C.

3.4.4 *In vitro* transcription assay

Linear DNA templates for *rpsD* and *spx* promoters were generated by PCR with primer oSN86 and oSN87 (Nakano et al., 2005) (encoding 71-base transcript), and with oML02-37 and oML02-22 (encoding a 50-base transcript) (Leelakriangsak & Zuber, 2007) respectively. Linear DNA templates for *spx* point mutation promoters were generated by PCR with primer oML02-37 and oMLbgaB encoding 110-base transcript using pML42, pML43, pML44 and pML46 as templates for *spx* T-26A, T-20G, T-19G and A-14T respectively. Primer oML02-37 and oML02-22 (Leelakriangsak & Zuber, 2007) were used to generate T+24C linear DNA templates using pML48 as templates encoding 50-base transcript. The 3′ *spx* promoter deletions DNA templates were also generated by PCR with primer oML02-37 and oMLbgaB (Leelakriangsak & Zuber, 2007) encoding 100-base transcript for +40 and 65-base for +5 using pML34 and pML30 as template, respectively. The 20 nM of DNA templates were mixed with 50 nM RNAP and σ^A (Nakano *et al.*, 2006), then incubated with and without PerR or YodB in 10 mM Tris-HCl pH 8.0, 50 mM NaCl, 5 mM MgCl2, 50 μg/ml BSA, 5 mM DTT at 37 °C for 10 min. A nucleotide mixture (200 μM ATP, GTP and CTP, 10 μM UTP, 10 μcu α-^{32}P-UTP) was added to the reaction. The reaction mixtures (20 μl) were further incubated at 37 °C for 15 min and the transcripts were precipitated by ethanol. Electrophoresis was performed as described (Liu & Zuber, 2000)

3.4.5 DNase I footprinting

DNA probes for *spx* (position corresponding -100 to +70), *spx* T-19G, T-26A and T+24C (-100 to +50) were made by PCR amplification using primer oML02-15 and oML02-25, and primer oML02-25 and oML02-22 (Leelakriangsak & Zuber, 2007), respectively. Plasmids pSN16 (*spx* wild-type promoter), pML42 (*spx* T-19G), pML44 (*spx* T-26A) and pML48 (*spx* T+24C) were used as PCR templates. DNA probe for *yrrT* was made as described previously (Choi et al., 2006). The coding strand primer (oML02-25) (Leelakriangsak & Zuber, 2007) was treated with T4 polynucleotide kinase and [γ-^{32}P]-ATP before the PCRs. The PCR products were separated on a non-denaturing polyacrylamide gel and purified with Elutip-d columns (Schleicher and Shuell). Dideoxy sequencing ladders were obtained using the Thermo Sequenase cycle sequencing kit (USB) and the same primer used for the footprinting reactions. DNase I footprinting reactions were performed in 10 mM Tris-HCl pH 8.0, 30 mM KCl, 10 mM MgCl$_2$, 0.5 mM β-mercaptoethanol. 1 mM and 1.5 mM diamide were used to detect the effect of diamide on the DNA-binding. The concentrations of H$_2$O$_2$ used were 25 mM, 50 mM and 100 mM to detect the effect of H$_2$O$_2$ on the DNA-binding. Proteins were incubated with 100,000 cpm ^{32}P labeled probe at 37°C for 20 min prior to DNase I treatment. The reactions were then precipitated by ethanol and subjected to 8% polyacrylamide-8 M urea gel electrophoresis.

3.4.6 Primer extension analysis

JH642 (wild-type), ORB6208 (*yodB*::*cat*), ORB6267 (*perR*::*kan*) and ORB6324 (*yodB*::*cat*, *perR*::*kan*) were grown at 37°C in TSS medium. Primer extension analysis was performed as described in the accompanying paper (Leelakriangsak & Zuber, 2007).

Table 3.1 *Bacillus subtilis* strains and plasmids used

Strain or plasmid	Relevant phenotype or description	Reference or source
Strains		
JH642	*trpC2 pheA1*	J. A. Hoch
ZB278	*trpC2* SPβ*ᶜ*	(Zuber & Losick, 1987)
ZB307A	SPβ*c2del2*::Tn*917*::pSK10Δ6	(Zuber & Losick, 1987)
HB2078	*att*SPβ *trpC2 perR*::*kan*	(Fuangthong et al., 2002)
TF277	*trpC2 yodB*::*cat*	This study
ORB4563	*trpC2 pheA1* Spβ*c2del2*::Tn*917*::pML8-15	This study
ORB5058	*trpC2 pheA1 amyE*::*spx*P₃Δ+50(wt)-*bgaB cat*	(Leelakriangsak & Zuber, 2007)
ORB6030	*trpC2 pheA1 amyE*::*Pspx*(T−26A)-*bgaB cat*	(Leelakriangsak & Zuber, 2007)
ORB6031	*trpC2 pheA1 amyE*::*Pspx*(T−20G)-*bgaB cat*	(Leelakriangsak & Zuber, 2007)
ORB6032	*trpC2 pheA1 amyE*::*Pspx*(T−19G)-*bgaB cat*	(Leelakriangsak & Zuber, 2007)
ORB6033	*trpC2 pheA1 amyE*::*Pspx*(A−14T)-*bgaB cat*	(Leelakriangsak & Zuber, 2007)
ORB6034	*trpC2 pheA1 amyE*::*Pspx*(A+3G)-*bgaB cat*	(Leelakriangsak & Zuber, 2007)
ORB6035	*trpC2 pheA1 amyE*::*Pspx*(T+7C)-*bgaB cat*	(Leelakriangsak & Zuber, 2007)
ORB6036	*trpC2 pheA1 amyE*::*Pspx*(T+24C)-*bgaB cat*	(Leelakriangsak & Zuber, 2007)
ORB6118	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pML8-15	This study
ORB6208	*trpC2 pheA1 yodB*::*cat*	This study
ORB6267	*trpC2 pheA1 perR*::*kan*	This study
ORB6268	*trpC2 pheA1 amyE*::*spx*P₃Δ+*50*(wt)-*bgaB cat perR*::*kan*	This study

Strain or plasmid	Relevant phenotype or description	Reference or source
ORB6284	*trpC2 pheA1 amyE*::*spx*P$_3$Δ+50(wt)-*bgaB cat*::*tet*	This study
ORB6288	*trpC2 pheA1 amyE*::*spx*P$_3$Δ+50(wt)-*bgaB cat*::*tet yodB*::*cat*	This study
ORB6299	*trypC2 pheA1 yodB pcm*::*spc*	This study
ORB6324	*trpC2 pheA1 yodB*::*cat perR*::*kan*	This study
ORB6594	*trpC2 pheA1 amyE*::*spx*P$_3$Δ+50(wt)-*bgaB cat*::*tet yodB*::*cat perR*::*kan*	This study
ORB6616	*trpC2 pheA1 amyE*::*spx*P$_3$Δ+50(wt)-*bgaB cat*::*tet yodB*::*cat thrC*::*yodB*	This study
ORB6684	*trpC2 pheA1 amyE*::*Pspx*(T−26A)-*bgaB cat yodB*::*spc*	This study
ORB6685	*trpC2 pheA1 amyE*::*Pspx*(T−26A)-*bgaB cat perR*::*kan*	This study
ORB6686	*trpC2 pheA1 amyE*::*Pspx*(T−20G)-*bgaB cat yodB*::*spc*	This study
ORB6687	*trpC2 pheA1 amyE*::*Pspx*(T−20G)-*bgaB cat perR*::*kan*	This study
ORB6688	*trpC2 pheA1 amyE*::*Pspx*(T−19G)-*bgaB cat yodB*::*spc*	This study
ORB6689	*trpC2 pheA1 amyE*::*Pspx*(T−19G)-*bgaB cat perR*::*kan*	This study
ORB6690	*trpC2 pheA1 amyE*::*Pspx*(A−14T)-*bgaB cat yodB*::*spc*	This study
ORB6691	*trpC2 pheA1 amyE*::*Pspx*(A−14T)-*bgaB cat perR*::*kan*	This study
ORB6692	*trpC2 pheA1 amyE*::*Pspx*(A+3G)-*bgaB cat yodB*::*spc*	This study
ORB6693	*trpC2 pheA1 amyE*::*Pspx*(A+3G)-*bgaB cat perR*::*kan*	This study
ORB6694	*trpC2 pheA1 amyE*::*Pspx*(T+7C)-*bgaB cat yodB*::*spc*	This study
ORB6695	*trpC2 pheA1 amyE*::*Pspx*(T+7C)-*bgaB cat perR*::*kan*	This study
ORB6696	*trpC2 pheA1 amyE*::*Pspx*(T+24C)-*bgaB cat yodB*::*spc*	This study
ORB6697	*trpC2 pheA1 amyE*::*Pspx*(T+24C)-*bgaB cat perR*::*kan*	This study
Plasmids		
pML8-15	pSN16 derivative; *spx-lacZ* fusion	This study
pML34	*spx* promoter fragment (+40) in pDL	(Leelakriangsak & Zuber, 2007)
pML42	*spx*(*T−26A*) in pDL	(Leelakriangsak & Zuber, 2007)
pML43	*spx*(*T−20G*) in pDL	(Leelakriangsak & Zuber, 2007)
pML44	*spx*(*T−19G*) in pDL	(Leelakriangsak & Zuber, 2007)

Strain or plasmid	Relevant phenotype or description	Reference or source
pML46	*spx*(*A−14T*) in pDL	(Leelakriangsak & Zuber, 2007)
pML48	*spx*(*T24C*) in pDL	(Leelakriangsak & Zuber, 2007)
pML54	YodB expression plasmid in pPROEX-1	This study
pML63	*yodB* promoter fragment in pUC19	This study
pML64	*yodB* promoter fragment in pDG795	This study
pHis-PerR	pQE8 encoding His$_6$-PerR	(Hayashi et al., 2005)
pCm::Sp	Plasmid for replacing *cat* with *spc* (spectinomycin resistance) cassette	(Steinmetz & Richter, 1994)
pCm::Tc	Plasmid for replacing *cat* with *tet* (tetracycline resistance) cassette	(Steinmetz & Richter, 1994)

Table 3.2 Oligonucleotides used

Oligonucleotide	Sequence (5′-3′)	Reference or source
oMLbgaB	CCCCCTAGCTAATTTTCGTTTAATTA	(Leelakriangsak & Zuber, 2007)
oML02-15	TCCTCTAATTAGTAGGATGAACAT	(Leelakriangsak & Zuber, 2007)
oML02-22	CGGGATCCCGAACATCTATTTTATTC	(Leelakriangsak & Zuber, 2007)
oML02-25	GGAATTCCAGAATAAGAACATATC	(Leelakriangsak & Zuber, 2007)
oML02-37	GGAATTCCGGTACCGGCGCCGGTCATT	(Leelakriangsak & Zuber, 2007)
yodB-F1	ATTGCGCAAGTGTCATAACC	This study
yodB-R1	GTTATCCGCTCACAATTCTAAGTCTTCATCCCTTCATC	This study
yodB-F2	CGTCGTGACTGGGAAAACGCCTGGTGACACAGTGTGTG	This study
yodB-R2	TCATCATTAGCATGACAAGC	This study
pUC-F	GTTTTCCCAGTCACGACG	(Nakano et al., 2006)
pUC-R	GAATTGTGAGCGGATAAC	(Nakano et al., 2006)
oyodB-HisN	GGAATTCCATATGGGAAATACGATGTGCC	This study
oyodB-HisB	CGGGATCCGTGCCGCTTTCCTTATTTCTC	This study
oyodB-EcoRI	CGGAATTCGAGATCGGGGCATTT	This study

yezE	ydiH	rok	pucR	padR
ydhR	ydeP	ydeB	ydcN	ydcD
cymR	yrhO	yodB	yobV	ykvN
ywgB	yvlC	yvbF	yvaV	yvaP
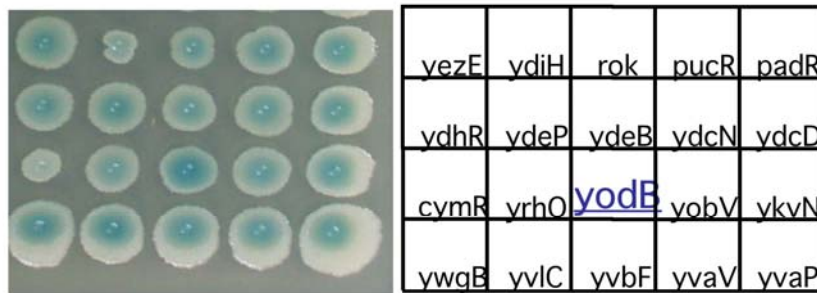
Figure 3.1 Transcription factor-transformation array using *spx-lacZ* fusion strain as recipient was performed by Kazuo Kobayashi. Colonies on the left are transformants of strain that were transformed with DNA from individual mutants with an insertion in genes encoding known or putative transcription factors. The genes mutated are indicated on the right and correspond to the pattern of colonies on the left.
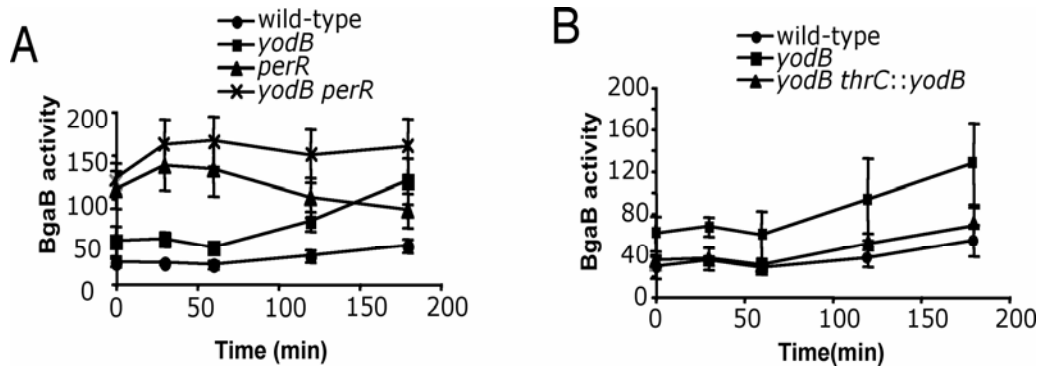
Figure 3.2 Effect of the *perR*, *yodB* and *perR yodB* disruption on the expression of *spx-bgaB* cells.The cells were grown in DSM and their β-galactosidase (BgaB) activities were determined as described in Material and Methods. Time 0 indicates the mid-log phase. Triplicate experiments were performed. ●, ORB6284 (wild-type); ■, ORB6288 (*yodB* mutant); ▲, ORB6268 (*perR* mutant); ✕, ORB6594 (*yodB perR* double mutants). B. Complementation experiment of *yodB*. β-galactosidase activity of strains containing *spx-bgaB*, *yodB::cat* complemented with *yodB*. Cells were grown in DSM. Samples were taken after OD600 reach 0.4-0.5 (time 0), 30 min, 60 min, 120 min and 180 min. Results are mean ± SD from three independent experiments. ●, ORB6284 (wild-type); ■, ORB6288 (*yodB::cat*) ; ▲, ORB6616 (*yodB::cat, thrC::yodB*)
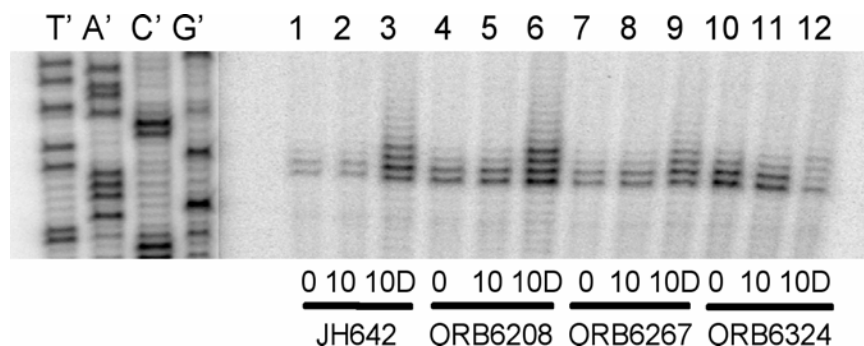
Figure 3.3 Primer extension analysis of RNA extracted from JH642, ORB6208 (*yodB* mutant), ORB6267 (*perR* mutant) and ORB6324 (*yodB perR* double mutants) cells in cultures subjected to diamide treatment. Cells were treated with 1 mM diamide for 10 min (10D) and without diamide (0 and 10 min) after OD_{600} reached 0.4-0.5. Labeled primer oML02-15 was used for primer extension reaction. The dideoxy sequencing ladders are shown on the left. For dideoxynucleotide sequencing, the nucleotide complementary to the dideoxy nucleotide added in each reaction is indicated above the corresponding lane (T′, A′, C′, G′).

	Δ+40	Δ+5	T24C	T-26A	T-20G	T-19G	A-14T	*rpsD*
PerR(µM)	- 3 6	- 3 6	- 3 6	- 3 6	- 3 6	- 3 6	- 3 6	- 2 4
Ratio of Transcription (%)	100 24 12	100 103 100	100 115 77	100 48 20	100 41 37	100 77 35	100 60 49	100 90 118
YodB(µM)	- 0.5 1	- 1 2	- 0.5 1	- 0.5 1	- 0.5 1	- 0.5 1	- 0.5 1	- 0.5 1
Ratio of Transcription (%)	100 17 10	100 17 15	100 27 20	100 92 65	100 112 108	100 61 64	100 57 34	100 86 65
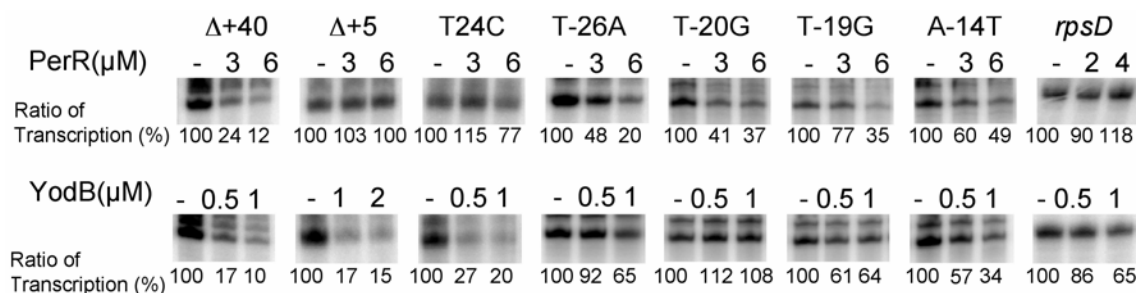
Figure 3.4 Run off in vitro transcription analysis showed the effects of purified PerR and YodB on the levels of *spx* transcripts. Linear DNA templates for *spx* (deletions; +40, +5, point mutations; T24C, T-26A, T-20G, T-19G and A-14T) promoters and *rpsD* were generated by PCR. RNAP and DNA templates were incubated with or without PerR or YodB as indicated. The major transcript (the bottom band) was quantified by using IMAGEQUANT data analysis software. The ratio of transcription (%) was measured by comparing the transcripts from the reaction without repressor protein (as 100%) and with repressor protein for each template.
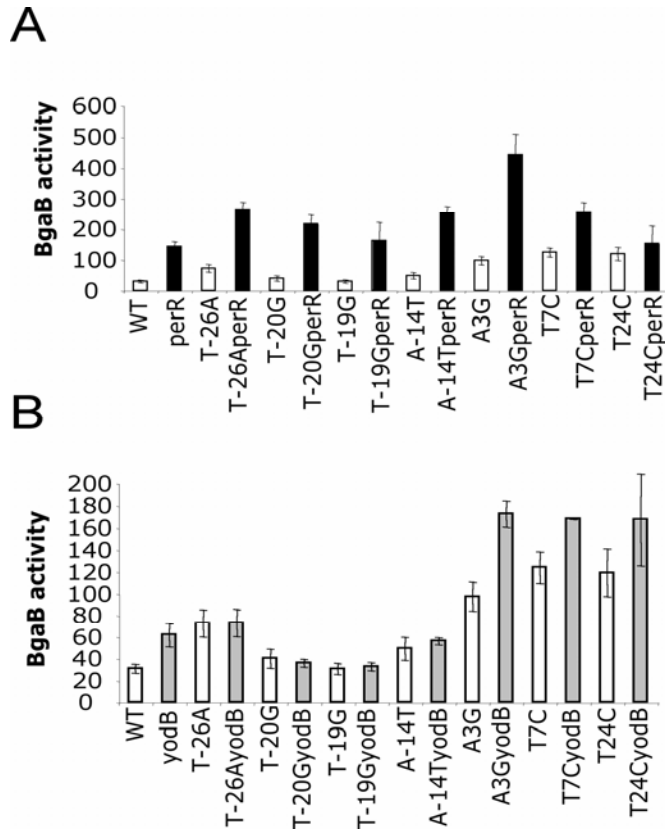
Figure 3.5 Effect of the *perR* (A) and *yodB* (B) disruption on the expression of *spx* promoter P$_3$ point mutations cells. Cells were grown in DSM. The expression was determined as BgaB activity in Miller units at 30 min after cultures reached mid-log phase. Cells containing *spx* wild-type and point mutations (T-26A, T-20G, T-19G, A-14T, A3G, T7C and T24C) promoters are shown in white bars. The expression of *perR* disruption and *perR* disruption in *spx* point mutations cells are shown in black bar (A). The expression of *yodB* disruption and *yodB* disruption in *spx* point mutations cells are shown in gray bars (B). Results are mean ± SD from at least three independent experiments.
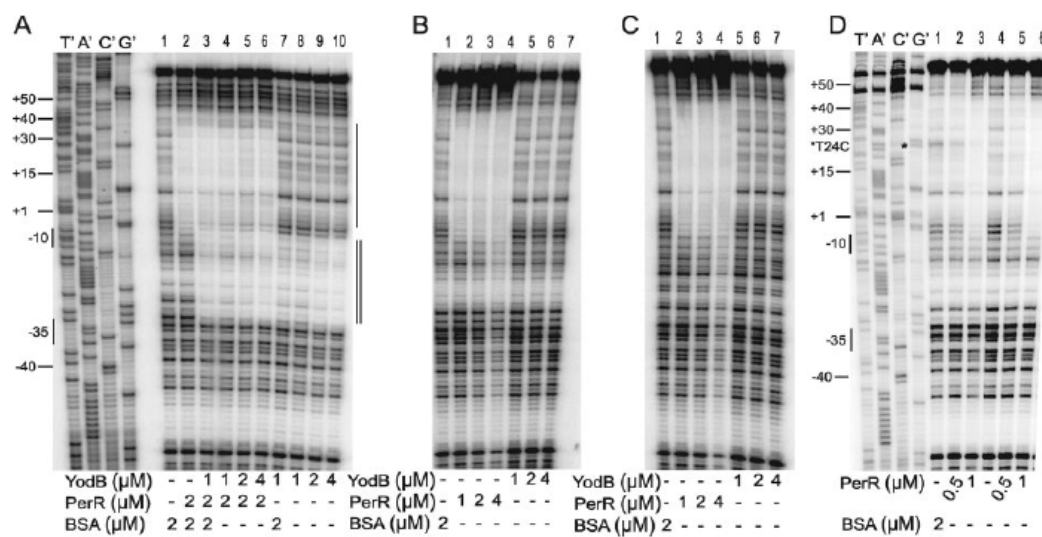
Figure 3.6 Result of DNaseI footprinting of PerR and YodB to the top strand of the *spx* promoter. The wild-type *spx* promoter (A), point mutation T-26A *spx* promoter (B), point mutation T-19A *spx* promoter (C), and point mutation T+24C (lane 1-3), wild-type (lane 4-6) (D) prepared by PCR was incubated in separate reactions with the increased amount of His-tagged PerR and YodB and subjected to DNase I cleavage. Lines along Fig. 5A indicate the protected regions, single line for PerR and double lines for YodB. The positions relative to the transcriptional start site are shown on the left (A, D). The nucleotide substitution (T24C) is indicated by asterisk (D).

Figure 3.7 Effect of diamide and hydrogen peroxide on the DNA-binding. DNase I footprinting was used to assess the effect of diamide on YodB, PerR binding to the *spx* promoter (A) on CymR binding to the *yrrT* promoter (B). The protected region by CymR is indicated by the dashed line on the right side. The positions relative to the transcriptional start site are shown. (C) Effect of H₂O₂ on the DNA-binding. PerR and YodB were incubated with DNA as follows: Lane1-2, DNA alone control; lane 3-6, 11, 12, 1 μM PerR; lane 7-12, 2 μM YodB.The concentrations of H₂O₂ used were: 25 mM, 50 mM and 100 mM.

TTAAGCTAATTGAGAATAAGAACATATCATGAACATTAAAAATTTGCTGGATT

GACACATTTTTTTCTTAGGATACTGTTCACACTTACTTTTTTATAGTATAATAC
-35   T-26A T-20,-19G A-14T   -10
                                        YodB

TSS ▷
CTGTAAAGATTCCTTTTTTAGAGTAATTTCAATTTAAGAATAAAATAGATGTT
                T24C
                PerR

CATCCTACTAATTAGAGGAGTGAAGATGA
            RBS

Figure 3.8 Summary of the protection patterns from DNaseI footprinting experiments. The nucleotide sequence of the *spx* promoter is shown. The regions protected by YodB (double solid lines) and by PerR (single solid line) are indicated. The putative -10 and -35 sequence are boxed. TSS, transcription start site. RBS, ribosome-binding site.

CHAPTER 4

MECHANISM OF NEGATIVE TRANSCRIPTIONAL CONTROL IN RESPONSE TO THIOL-REACTIVE AGENTS BY THE DUF24/MAR-LIKE REPRESSOR, YODB OF BACILLUS SUBTILIS

4.1   INTRODUCTION

The Gram-positive bacterium *Bacillus subtilis* is exposed to a variety of toxic and antimicrobial compounds in the soil that induce general and specific stress responses in growing cells. As part of the stress response, a collection of stress proteins confer resistance to reactive aromatic compounds to eliminate and alleviate the damage caused by such toxic agents. Catecholic and hydroquinone-like intermediates are formed within the catabolic pathways of many aromatic compounds (Vaillancourt et al., 2006). They are channeled into the *ortho*- or *meta* cleavage pathways of ring-fission by specific ring-cleavage dioxygenases. Proteome and transcriptome analyses showed a strong overlap in the response of *B. subtilis* to aromatic compounds catechol, 2-methylhydroquinone (MHQ) and the thiol-reactive compound diamide. This is reflected by the common induction of the Spx, CtsR, PerR and CymR regulons and four paralogous glyoxalase/dioxygenase systems (Duy N.V., 2007, Leichert et al., 2003, Nakano et al., 2003a, Tam et al., 2006a, Zuber, 2004). Recently, we have shown that the MarR-type repressor MhqR (YkvE) controls three hydroquinone-specific glyoxalases/dioxygenases (MhqA, MhqNOP and MhqED) and the azoreductase YvaB (AzoR2) and, thus, functions in the governance of MHQ and catechol resistance in *B. subtilis* (Töwe *et al.*, 2007).

Several regulatory factors participate in controlling the complex responses to toxic compounds. The Spx protein of *B. subtilis* is a global transcriptional regulator that controls genes whose products function in maintenance of thiol-homeostasis under disulfide stress conditions (Nakano et al., 2005, Nakano et al., 2003a, Zuber, 2004).

Regulation by Spx requires interaction with the C-terminal domain of the RNA polymerase α subunit (Nakano et al., 2003a, Nakano et al., 2003b). Recent studies showed that *spx* transcription initiation at the P_3 promoter located upstream of *spx* is induced in response to diamide treatment (Leelakriangsak *et al.*, 2007, Leelakriangsak & Zuber, 2007). Two negative transcriptional regulators, PerR and YodB, were discovered to interact directly with and repress transcription initiation from the *spx* P_3 promoter (Leelakriangsak & Zuber, 2007). PerR was previously characterized as a repressor of the peroxide stress regulon (Mongkolsuk & Helmann, 2002). The novel DUF24/MarR-type family transcriptional regulator YodB also controls the divergently transcribed *yodC* gene which encodes a putative nitroreductase and is induced in response to disulfide stress conditions (Duy N.V., 2007, Leelakriangsak et al., 2007). Moreover, three cysteine residues are present in the YodB protein, these residues could be the targets for the redox-sensitive control of repressor activity that responds to thiol-reactive compounds. Thus, YodB might be a novel regulator of the thiol-specific stress response in *B. subtilis*.

In this study, we have identified the azoreductase-encoding *azoR1* (*yocJ*) gene as the most strongly derepressed member of the YodB regulon under thiol-specific stress conditions. Transcriptional studies verified the increased transcription of *azoR1* in response to diamide, catechol, MHQ and nitrofurantoin stress and provided evidence for co-transcription of *azoR1* and the downstream 6S RNA *bsrB* under thiol-stress conditions. In addition, the synthesis of YodC and Spx are also up-regulated in response to the toxic oxidants. Mutational analyses further indicate that the conserved Cys6 is required for optimal repression of *spx* and *azoR1* transcription by YodB. Finally, we show that the azoreductase AzoR1 confers resistance to thiol-specific compounds. Thus YodB regulon involves in detoxification and maintenance the thiol-homeostasis in response to the toxic oxidants.

4.2   RESULTS

4.2.1   Microarray and proteome analyses identified genes that are induced by diamide, catechol, 2-MHQ and nitrofurantoin stress, and controlled by YodB

The novel MarR-type repressor, YodB was shown to control the expression of *spx* and that of the nitroreductase-encoding *yodC* gene in response to thiol-specific oxidative stress conditions in *B. subtilis* (Duy N.V., 2007, Leelakriangsak et al., 2007). To further define the YodB regulon, other genes controlled by YodB were identified through microarray and proteome analyses. Cultures of *B. subtilis* wild-type (either 168 or JH642 strains of *B. subtilis*) and *ΔyodB* mutant derivatives were grown in minimal medium and used for proteome and transcriptome comparisons. RNA from a triplicate set of samples was purified and used to generate dye-labeled cDNA probes for microarray hybridization. Table 1 lists genes that are at least 3-fold induced or repressed in the *ΔyodB* mutant. The most strongly derepressed, YodB-controlled gene was identified as *yocJ*, which encodes a putative FMN-dependent NADH-azoreductase and is also referred as AzoR1 according to Swiss-Prot database annotations (accession number O35022). The *azoR1* (*yocJ*) transcriptional start site was mapped previously and the concentration of *azoR1* transcript was observed to increase after treatment with the thiol-specific oxidant, diamide (Nakano et al., 2003a). In addition, protein synthesis of AzoR1 is strongly increased by diamide, catechol, 2-MHQ and nitrofurantoin treatment as shown by the proteome analysis (Fig. 4.1, Table 4.1). The proteome analyses verified the observed derepression of AzoR1 production in the *ΔyodB* mutant in addition to that of the nitroreductase YodC (Fig. 4.1). The proteomic data also confirmed that AzoR1 production is several-fold more derepressed than that of YodC (Fig. 4.1, Table 4.1). In a previous study, we had shown that production of the paralogous azoreductase, AzoR2 (YvaB), is induced by MHQ and catechol stress (Töwe et al., 2007) (Fig. 4.1). However, in contrast to AzoR1, induction of AzoR2 synthesis was not observed after diamide or nitrofurantoin stress (Fig. 4.1). The MarR-type repressor, MhqR (YkvE), was shown to regulate expression of *azoR2*. Interestingly, synthesis of AzoR2 was reduced in the *ΔyodB* mutant and, conversely, synthesis of AzoR1 was lower in the *ΔmhqR* mutant under control conditions (no

chemical treatment) (Fig. 4.1). Furthermore, the level of YodC was also reduced in the *ΔmhqR* mutant, which provides evidence for regulatory interactions between the YodB and MhqR regulons. In conclusion, the microarray and proteome results show that YodB negatively controls the expression of *azoR1, yodC* and *spx*.

4.2.2 Transcription of *azoR1* is induced by catechol and 2-MHQ and derepressed in the YodB mutant

To validate the microarray and proteome data, an *azoR1-lacZ* fusion was constructed and its expression was examined in wild-type and *ΔyodB* mutant cells. The expression of *azoR1-lacZ* was low and constant throughout growth in wild-type *B. subtilis* cells (ORB6734). A very high level of derepression was observed in the *ΔyodB* mutant (ORB6735) (Fig.4.2).

In addition, northern blot analyses were performed to examine the level and size of *azoR1* transcript in wild-type and *ΔyodB* mutant cells after exposure to 2-MHQ and catechol. The results verified the very strong transcription of *azoR1* in response to catechol and 2-MHQ stress in the wild-type and derepression in the *ΔyodB* mutant (Fig. 4.3). Two different *azoR1*-specific transcripts were detected using an *azoR1* specific RNA probe (Fig. 4.3A). The smaller 0.7kb transcript is most strongly elevated after catechol and MHQ stress and corresponds to a *azoR1*-specific monocistronic transcript. The larger 1.1 kb transcript corresponds to the *azoR1-bsrB* specific transcript which is only visible after catechol and MHQ stress in the wild-type parent and derepressed in the *yodB* mutant. The *bsrB* gene encodes a highly abundant small 6S RNA that is transcribed during exponential growth and decreases in concentration during stationary phase (Ando *et al.*, 2002, Barrick *et al.*, 2005). The conserved features of this 6S RNA suggest that it binds RNA polymerase by mimicking the structure of an open promoter complex, thereby regulating gene expression (Barrick et al., 2005). Transcription of *azoR1-bsrB* mRNA in treated wild-type cells was also verified using a *bsrB* specific mRNA probe (Fig. 4.3B). These different *azoR1* specific transcripts observed in the northern blot suggests that the *azoR1-bsrB* mRNA is subjected to RNA processing. However, from northern analysis using the *bsrB*-specific probe, expression of *bsrB* can be attributed largely to constitutive transcription from its own promoter that resides downstream of the

azoR1 coding sequence. Thus, the *azoR1-bsrB* transcript makes only a minor contribution to *bsrB* transcription under thiol-stress conditions. Transcription of *bsrB* as part of the *azoR1-bsrB* mRNA under thiol-stress conditions might result from readthrough past the *azoR1* terminator due to the very high induction of *azoR1* transcription.

In summary, our *azoR1-lacZ* and northern data indicated that the expression of *azoR1* transcription initiation was induced by catechol and 2-MHQ stress in wild-type cells and is regulated by YodB.

4.2.3 Diamide, hydrogen peroxide and 2-MHQ impair binding of YodB protein to *azoR1* promoter DNA

To identify the region of the *azoR1* promoter DNA required for transcriptional repression by YodB interaction, DNase I footprinting was performed. The end-labeled top strand of the *azoR1* promoter was mixed with YodB protein (Leelakriangsak et al., 2007), followed by digestion with DNase I. YodB protected a region from approximately -20 to +10 relative to the transcription start site (Fig.4.4A lane 3 and 8). Promoter alignments identified putative sites of YodB recognition (YodB boxes) that were uncovered by DNase I footprinting experiments of YodB with the *spx* (Leelakriangsak et al., 2007) and *azoR1* promoters. YodB boxes, which contain a common 15 bp consensus sequence of TACTWTTTgWWAGTA, consist of a 4 bp palindromic sequence separated by 7 bp. In previous work, mutations in the *spx* promoter at positions corresponding to the nucleotides 6 (T), 7 (T) and 12 (A) of the consensus sequence impaired YodB binding (Leelakriangsak et al., 2007).

As shown previously, YodB binding activity on *spx* P$_3$ promoter DNA was impaired in the presence of diamide and H$_2$O$_2$ (Leelakriangsak et al., 2007). We further determined whether the binding activity of YodB on the *azoR1* promoter is reversed by the addition of diamide, H$_2$O$_2$ and 2-MHQ in varying concentrations to the footprinting reactions. As shown in lane 4, 5, 9 to 11 in Fig. 4.4A and lane 4 to 6 in Fig. 4.4B, YodB bound poorly to the operator sequence when diamide, H$_2$O$_2$ or 2-MHQ was present compared to the reactions that contained YodB alone (lane 3, 8 Fig. 4.4A and lane 3 Fig. 4.4B). However, the presence of diamide, 2-MHQ and H$_2$O$_2$ at concentrations that inhibit YodB-target DNA interaction did not effect DNA binding of CymR repressor to the *yrrT*

promoter (Leelakriangsak et al., 2007, Töwe et al., 2007)Leelakriangsak M. and Zuber P., unpublished) showing that these compounds do not have a general effect on protein-DNA interaction. In addition, neither diamide, H_2O_2 nor 2-MHQ at the higher concentrations tested in the DNA-protein-binding experiments affected DNase I activity (Fig. 4.4A lane 2 and 7 and Fig. 4.4B lane 2). These results confirmed that YodB acts as the repressor by interaction with *azoR1* promoter DNA and impairment of YodB–DNA interaction occurs when toxic oxidants and phenolic compounds are present.

4.2.4 Full repression of *spx* and *azoR1* transcription by YodB requires the Cys6 residue *in vivo*

The YodB protein contains three cysteine (Cys or C) residues, one at the N-terminal- position 6 (C6), and two at the C-terminal- positions 101 (C101) and 108 (C108). The N-terminal Cys residue (C6) is conserved among *yodB* homologs in low GC Gram-positive bacteria. These Cys residues may be involved in the redox-sensitive control of YodB activity, as has been shown for Cys residues of other regulatory factors involved in the oxidative stress response such as OxyR, Hsp33, Spx and OhrR (Barbirz et al., 2000, Fuangthong et al., 2002, Graumann et al., 2001, Hong et al., 2005, Lee et al., 2007, Nakano et al., 2005). To investigate the role of Cys residues in YodB-mediated regulation, each was replaced with alanine, thus generating the *yodBC6A*, *yodBC101A*, and *yodBC108A* alleles, along with the double mutant *yodBC101,108A*. The expression of the *spx-bgaB* fusion was examined in partial diploid strains bearing the Δ*yodB* allele and ecotopically expressed versions of the C-to-A alleles integrated at the *thrC* locus. (Fig. 4.5A). In *yodB* mutant cells (ORB6288), *spx-bgaB* activity was again higher than that of wild-type cells (ORB6284) (Leelakriangsak et al., 2007). The *spx-bgaB* activity in strains ORB6715 (Δ*yodB/yodBC101A*), ORB6716 (Δ*yodB/yodBC108A*) and ORB6777 (Δ*yodB/yodBC101,108A*) was similar to the wild-type (ORB6284). However, the expression of *spx* was derepressed in the ORB6599 (Δ*yodB/yodBC6A*) strain. The activity observed in the Δ*yodB* and *yodBC6A* mutant cells was approximately 2-fold and 1.5-fold higher than that of wild-type cells, respectively.

Primer extension analysis was performed to determine the level of *spx*P_3 and *azoR1* transcripts after treatment of *yodB* Cys-to-Ala mutants with diamide (Fig. 4.5B).

As reported previously (Leelakriangsak & Zuber, 2007, Nakano et al., 2003a), the levels of *spx*P$_3$ and *azoR1* transcripts increased after diamide treatment (Fig. 4.5B, lane 3). The transcript concentration of *azoR1* and *spx*P$_3$ was high in the untreated *ΔyodB* mutant (Fig. 4.5B, lane 4-5) compared to untreated wild-type cells (Fig. 4.5B, lane 1-2). A very high level of *azoR1* transcript was observed in the *ΔyodB* mutant (ORB6288), which validates the microarray and Northern blot results (Fig. 4.5B, lane 4-6). The induction of the *spx*P$_3$ and *azoR1* transcription was detected for all *yodB* Cys to Ala mutation, although not as pronounced in the C6A mutant (ORB6599). Importantly, higher levels of *spx*P$_3$ and *azoR1* transcripts were observed in the untreated *yodBC6A* mutant (ORB6599) (Fig. 4.5B, lane 7-8) compared to untreated wild-type cells (Fig. 4.5B, lane 1-2), which is consistent with the β-galactosidase activity data (Fig. 4.5A). The loss of diamide response of *azoR1* transcript was observed in *yodBC101,108A* mutant (ORB6777) (Fig. 4.5B, lane 16-17) compared to diamide treated cells (Fig. 4.5B, lane 18).

To examine YodBC6A protein binding activity to *spx* and *azoR1* promoter DNA, the YodBC6A protein was purified (see Experimental Procedures) and used in DNase I footprinting experiments to compare mutant DNA-binding protein activity to that of wild-type YodB. YodBC6A protein exhibited reduced binding activity to the target DNA when compared to the wild-type YodB protein (Fig. 4.6 A, B lane 3 and 6). To further clarify whether the binding of YodBC6A protein was sensitive to a toxic oxidant, diamide was added to the footprinting reactions. As had been shown previously, diamide reduced YodB binding to target DNA (Leelakriangsak et al., 2007). Interestingly, diamide also caused a loss of YodBC6A binding activity to *spx* and *azoR1* promoter DNA *in vitro* (Fig. 4.6 A, B lane 7-8). These data indicated that YodBC6A could still function and respond somewhat to oxidative stress despite its reduced DNA-binding activity.

4.2.5   *ΔyodB* mutant is resistant to catechol and 2-MHQ stress which is attributed to derepression of the *azoR1* gene

To investigate the *ΔyodB* mutant phenotype with respect to thiol-reactive oxidant sensitivty, we analyzed the growth of the *ΔyodB* mutant after addition of different concentrations of diamide, catechol and 2-MHQ to exponentially growing cells. The

growth of the *ΔyodB* mutant was similar to that of the wild-type parent after exposure to diamide (data not shown). As reported earlier, the growth of wild-type cells was inhibited in the presence of 4.8 mM catechol and 0.66 mM MHQ (Duy N.V., 2007, Tam et al., 2006a). The *ΔmhqR* mutant displayed a catechol and 2-MHQ resistance phenotype since it was able to grow with 12 mM catechol and 1 mM 2-MHQ (Marnett *et al.*, 2003). Interestingly, the *ΔyodB* mutant could grow in the presence of 9.6 mM catechol and 0.83 mM 2-MHQ, suggesting that YodB-controlled genes also confer resistance to these phenolic compounds (Fig. 4.7A). Moreover, ΔyodBΔmhqR double mutant cells showed an additive hyper-resistance to catechol and 2-MHQ since growth was still possible in the presence of 19.2 mM catechol and 2 mM 2-MHQ (Fig. 4.7B).

The next step was to investigate whether the paralogous azoreductases AzoR2 and AzoR1 controlled by MhqR and YodB, respectively, contribute to catechol and MHQ resistance. As reported previously, the growth of the Δ*azoR2* mutant was impaired in the presence of 3.6 mM catechol and 0.5 mM 2-MHQ (Fig. 4.7C; (Töwe et al., 2007). Surprisingly, no growth difference was detected for the *azoR1* mutant compared to the wild-type parent after exposure to 2-MHQ, catechol and diamide (data not shown). However, deletion of both azoreductases in the Δ*azoR2*Δ*azoR1* double mutant resulted in a cessation of growth after exposure to 2.4 mM catechol and cellular lysis after treatment with 3.6 mM 2-MHQ (Fig.4.7C). Thus, the Δ*azoR2*Δ*azoR1* double mutant was observed to be more sensitive to catechol and 2-MHQ stress compared to the Δ*azoR2* single mutant (Fig. 4.7C). Moreover, Δ*azoR2*Δ*azoR1* mutant cells showed a growth defect in the presence of 1 mM diamide compared to wild-type cells whereas the Δ*yodB*Δ*mhqR* double mutant grew faster in the presence of 1mM diamide than the wild-type parent (Fig. 4.8). These data confirmed that paralogous azoreductases encoded by *azoR2* and *azoR1* are major MhqR- and YodB-controlled determinants contributing to catechol and MHQ resistance. The increased sensitivity of the Δ*azoR2*Δ*azoR1* double mutant towards thiol-reactive compounds compared to the Δ*azoR2* single mutant suggests that AzoR2 could complement AzoR1 deficiency with respect to detoxification.

To verify that the resistance of the Δ*yodB* mutant to catechol and MHQ was attributed to *azoR1* derepression, the Δ*azoR1* mutation was introduced into the Δ*yodB*

mutant. Notably, the $\Delta yodB\Delta azoR1$ double mutant was similarly sensitive to catechol and 2-MHQ as the wild-type strain (Fig. 4.9). These data confirmed that the resistance of the $\Delta yodB$ mutant was caused by the overproduction of AzoR1.

4.3   DISCUSSION

A variety of toxic substances and antimicrobial compounds are released into the environment by microorganisms, decaying plants, fungi and animals or derived from industrial contamination. *B. subtilis* has evolved a complex regulatory network to maintain its functional homeostasis and to handle different types of environmental and cellular stresses (Hecker & Volker, 2004, Matic *et al.*, 2004). A common threat for all aerobic organisms is oxidative stress (Liu *et al.*, 2005). Reactive oxygen species often lead to oxidation of protein thiolates resulting in (i) non-native disulfide bond formation or mixed disulfides with glutathione or other low molecular weight thiols, (ii) sulfenic, sulfinic or sulfonic acid formation and (iii) cyclic sulfenamide covalent bonds (Barford, 2004, Paget & Buttner, 2003).

Due to the high reactivity of the thiol group, cysteine has evolved as the amino acid that forms redox centers in an emerging group of oxidative stress transcription factors such as the *Eschericia coli* oxidative stress regulator OxyR and the *B. subtilis* organic hydroperoxide repressor OhrR (Fuangthong et al., 2002, Hong et al., 2005, Lee et al., 2007, Paget & Buttner, 2003). Redox-sensitive cysteines define the active sites in many metabolic enzymes such as glyceraldehyde-3-phosphate dehydrogenase (GapDH), urease, FolE and MetE (Krajewska & Zaborska, 2007, Leichert & Jakob, 2006). In addition, cysteines function as ligands for binding metal ions, such as $Fe^{2+/3+}$, Zn^{2+}, Cd^{2+} and Cu^+ required for conformational changes and structure stabilization (Giles *et al.*, 2003). Protein thiolates are strong nucleophiles which are easily conjugated with chemically-diverse electrophilic compounds such as iodoacetamide, acrylonitrile, N-acetyl-p-benzoqinone imine and 1,4 napthoquinone to form thiol-S-adducts (Giles et al., 2003, Rodriguez *et al.*, 2005). Depending on their chemistry, electrophilic compounds react with thiols by different mechanisms (Marnett et al., 2003). A wide range of such thiol-reactive agents, including iodoacetamide, acrylonitrile, N-acetyl-p-benzoqinone

imine and 1,4 napthoquinone, are described as irreversible inhibitors of the active site cysteine of GapDH and urease (Rodriguez et al., 2005). Alkylating agents, such as iodoacetamide, aliphatic epoxides and alkyl halides display S_N2 electrophilic chemistry. The reaction of quinones, hydroquinones and α,β-unsaturated carbonyls involves a 1,4-reductive nucleophilic addition of thiols to quinones (Michael-type addition). Thus, modifications of redox-sensitive cysteines include either oxidation to disulfide bonds, sulfenic, sulfinic or sulfonic acids or direct nucleophilic addition of thiols to electrophiles. Of these modifications, disulfide bonds and sulfenic acids, but not the higher oxidation states (sulfinic and sulfonic acid) can be reversibly reduced by thiol-disulfide exchange reactions as catalyzed by the thioredoxin-thioredoxin reductase system. Thioredoxin generally functions as a thiol-disulfide oxidoreductase for maintaining the reducing environment in the cell (Arner & Holmgren, 2000, Holmgren *et al.*, 2005). Many enzymes that use thiol-disulfide exchange reactions, such as the arsenate reductase (ArsC) family (Li *et al.*, 2007, Mukhopadhyay & Rosen, 2002), are dependent on the thioredoxin system. The transcriptional induction of the thioredoxin system in *B. subtilis* requires the global redox-sensitive regulator Spx, an ArsC family member, which is activated by disulfide-bond formation in the CXXC motif in response to the thiol-specific reagent diamide (Nakano et al., 2005).

Diamide was shown to trigger oxidative protein damage by non-native disulfide bond formation in several cytoplasmic proteins of *B. subtilis* (Hochgrafe et al., 2005, Leichert et al., 2003). Later reports showed that a wide range of chemically-diverse compounds are able to induce the thiol-specific oxidative stress response in *B. subtilis* including furanone that affects biofilm formation, the antibiotic nitrofurantoin, or the phenolic compounds catechol and MHQ (Duy N.V., 2007, Ren *et al.*, 2004, Tam et al., 2006a). This global thiol-specific oxidative stress response is governed by the Spx, CtsR, PerR and CymR regulons as revealed by microarray and proteome analyses (Leichert et al., 2003, Nakano et al., 2003a, Zuber, 2004).

In the study reported herein, we uncovered the regulation of the novel thiol-reactive compound specific repressor YodB as one further global regulator of this thiol-specific stress response in *B. subtilis*. YodB is conserved among Gram-positive bacteria and several paralogs are encoded within the *B. subtilis* genome, which defines a

conserved family of DUF24/MarR-like regulatory proteins. Common among these YodB-like regulators is the conserved N-terminal cysteine in position 5 or 6 followed by a conserved proline residue. As reported herein, this conserved Cys6 is required for optimal repression of YodB-controlled target genes *in vivo* and *in vitro*. Inactivation of Cys6 either in a YodBC6A mutant or by irreversible modification of the Cys6 thiol with electrophilic compounds resulted in a reduction of DNA binding activity of YodB to the *azoR1* and *spx* promoters. In addition, the Cys residues Cys101 and Cys108 might require for sensing the thiol-reactive compounds due to loss of diamide induction *in vivo* was observed in *yodBC101,108A* mutant (*azoR1* transcription Fig. 4.5B, lane 16-18). Using mass spectrometry we observed the formation of Cys-S-adducts upon treatment of YodB with 2-MHQ and catechol *in vitro* (data not shown). We detected all three Cys-S-conjugates of 2-MHQ and catechol after the treatment of YodB with 1 mM 2-MHQ and 10 mM catechol respectively *in vitro* by mass spectrometry analyses (data not shown). These results show that 2-MHQ and catechol are reactive to protein thiolates. Thus, the derepression of YodB in response to diverse aromatic compounds might be caused by the electrophilic nature of these compounds which all lead to covalent cysteine modifications. However, the mechanism of YodB in response to the thiol-reactive compounds is not clear. Future studies are underway to elucidate the function of Cys in *yodBC6,101,108A* mutant in response to thiol-reactive compounds. An alternative mechanism of thiol modification by electrophilic compounds controls the oxidative stress response in *E. coli*. In this case, OxyR is activated by formation of an intramolecular disulfide bond between Cys199 and Cys208 (Choi *et al.*, 2001, Kim et al., 2002). However, specific Cys modifications of OxyR that include S-nitrosylation, S-glutathionylation and sulfenic acid formation caused by thiol-reactive agents also lead to induction of the OxyR regulon (Kim et al., 2002). Recently, it has been shown that the organic hydroperoxide repressor OhrR is regulated by oxidation at Cys15 leading to formation of mixed disulfides (S-thiolated OhrR) with low molecular weight thiols (cysteine, CoASH and a novel thiol) or by cyclic sulfenamide formation (Lee et al., 2007). All attempts to detect YodB modifications upon treatment with diamide failed using mass spectrometry. We only observed that the peptide containing Cys6 was absent in the MS spectrum after diamide treatment (data not shown). Thus, the mechanism of YodB inactivation upon diamide

treatment is currently unknown. Since S-thiolation has been shown as one mechanism of OhrR and OxyR regulation (Kim et al., 2002, Lee et al., 2007), modification of YodB could occur *in vivo* in response to diamide *via* the formation of mixed disulfides containing cysteine, CoASH or other LMW thiols. The identification of the Cys6 modifications leading to the loss of DNA binding activity of YodB upon exposure to diamide *in vivo* are the subject of future studies.

Previous transcriptome and proteome analyses have uncovered many new putative detoxification systems which are induced as part of the thiol-specific stress response (Duy N.V., 2007, Leichert et al., 2003, Tam et al., 2006a). Thus, it appears that *B. subtilis* has evolved an array of different strategies to protect against thiol-modifications by electrophiles and to detoxify such compounds. YodB represents an additional component in this complex network that likely senses thiol-reactive compounds. Expression of *spx* and *yodC*, encoding nitroreductase, was observed to be regulated by YodB in response to thiol-specific reagents (Duy N.V., 2007, Leelakriangsak & Zuber, 2007). The classical nitroreductase from *Enterobacter cloacae* can utilize either NADH or NADPH as a source of reducing equivalents and can catalyze the reduction a series of nitroaromatic compounds including nitrobenzenes and nitrofurantoin as well as quinones (Bryant & DeLuca, 1991, Nivinskas *et al.*, 2000). A member of *soxRS* regulons, *nfsA* encoding nitroreductase in *E coli*, has been shown to be up-regulated in response to oxidative stress (Liochev *et al.*, 1999, Paterson *et al.*, 2002). Two-electron reduction of nitroaromatic compounds proceed through the nitroso and subsequently hydroxylamine intermediates to the fully reduced amino adduct while the reduction of nitroaromatic compounds by one-electron step give rise the anion-free radical intermediates which cause oxidative stress (Bryant & DeLuca, 1991, Koder *et al.*, 2002). Thus, the nitroreductase was suggested to contribute to the defenses against oxidative stress by decreasing the free radical upon the one-electron reduction of nitrocompounds, quinone and dyes (Liochev et al., 1999, Paterson et al., 2002).

In this study, we uncovered the azoreductase -encoding *azoR1* gene as the most strongly derepressed member of the YodB-regulon observed in the *yodB* mutant background. Azoreductase catalyzes reductive cleavage of the azo group (-N=N-) resulting in azo dye degradation (Chen, 2006, Nakayama *et al.*, 1983). In addition, a

thiol-reactive diamide is azo-bearing compound that could be a substrate for azoreductases. Autooxidation of active metabolites azo compounds could generate reactive oxygen species (Nakayama et al., 1983). Moreover, the oxidation of catechol and 2-MHQ generate reactive oxygen species (Cavalieri et al., 2002, Murata et al., 1999, Oikawa et al., 2001). Transcriptional studies revealed that *azoR1* is co-transcribed with the downstream *bsrB* gene encoding a 6S RNA in response to thiol-specific stress, which might be caused by readthrough past the *azoR1* terminator. DNase I footprinting experiments uncovered a 15 bp consensus sequence (YodB box) within the *azoR1* and *spx* promoters that is protected by YodB. The putative YodB box is also present in the *yodC* promoter region 49 bp upstream of the start codon. YodB binds with higher affinity to the *azoR1* promoter compared to the *spx* promoter as shown in DNase I footprinting experiments (Fig. 4.6) since less YodB protein was required for protection of the *azoR1* promoter than for the *spx* promoter.

Previous studies showed that the global thiol-specific oxidative stress response is controlled by the Spx, PerR, CtsR and CymR regulators. These transcription factors possess cysteine residues that are likely the targets for inactivation in response to thiol-reactive agents. The N-terminal CXXC motif of Spx is involved in the redox-control of as the regulator's transcription-stimulating activity (Nakano et al., 2005). The peroxide regulon repressor PerR has four cysteine residues that form a Zn^{2+} binding site and were shown to be essential for DNA binding *in vivo* (Lee & Helmann, 2006a). The derepression of the CtsR-regulated Clp protease genes in response to thiol-stress could be explained by the inactivation of redox-sensitive cysteine residues in the zinc-finger motif of the regulatory McsA protein (Krüger *et al.*, 2001). In light of our results, we speculate that the complex thiol-specific oxidative stress response is governed by several transcription factors that all share reactive cysteine residues that are essential for their regulatory functions. Further examples supporting this notion are the derepression of the ArsR-dependent arsenate reductase operon in response to diamide and MHQ, which encodes also another glyoxalase (YqcK) (Duy N.V., 2007, Leichert et al., 2003). Inactivation of the ArsR repressor also involves conserved cysteine residues in the helix-turn-helix DNA binding region. The Cys residues of ArsR are candidate metal ligands (Morby *et al.*, 1993). Interestingly, derepression of genes regulated by the cysteine-

containing ArsR/SmtB paralog YczG is also reported in response to thiol-specific agents (our unpublished data).

In summary, our studies uncovered the regulation of the YodB repressor that controls Spx and the oxidoreductases AzoR1 and YodC in response to toxic oxidants. We detected the S-conjugation with conserved Cys6 residue of YodB via electrophile thiol-reactive compounds resulting in a reduction of DNA binding activity. In response to aromatic and thiol-specific compounds, YodB up-regulates *yodC* and *azoR1* gene which involve in detoxification and *spx* gene which involve in the damage repair oxidized proteins.

4.4   EXPERIMENTAL PROCEDURES

4.4.1   Bacterial strains and growth conditions

Bacillus subtilis strains used in this study are derivatives of JH642 and 168 and are listed in Table 4.2. Cells were cultivated in a shaking water bath at 37°C in Difco sporulation medium (DSM) for β-galactoisidase assays or TSS minimal medium and Belitsky minimal medium (Stulke *et al.*, 1993) for treatments with thiol-reactive compounds. Diamide and hydrogen peroxide were purchased from Sigma-Aldrich. 2-Methylhydroquinone (2-MHQ) was purchased from Acros.

The isogenic *azoR1* mutant was constructed by transformation of JH642 with chromosomal DNA of the *azoR1D* (*yocJ::pMutin4*) strain, which carries *a azoR1-lacZ* fusion, resulting in a strain designated ORB6734. The *yodB* mutant bearing a *azoR1-lacZ* fusion was constructed by transformation of ORB6734 with chromosomal DNA of ORB6208 (*yodB* mutant) (Leelakriangsak et al., 2007) to generate ORB6735. The *yodB*::pMutin4 and *azoR2*::pMutin4 mutants were constructed in the course of the European and Japanese *B. subtilis* functional analysis project. Chromosomal DNA of the *azoR2*::pMutin4 (ST8) and *yodB*::pMutin4 (ST5) mutants were introduced by transformation into the Δ*azoR1* mutant (ST7) to generate the Δ*azoR1*Δ*azoR2* (ST9) and Δ*azoR1*Δ*yodB* (ST10) double mutants, respectively. The Δ*mhqR* mutant (TF176) was transformed with chromosomal DNA of the *yodB*::pMutin4 (ST5) mutant to generate the

Δ*yodB*Δ*mhqR* double mutant (ST6). The lesions harbored by the mutants were verified by PCR.

When necessary, the following antibiotics were added (at a concentration shown in parenthesis): ampicillin (25 μg/ml), chloramphenicol (5 μg/ml), erythromycin plus lincomycin (1 and 25 μg/ml, respectively), and tetracycline (12.5 μg/ml).

4.4.2   Construction of *yodBC6A*, *C101A*, *C108A* and *C101,108A* mutants

Plasmids pML60 (C6A), pML65 (C101A), pML66 (C108A) and pML69 (C101,108A) were produced by using PCR mutagenesis. First-round PCR was performed in two separate reactions with primers oyodB-EcoRI with oyodB-C6AR and primers oyodB-C6AF with oyodB-HisB (Table 4.3) using JH642 chromosomal DNA as template. The PCR products were hybridized and subsequently amplified by a second round of PCR using primers oyodB-EcoRI and oyodB-HisB. The product from the second round PCR were then digested with EcoRI and BamHI restriction enzymes and inserted into plasmid pUC19 that was digested with the same enzymes to generate pML60. PCR product ($\approx$60 bp) that was synthesized using primer oyodB-C101A and oyod-HisB was used as the primer together with oyodB-EcoRI for second round PCR to generate pML65. pML66 was generated by using primers oyodB-EcoRI and oyodB-C108A. Primers oyodB-EcoRI and oyodB-C108A (Table 4.3) were used to produce pML69 using pML65 as the template. The *yodB* sequences were verified by DNA sequencing. The plasmids pML60, pML65, pML66 and pML69 were digested with EcoRI and BamHI and the released fragments bearing the mutations were then inserted into pDG795 that was digested with the same enzymes to generate pML61, pML67, pML68 and pML72, respectively. pML61, pML67, pML68 and pML72 were introduced by transformation with selection for erythomycin-lincomycin, into JH642, where yodBC6A, C101A, C108A and C101,108A fragments integrated into the *thrC* locus. The resulting strains were designated ORB6592 (*thrC::yodBC6A*), ORB6713 (*thrC::yodBC101A*), ORB6714 (*thrC::yodBC108A*) and ORB6775 (*thrC::yodBC101,108A*). Chromosomal DNA of ORB6592, ORB6713, ORB6714 and ORB6775 were used to transform ORB6288 to generate ORB6599, ORB6715, ORB6716 and ORB6777 respectively.

4.4.3   Transcriptome analysis

For microarray analysis, the *B. subtilis* 168 wild-type and isogenic ΔyodB (TF277) mutant were grown in minimal and harvested at OD500 of 0.4. Total RNA was isolated by the acid phenol method as described (Majumdar *et al.*, 1991). Generation of fluorescence-labeled cDNA and hybridization with *B. subtilis* whole-genome microarrays (Eurogentec) was performed as described previously (Jurgen *et al.*, 2005). Two independent hybridization experiments were performed using RNAs from two independent experiments. Genes showing induction ratios of at least three-fold in two independent experiments were considered as significantly induced. Another independent microarray experiment was also conducted using strains ORB6208 (Δ*yodB::cat*) and the JH642 parent. Procedures for RNA purification and microarray analysis were described previously (Choi et al., 2006, Nakano et al., 2003a).

4.4.4   Assay of β-galactosidase activity

Toluene treatments and galactosidase assays using *o*-nitrophenyl-β-D-galactopyranoside were performed as previously described (Nakano *et al.*, 1988, Schrogel & Allmansberger, 1997). β-galactosidase activity is expressed as Miller Units (Miller, 1972).

4.4.5   Protein purification

The *yodB* coding sequences was amplified by PCR using primers oyodB-HisNC6A and yodB-HisB. The PCR products were digested with NdeI and BamHI restriction enzymes and inserted into pPROEX-1(Life Technologies) digested with the same enzymes to generate pML74. Cells were cultured and the protein was purified followed by the removal the N-terminal His_6 tag from ptorein as described previously (Leelakriangsak et al., 2007).

4.4.6   Primer extension analysis

ORB6284, ORB6288, ORB6599, ORB6715, ORB6716 and ORB6777 were grown at 37°C in TSS medium. Primer extension analysis was performed as described

previously (Leelakriangsak & Zuber, 2007). Primers oML02-15 and oSN03-63 were used to synthesize the primer extension products to examine the transcripts of *spx* and *azoR1* respectively.

4.4.7 DNase I footprinting

A DNA probe for *yocJ* (position corresponding -160 to +110 with respect to transcription start site) was made by PCR amplification using primers oSN6-03-64 and oSN03-65. Dideoxy sequencing ladders were obtained using the Thermo Sequenase cycle sequencing kit (USB) and the PCR product specifying *yocJ* as template. The same primers used for the footprinting reactions were also used for sequencing. DNase I footprinting reactions were performed as described previously (Leelakriangsak et al., 2007).

4.4.8 Proteome analysis

Cells grown in minimal medium to an OD_{500} of 0.4 were pulse-labeled for 5 min each with 5 μCi of L-[^{35}S]methionine per ml before (control) and 10 min after exposure to 0.33 mM 2-MHQ, 2.4 mM catechol, 1 mM diamide or 0.1 mM nitrofurantoin. Preparation of cytoplasmic L-[^{35}S]methionine-labelled proteins and separation by two-dimensional gel electrophoresis (2D-PAGE) using the immobilized pH gradients (IPG) in the pH range 4-7 was perfomed as described (Tam et al., 2006a). The quantitative image analysis was performed with the DECODON Delta 2D software (http://www.decodon.com).

4.4.9 Northern blot analysis

Northern blotting was performed as described (Wetzstein *et al.*, 1992) using RNA isolated from *B. subtilis* wild-type and Δ*yodB* (TF277) mutant cells before (control) as well as 10 and 20 min after the treatment with 2.4mM catechol or 0.33 mM 2-MHQ, respectively. Hybridizations specific for *azoR1* and *bsrB* were performed with the digoxigenin-labeled RNA probes synthesized *in vitro* using T7 RNA polymerase from T7 promoter containing PCR products amplified with *azoR1*-specific primers (yocJ_for and yocJ_rev) and *bsrB*-specific primers (bsrB-for and bsrB-rev).

Table 4.1 Induction of genes after 2-MHQ, cetechol, diamide and nitrofurantoin stress in the wild-type (wt) and derepression in the *ΔyodB* mutant[a] by Antelmann group.

Operon	Gene	Transcriptome (wt) [b]					Proteome (wt) [c]								Transcriptome ΔyodB/wt		Proteome ΔyodB/wt		Function or similarity
		Catechol		2-MHQ		Dia	Catechol		2-MHQ		Diamide		NT						
azoR1-bsrB	azoR1(yocJ)	36.7	42.1	106	66.3	38.4	18	19.6	11.9	19.3	8.86	7.42	5.26	8.475	70.9	82.4	19.6	21.3	FMN-dependent NADH-azoreductase 1
	yodC	5.1	9.2	13.6	10.1	12.4	3.7	3.8	2.8	3.9	7.26	6.89	7.3	7.623	3.9	3.3	3.5	3.7	NAD(P)H nitroreductase
yjbC-spx	spx	4.1	5.6	3.4	6.7	7.6									2.2	3.7			regulator of thiol-specific oxidative stress response
ribDEAHT	ribD			4.5	3.3										1.9	5.2			riboflavin-specific deaminase
	ribE			6.3	3.3										2.2	5.4			riboflavin synthase (alpha subunit)
	ribA			6.2	3.3										2.2	4.8			GTP cyclohydrolase II
	ribH			4.8	3.6										2.2	4.6			riboflavin synthase (beta subunit)

a)All genes with induction ratios of at least three-fold in two transcriptome experiments and proteins with protein synthesis ratios of at least two-fold in two proteome experiments which are induced in the wild-type (wt) after 10 min in response to 63 µg/ml 2-MHC, 2.4mM catechol, 1mM diamide and 0.1mM nitrofurnatoin (NT) stress and derepressed in the *ΔyodB* mutant under control conditions (*ΔyodB*/wt) were listed.The gene function or similarity is derived from the SubtiList database (http://genolist.pasteur.fr/SubtiList/index.html).

b)The transcriptome data for the induction ratios of YodB-dependent genes in response to catechol, 2-MHQ and diamide were derived from previous publications (Tam et al., 2006; Duy et al., 2007; Leichert et al., 2003).

c)The protein synthesis ratios of YodB-dependent proteins in response to catechol, 2-MHQ, diamide and nitrofurantoin were calculated from proteome experiments described in this paper.

Table 4.2 *Bacillus subtilis* strains and plasmids used

Strain or plasmid	Genotype	Reference or source
Strains		
JH642	*trpC2 pheA1*	J.A. Hoch
168	*trpC2*	
TF176	*trpC2 mhqR::cat*	(Töwe et al., 2007)
TF277	*trpC2 yodB::cat*	(Leelakriangsak et al., 2007)
ST5	*trpC2 yodB::Erm (yodB::pMutin4)*	(Duy N.V., 2007)
ST6	*trpC2 yodB::Erm ykvE::cat*	This study
ST7	*trpC2 azoR1::cat*	This study
ST8	*trpC2 azoR2::Erm (azoR2::pMutin4)*	(Töwe et al., 2007)
ST9	*trpC2 azoR1::cat azoR2::Erm (azoR2::pMutin4)*	This study
ST10	*trpC2 yodB::Erm azoR1::cat*	This study
ORB6208	*trpC2 pheA1 yodB::cat*	(Leelakriangsak et al., 2007)
ORB6284	*trpC2 pheA1 amyE::Pspx-bgaB pcm::tet*	(Leelakriangsak et al., 2007)
ORB6288	*trpC2 pheA1 amyE::Pspx-bgaB pcm::tet yodB::cat*	(Leelakriangsak et al., 2007)
ORB6592	*typC2 pheA1 thrC::yodBC6A*	This study
ORB6599	*trpC2 pheA1 amyE::Pspx-bgaB pcm::tet yodB::cat thrC::yodBC6A*	This study
ORB6713	*typC2 pheA1 thrC::yodBC101A*	This study
ORB6714	*typC2 pheA1 thrC::yodBC108A*	This study
ORB6715	*trpC2 pheA1 amyE::Pspx-bgaB pcm::tet yodB::cat thrC::yodBC101A*	This study
ORB6716	*trpC2 pheA1 amyE::Pspx-bgaB pcm::tet yodB::cat thrC::yodBC108A*	This study
ORB6734	*typC2 pheA1 azoR1D::Erm (yocJ::pMutin4)*	This study
ORB6735	*typC2 pheA1 azoR1D::Erm (yocJ::pMutin4) yodB::cat*	This study
ORB6775	*typC2 pheA1 thrC::yodBC101,108A*	This study
ORB6777	*trpC2 pheA1 amyE::Pspx-bgaB pcm::tet yodB::cat thrC::yodBC101,108A*	This study

Strain or plasmid	Genotype	Reference or source
Plasmids		
pML60	yodBC6A in pUC19	This study
pML61	yodBC6A in pDG795	This study
pML65	yodBC101A in pUC19	This study
pML66	yodBC108A in pUC19	This study
pML67	yodBC101A in pDG795	This study
pML68	yodBC108A in pDG795	This study
pML69	yodBC101,108A in pUC19	This study
pML72	yodBC101,108A in pDG795	This study
pML74	YodBC6A expression plasmid in pPROEX-1	This study

Table 4.3 Oligonucleotide primers used

Name	Sequence (5′-3′)	Reference or source
oML02-15	TCCTCTAATTAGTAGGATGAACAT	(Leelakriangsak & Zuber, 2007)
oyodB-EcoRI	CGGAATTCGAGATCGGGGCATTT	(Leelakriangsak et al., 2007)
oyobB-C6AR	TTAGGTGCCATCGTATTTCCCAT	This study
oyodB-C6AF	TACGATGGCACCTAAAATGGAAT	This study
oyodB-C101A	TGGGCCGATCAATTTGCAGAGCCTGGT	This study
oyodB-C108A	CGGGATCCGTGCCGCTTTCCTTATTTCTCTT CTTCTGCCACTGTGT	This study
oyodB-HisNC6A	GGAATTCCATATG GGAAATACGATGGCAC	This study
oyodB-HisB	CGGGATCC GTGCCGCTTTCCTTATTTCTC	(Leelakriangsak et al., 2007)
oSN03-63	AGTTGAAACGCCTTCTTCAGCTGTACGGTC	(Nakano et al., 2003a)
oSN03-64	GAGAATTCGCAGACGTATGAAATCTTGGAT C	(Nakano et al., 2003a)
oSN03-65	GAGGATCCTTCGTAAAGTTTAGTTGAAACG CC	(Nakano et al., 2003a)
yocJ_for	GGAAAACCTTCCTTACCTTG	This study
yocJ_rev	CTAATACGACTCACTATAGGGAGAACTGGT CCTTCTTGTGTGTA	This study
bsrB_for	CGAATTGTCAGGTATTTTGG	This study
bsrB_rev	CTAATACGACTCACTATAGGGAGACGGCCT TCATTTCTTTCACAC	This study
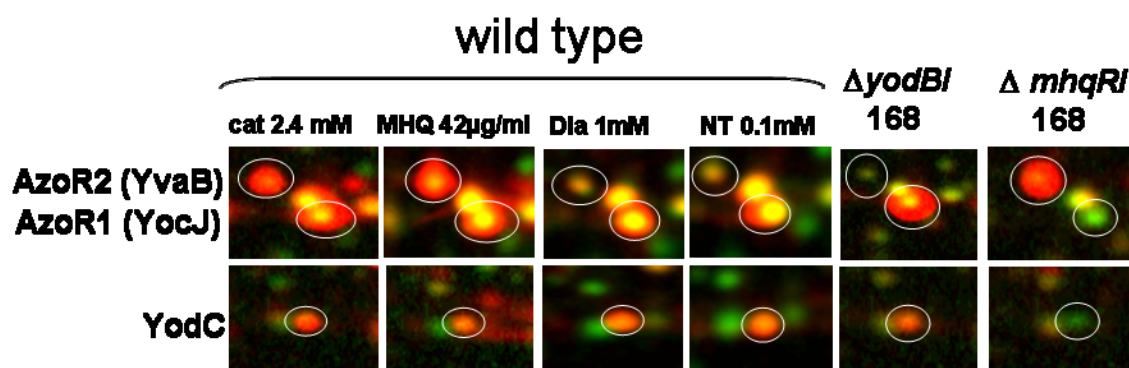
Figure 4.1 Proteome analysis of the YodB-regulon in *B. subtilis*. Induction of AzoR1 and YodC by catechol, 2-MHQ, diamide and nitrofurantoin in *B. subtilis* wild-type cells, derepression in the Δ*yodB* mutant and repression in the Δ*mhqR (ykvE)* mutant. *B. subtilis* wild-type cells grown in minimal medium to an OD500 of 0.4 were pulse-labeled for 5 min each with L-[35S]methionine at control conditions (green image) and in the wild-type 10min after 2-MHQ, catechol, diamide and nitrofurantoin challenge or in the Δ*yodB* and Δ*mhqR* mutant at control conditions (red image). Preteome analysis is performed by Antelmann group.
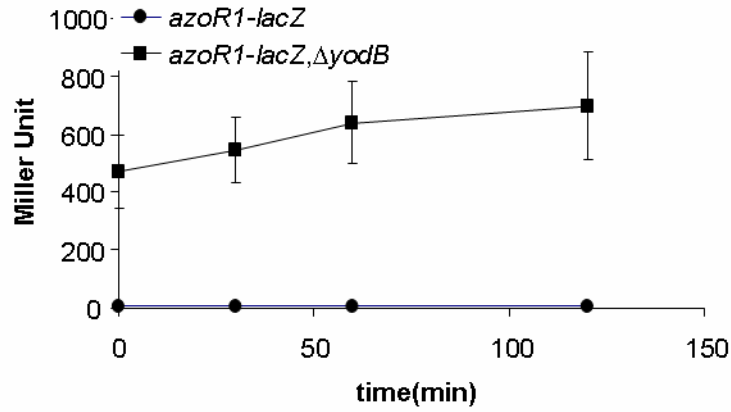
Figure 4.2 Effect of the disruption of *yodB* on the transcriptional expression of *azoR1-lacZ*. The cells were grown in DSM, and their β-galactosidase activities were determined as described in Experimental procedures. Samples were taken after OD600 reached 0.4-0.5 (time zero) and at 30 min, 60 min and 120 min. Results are means ± standard deviations from four independent experiments. ●, ORB6734 (*yocJ-lacZ*); ■, ORB6735 (*yocJ-lacZ,yodB*).
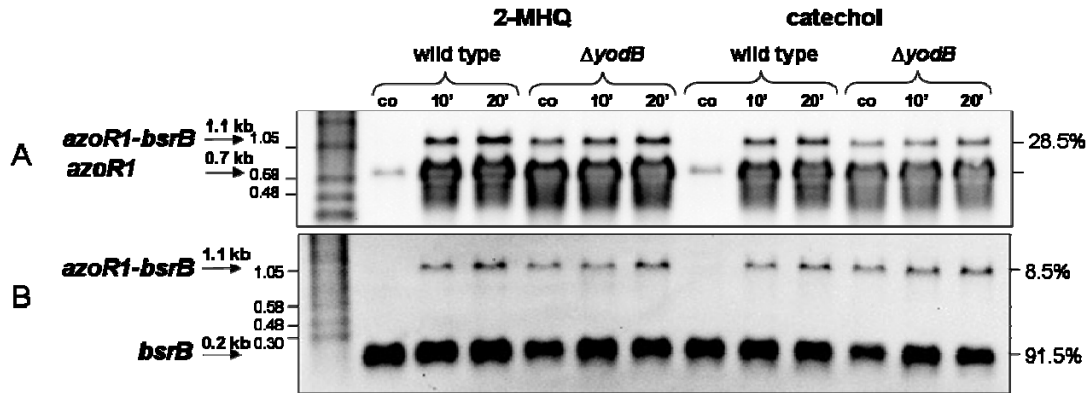
Figure 4.3 Transcript analyses of *azoR1* (A) and *bsrB* (B) in the wild-type and Δ*yodB* mutant in response to 2-MHQ (left) and catechol stress (right). For northern blot experiments 5 μg RNA each were applied isolated from the *B. subtilis* strains before (co) and at different times (10, 20 min) after the exposure to 2.4 mM catechol or 0.33 mM 2-MHQ. The northern blots were hybridized with *yocJ* (A) and *bsrB* (B) specific mRNA probes. The arrows point toward the sizes of the *azoR1, azoR1-bsrB* and *bsrB* specific transcripts. The percentage amounts of *azoR1* and *azoR1-bsrB* specific mRNAs are calculated with the ImageJ software and are shown on the right. Northern blot analysis was performed by Antelmann group.
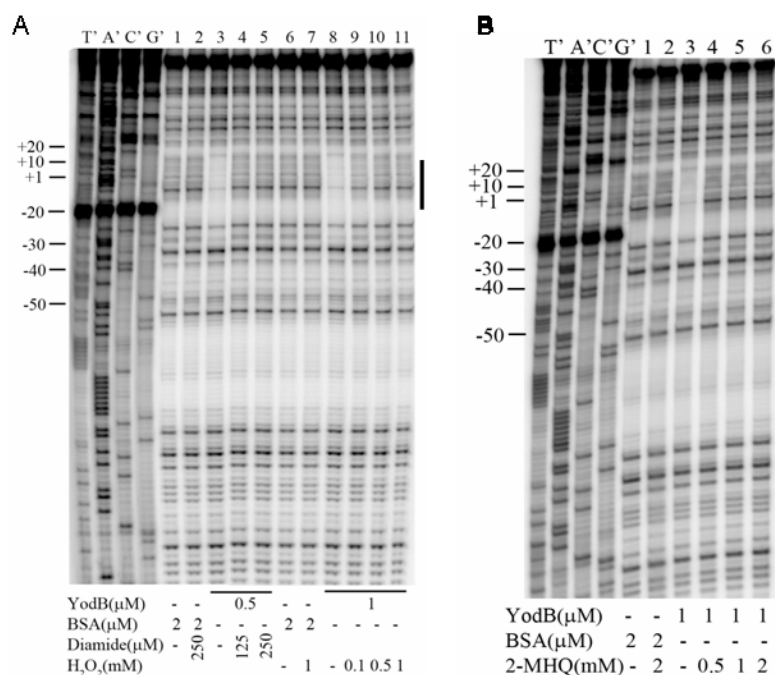
Figure 4.4 Effect of diamide, hydrogen peroxide(A) and 2-MHQ (B) on DNA binding to *azoR1* promoter. DNaseI footprinting was used to assess the effect of diamide, hydrogen peroxide and 2-MHQ on YodB binding to the top strand of the *azoR1* promoter. The region protected by YodB is indicated by the solid line. The positions relative to the transcriptional start site are shown on the left. For the dideoxynucleotide sequencing shown on the left, the nucleotide complementary to the dideoxynucleotide added in each reaction mixture is indicated above the corresponding lane (T′, A′, C′ and G′).
BSA, bovine serum albumin.
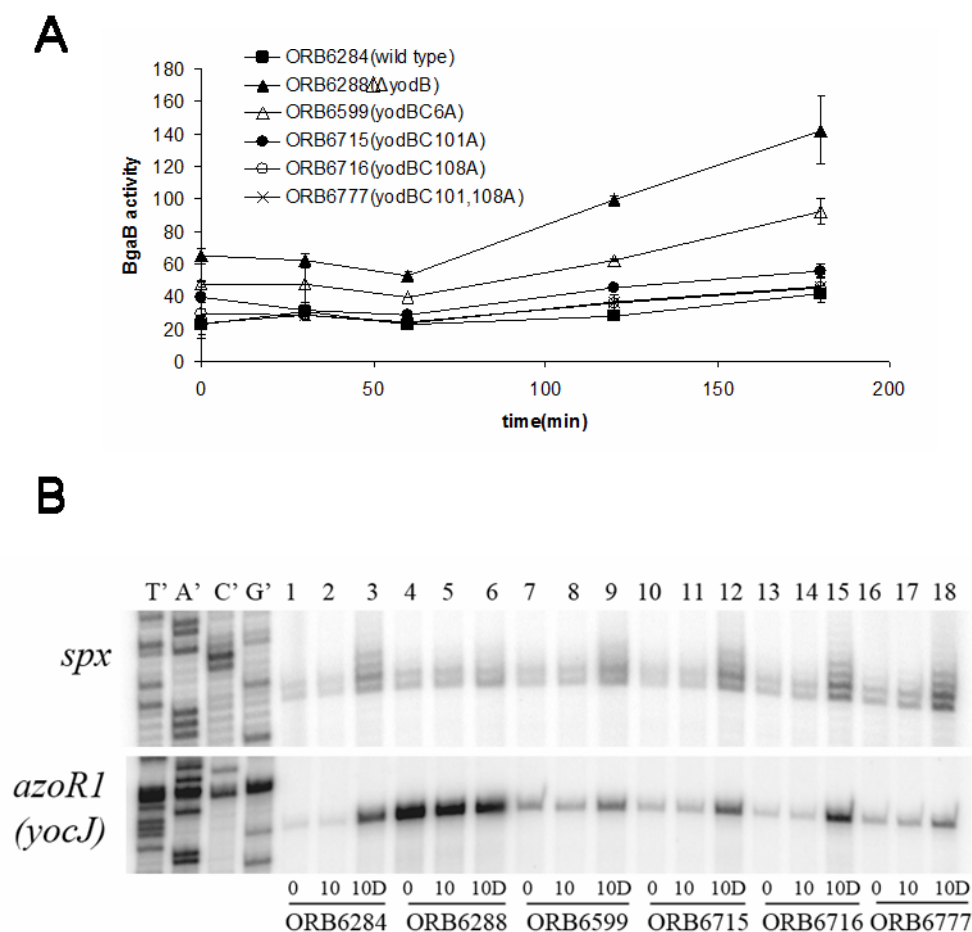
Figure 4.5 Role of YodB Cys mutants on regulation of *spx-bgaB* activity (A) and *spx* and *azoR1* transcription (B) *in vivo*. (A) Expression of *spx-bgaB* fusion cells containing wild-type *yodB* (■,ORB6284), *ΔyodB* mutant (▲,ORB6288), *yodBC6A* (△,ORB6599), *yodBC101A* (●,ORB6715), *yodBC108A* (○,ORB6716) and *yodBC101,108A* (×,ORB6777) were determined. The cells were grown in DSM, and their β-galactosidase (BgaB) activities were determined. Time zero indicates the mid-log phase. (B) Primer extension analysis of RNA extracted from ORB6284 (wild-type), ORB6288 *(ΔyodB* mutant), ORB6599 (*yodBC6A*), ORB6715 (*yodBC101A*), ORB6716 (*yodBC108A*) and ORB6777 (*yodBC101,108A*) cells in cultures subjected to 1mM diamide treatment for 10 min (10D) and without diamide (0 and 10 min). Labeled primer oML02-15 and oSN03-63 were used to examine the level of *spx* and *azoR1* transcript respectively. For the dideoxynucleotide sequencing shown on the left, the nucleotide complementary to the dideoxynucleotide added in each reaction mixture is indicated above the corresponding lane (T′, A′, C′ and G′)
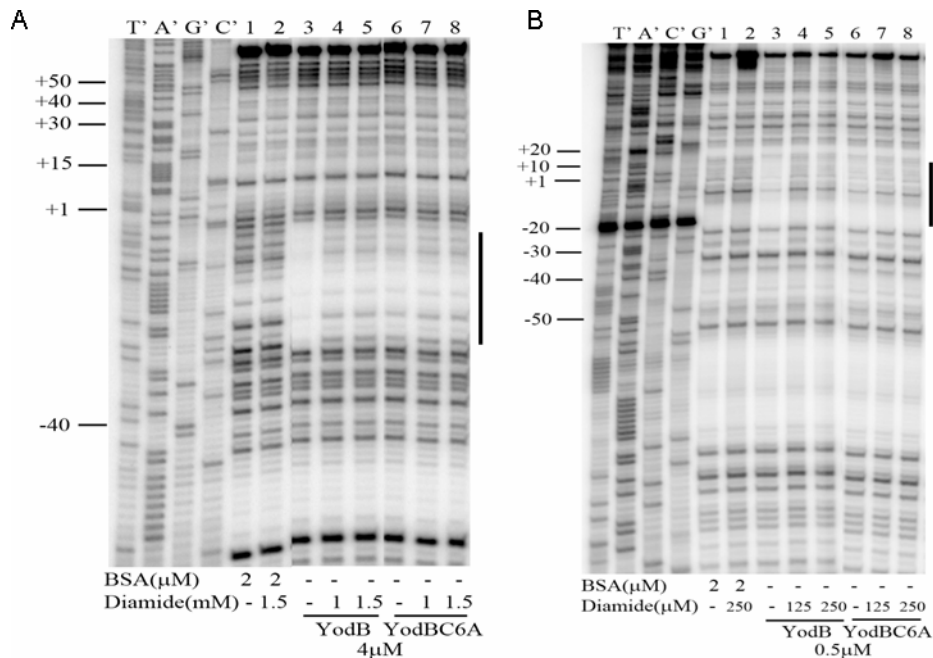
**Figure 4.6** Reduction of YodBC6A protein binding activity to the *spx* promoter (A) and *azoR1* promoter (B). DNase I footprinting was used to assess the binding activity of YodB and YobBC6A protein and the effect of diamide on DNA binding. The concentrations of proteins used were 4 µM (A, lane 3-8) and 0.5 µM (B, lane 3-8).The protected region by YodB is indicated by the solid line. The positions relative to the transcriptional start site are shown. The concentrations of diamide used were 1 mM and 1.5 mM for *spx* , 125 µM and 250 µM for *azoR1*. BSA, bovine serum albumin.
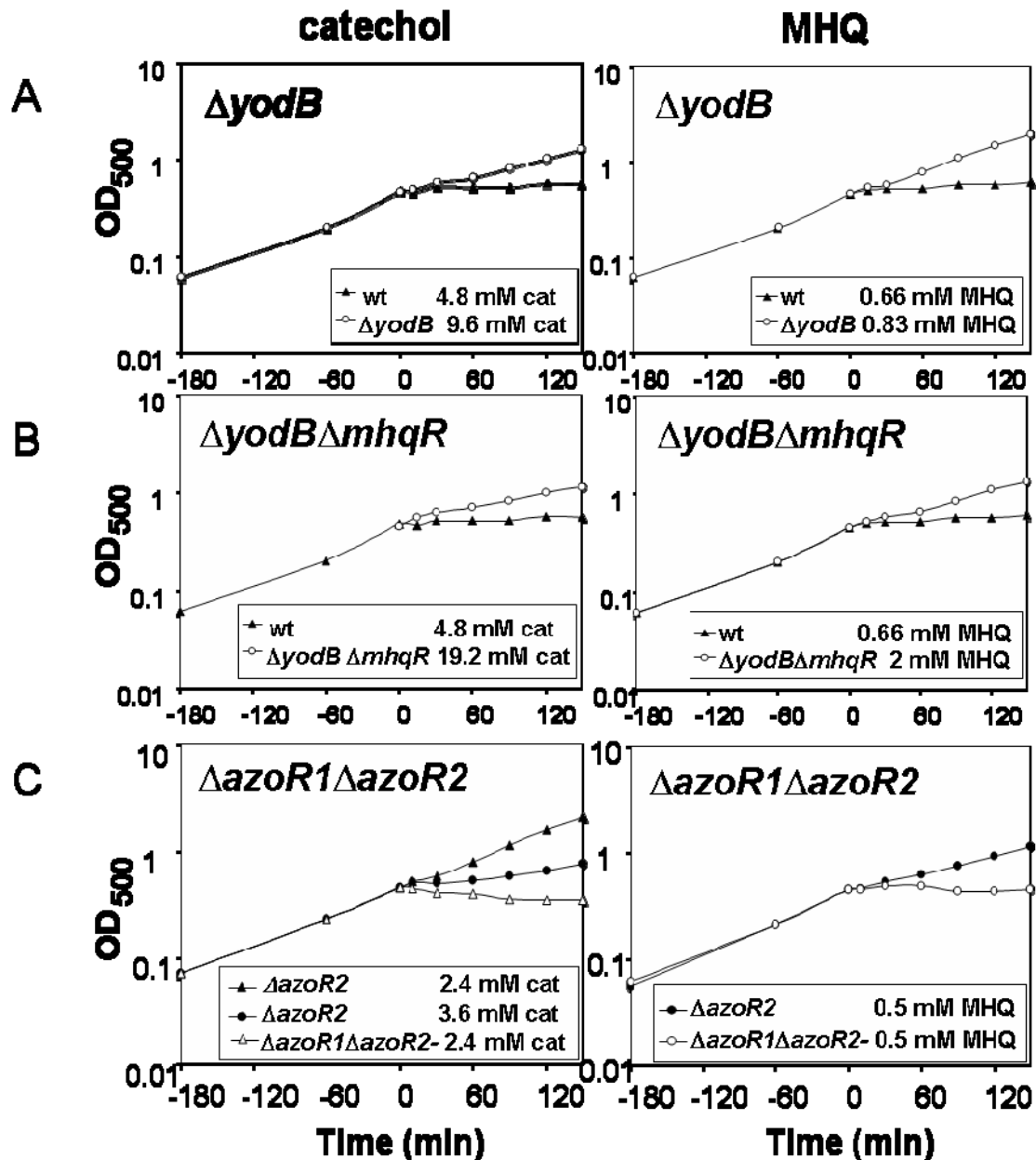
Figure 4.7 The Δ*yodB* mutant is resistant (A), the Δ*yodB*Δ*mhqR* double mutant is hyperresistant (B) and the Δ*azoR2*Δ*azoR1* double mutant is hypersensitive (C) to catechol and MHQ stress. *B. subtilis* wild type (wt), Δ*yodB*, Δ*yodB*Δ*mhqR,* Δ*azoR2* and Δ*azoR2*Δ*azoR1* mutant strains were grown in minimal medium to an OD500 of 0.4 and treated with catechol and MHQ at the time points that were set to zero. OD500 of the cultures was then measured at the time intervals indicated. Growth phenotype was performed by Antelmann group.
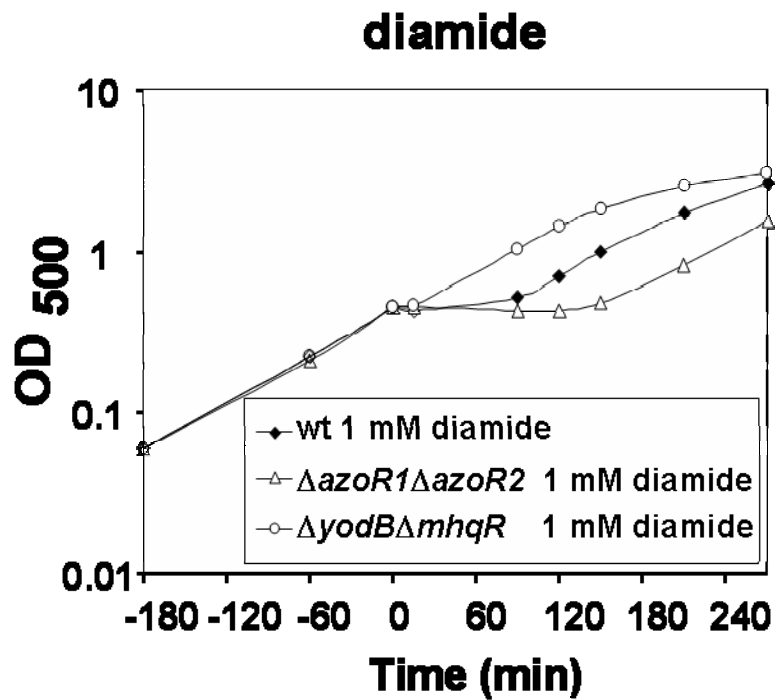
Figure 4.8 The Δ*azoR2*Δ*azoR1* double mutant is more sensitive and the Δ*yodB*Δ*mhqR* double mutant is more resistant to 1mM diamide compared to the wild-type. *B. subtilis* wild-type, Δ*azoR2*Δ*azoR1* and Δ*yodB*Δ*mhqR* mutant strains were grown in minimal medium to an OD500 of 0.4 and treated with 1mM diamide at the time points that were set to zero. Growth phenotype in the present of diamide was performed by Antelmann group.
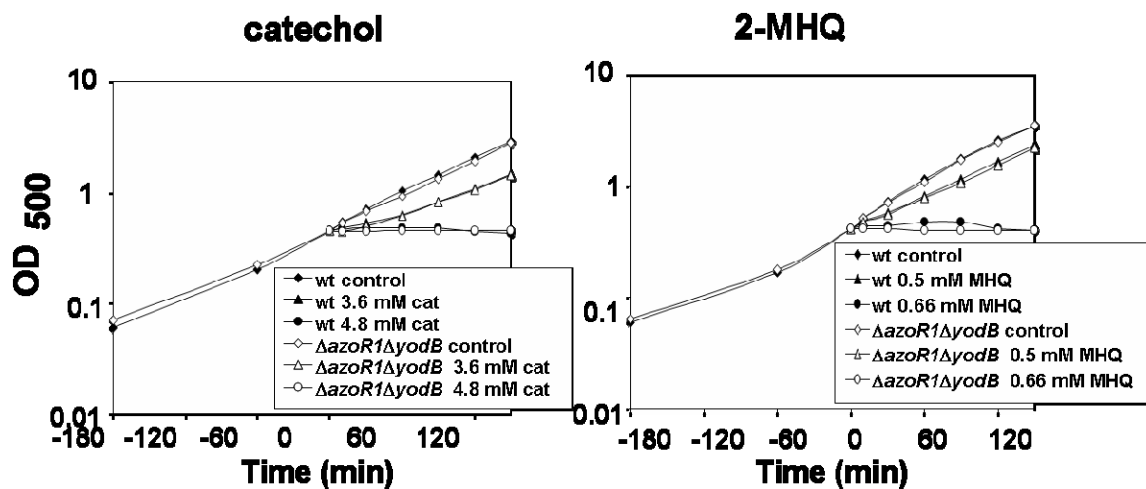
Figure 4.9 The Δ*yodB*Δ*azoR1* double mutant is not resistant to catechol and 2-MHQ. *B. subtilis* wild-type and Δ*yodB*Δ*azoR1* mutant strains were grown in minimal medium to an OD500 of 0.4 and treated with different concentrations of catechol and 2-MHQ at the time points that were set to zero. Growth phenotype of Δ*yodB*Δ*azoR1* double mutant in response to catechol and 2-MHQ is performed by Antelmann group.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 SUMMARY OF RESEARCH

5.1.1 Identification of *spx* P$_3$ promoter which is induced by the thiol-specific oxidant diamide

The transcription start site of the newly identified *spx* P$_3$ promoter was determined by primer extension analysis. Transcription from the *spx* P$_3$ promoter initiated from an A nucleotide 79 bp upstream of the *spx* start codon. The *spx* promoter region contains candidate recognition elements for σ^A RNAP. The transcription of *spx* from the P$_3$ promoter *in vitro* suggested that a positive transcriptional regulator was not required for transcription initiation. Primer extension showed that transcript levels of wild-type cells, *spx* mutant cells and *rpoA^{cxs-1}* cells increased after diamide treatment. Thus, the induction of *spx* transcription by diamide treatment does not require Spx-RNAP interaction. The *spx* expression is contributed from active promoters upstream of the *yjbC* coding sequence.

5.1.2 Discovery of two *cis*-acting negative control elements within the P$_3$ promoter sequence

Deletion analyses were performed to identify *cis*-acting elements associated with the P$_3$ promoter. Deletions of the sequence either 5′ or 3′ relative to P$_3$ were constructed and inserted in to *bgaB* reporter gene fusion construct. Levels of BgaB activity and the P$_3$ transcript were examined in cells after diamide treatment by measuring β-galactosidase activity and by performing primer extension analysis. Deletions of the 5′ end show

modest effects on basal-level expression although diamide induction is still detected. In contrast, higher basal-level expression of *spx* P$_3$ was detected in 3′ deletion mutants, as was loss of diamide induction. The analysis of *spx* P$_3$ point mutations obtained by UV and random PCR mutagenesis suggested the existence of a second *cis*-acting negative control region within the P$_3$ promoter. Several approaches including UV mutagenesis, plasmid-Erm insertion library screening, transposon mutagenesis and chemical mutagenesis were conducted to identify *trans*-acting factors. The absence of *trans*-acting mutations using those searches suggested that more than one regulator was controlling *spx* P$_3$ transcription. All of mutations obtained by UV mutagenesis and error-prone PCR contained multiple nucleotide substitutions in the intergenic region of *yjbC-spx*. Seven single-site mutants (T-26A, T-20G, T-19G, A-14T, A3G, T7C and T24C) were constructed by random PCR and site-directed oligonucleotide mutagenesis and were analyzed by measuring *spx*-directed BgaB activity. Four mutations within the P$_3$ promoter sequence, T-26A, A-14T, T7C and T24C, exhibit reduction of diamide induction ratio and/or elevated basal-level transcription. These finding suggested the existence of two *cis*-acting negative control sites within the P$_3$ promoter.

5.1.3 PerR and YodB negatively control *spx* P$_3$ transcription both *in vivo* and *in vitro*

A previously published study (Hayashi et al., 2005) and analyses using transcription factor/transformation array technology (Leelakriangsak et al., 2007) had uncovered proteins PerR and YodB as candidates for negative control factors affecting *spx*P$_3$ transcription. The *spx-bgaB* fusion was introduced into a *perR* and a *yodB* mutant strain to validate the previous results. *perR* and *yodB* mutations lead to derepression of *spx* transcription as observed by the increase of *spx-bgaB* expression. A *perR yodB* double mutant exhibited an additive effect on *spx-bgaB* transcription. In complementation experiments, the *yodB* wild-type allele complemented a *yodB* mutant, which confirms that the *yodB* product represses *spx-bgaB* expression. Primer extension analysis was carried out to determine the level of P$_3$ transcript in wild-type, *yodB* mutant, *perR* mutant and *yodB perR* mutant cells before and after diamide treatment. Diamide

treatment increased *spx* P$_3$ transcript levels in wild-type, *yodB* mutant and *perR* mutant cells. The levels of P$_3$ transcript were high in *yodB* and *perR* mutant cells compared to wild-type cells when the cultures were not treated with diamide. Unexpectedly, a reduction of P$_3$ transcript is observed in diamide treated *perR yodB* double mutant cells although untreated cells exhibit an increase of P$_3$ transcript levels, the latter result was consistent with the results from *spx-bgaB* fusion experiments.

Runoff *in vitro* transcription analysis was carried out to confirm the role of YodB and PerR in vitro. Purified YodB and PerR proteins were added to *in vitro* transcription reaction mixtures containing purified RNAP and various linear *spx* P$_3$ DNA fragments from *spx* P$_3$ promoter mutants, including T24C, T-26A, T-20G, T-19G, A-14T and *spx* deletion +5 and wild-type +40. YodB represses transcription of DNA templates made from wild-type and +5 deletion mutant promoter DNA, but not from T-26A, T-20G and T-19G. The A-14T template shows reduction of YodB-dependent repression compared to wild-type template. In contrast, the reduction of PerR-dependent repression was observed from T24C and +5 deletion templates whereas PerR repression is observed from the reaction mixtures containing wild-type, T-26A, T-20G, T-19G and A-14T. These results confirm that YodB interacts with a *cis*-acting element within the P$_3$ promoter, while PerR interacts at the operator located downstream of the P$_3$ transcriptional start site (Fig. 3.8 and see below).

5.1.4 Footprinting experiments revealed the binding sites of YodB and PerR proteins and showed that the proteins' binding activity is impaired in the presence of oxidants

The result of DNase I footprinting of YodB and PerR to the top strand of the *spx* promoter confirmed the binding sites of YodB and PerR transcriptional repressors. YodB protects an area from approximately positions -3 to -32 while PerR binds a region approximately between positions from -3 to +35, which contain the nucleotide positions that are the sites of mutations affecting the repression by YodB and PerR. The *spx* promoter DNA containing either T-26A or T-19G shows reduction of YodB binding activity without affecting PerR binding as determined by footprinting analysis. The

reduction of PerR binding and altered DNase I digestion pattern were observed in footprinting reactions that contained the T24C mutant promoter DNA. To examine the effect of toxic oxidants on DNA-binding activity of YodB and PerR, diamide and H_2O_2 were added to the footprinting reactions. DNA binding activity of YodB and PerR to the operator sequences is reduced in the presence of diamide or H_2O_2. These findings support the prediction that toxic oxidants impair YodB/PerR-dependent negative control, resulting in derepression of *spx* transcription.

5.1.5   The thiol-reactive compounds induce the YodB-controlled gene, *yocJ*(*azoR1*)

We identified other genes controlled by YodB by microarray and proteome analyses. The most strongly derepressed in *yodB* mutant cells is *yocJ* (*azoR1*), which encodes a putative FMN-dependent NADH-azoreductase. The proteome analysis showed an increase in AzoR1 protein synthesis by diamide, catechol, MHQ and nitrofurantoin treatment. In contrast, protein synthesis of the paralogous azoreductase AzoR2 which regulated by MarR-type repressor MhqR is not induced by addition of diamide and nitrofurantoin. The *bsrB* gene, encoding a small 6S RNA, resides downstream of *azoR1*. Transcription of monocistronic *azoR1* mRNA is induced in the presence of catechol and MHQ as determined by northern blot analysis. The increase in the level of a weak *azoR1-bsrB* transcript under thiol-stress conditions is due to a high induction of *azoR1* transcription and read-though past the *azoR1* terminator. Moreover, the expression of *azoR1-lacZ* is very high in the *yodB* mutant cells. These data confirm that YodB negatively regulates the expression of the *azoR1-bsrB*.

The *yodB* and *mhqR* mutants display a catechol and MHQ resistance phenotype. The *yodB mhqR* double mutant confers hyper-resistance to catechol, MHQ and diamide. Thus, *yodB* and *mhqR* regulons are very closely related in their responses to phenolic and azo-bearing thiol-reactive compounds. In contrast, the growth of *azoR1azoR2* double mutant is impaired in the presence of catechol and MHQ. This result suggests that AzoR1 and AzoR2 function in detoxification of the toxic thiol-reactive compounds.

5.1.6 YodB boxes residing within the *spx* and *azoR1* promoters are required for transcriptional repression by YodB

DNase I footprinting experiments identified the YodB protected region in the *azoR1* promoter from approximately -20 to +10 relative to the transcription start site. Alignments of the *spx* and *azoR1* promoter sequences identified a common 15 bp consensus sequence referred to as YodB boxes that are required for transcriptional repression by YodB. As shown previously, YodB binding activity on the *spx* P_3 promoter was impaired upon addition of diamide and H_2O_2. As expected, in the presence of diamide, H_2O_2 or MHQ, reduced binding of YodB to the *azoR*1 promoter was observed.

5.1.7 The Cys6 residue of YodB is required for full repression of *spx* and *azoR1* transcription

The YodB protein contains three cysteine residues Cys6, Cys101 and Cys108. Each of Cys residues was mutated to Ala, and a double mutant C101,108A was constructed. All mutant alleles of *yodB* were then integrated into the *thrC* locus. The expression of the *spx-bgaB* fusion was examined in *yodB* mutant background and each C-to-A alleles. The BgaB activity in the *yodBC6A* mutant is 1.5-fold higher than that of wild-type cells. Thus, the conserved N-terminal Cys6 is required for full repression of *spx* expression. DNase I footprinting analysis was carried out to examine YodBC6A protein binding activity on *spx* and *azoR1* promoters. A reduction of YodBC6A protein binding activity is observed compared to the wild-type YodB protein. However, YodBC6A still responds to diamide by losing its binding activity to *spx* and *azoR1* promoter DNA *in vitro*.

5.2 FUTURE DIRECTIONS

In these studies, we have elucidated the mechanism of *spx* gene transcription under oxidative stress. The expression of *spx* is under dual negative control by PerR and

YodB and responds to oxidative stress. Inactivation of repressors leads to the induction of *spx* expression. We have further identified the novel DUF24/MarR-like repressor YodB which controls genes, including *yodC* (encoding putative nitroreductase), *azoR1* (encoding azoreductase), in response to thiol-reactive compounds. *B. subtilis* YodB contains a conserved Cys residue, Cys6, like OhrR which belongs to MarR family protein. The structure of OhrR is similar to other MarR family which consists of six α helices and three β strands with topology: $\alpha1$, $\alpha2$, $\beta1$, $\alpha3$, $\alpha4$, $\beta2$, $\beta3$, $\alpha5$ and $\alpha6$ (Hong et al., 2005). OhrR is homodimer, each subunit consists of two functional domains: $\alpha1$, $\alpha2$, $\alpha5$ and $\alpha6$ which involve in dimerization between N and C termini and $\beta1$, $\alpha3$, $\alpha4$, $\beta2$ and $\beta3$ contain winged helix-turn-helix (wHTH) DNA binding domain (Fig. 5.1A). The comparison of sequence alignment of OhrR and YodB has revealed the conserved Cys at the N termini (Fig. 5.1B). The conserved Arg residue among MarR protein family which contacts DNA is also conserved in YodB. To investigate the importance of this Arg in DNA binding, substitution of Arg to Ala will be carried out to determine DNA binding affinity of YodB. Moreover, the predicted secondary structure of YodB also contains six α helices and at least two β strands with similar topology compared to OhrR (Fig. 5.1C). A BLAST search of YodB revealed the conserved Cys and Pro among Gram positive bacteria (Fig. 5.2).

The investigation of YodB Cys6 modifications by diamide is crucial in understanding the mechanism of diamide controlled YodB DNA binding activity. We can monitor *in vivo* oxidation of YodB by MALDI-TOF MS analysis (Lee & Helmann, 2006a, Lee et al., 2007). By using FLAG and anti-FLAG system, *yodB* ORF will be cloned to epitope protein tagging integration vector such as pMUTIN-FLAG. The *yodB-FLAG* will be integrated in the *amyE* locus of *B. subtilis*. Cells will be treated with 1mM diamide, and a parallel culture of cells will be left untreated as a control. Cell lysate containing YodB-FLAG will be incubated with anti-FLAG then modified with iodoacetamide to alkylate free cysteine. YodB-FLAG proteins will be recovered using SDS-PAGE and analyze the Cys6 modification by MALDI-TOF MS after in-gel tryptic digestion.

Azo dyes are synthetic organic colorants that are widely used for industrial, printing, cosmetic and clinical purposes. They are regarded as pollutants once they are released into the environment. Therefore, the biological degradation of these dyes by

azoreductases from microorganisms is a better alternative than uses of chemical degradation system for the research and development of biodegradation systems. Azoreductase catalyzes reductive cleavage of the azo group (-N=N-) resulting in azo dye degradation. We discovered the putative azoreductase AzoR1, the expression of which is negatively controlled by YodB. AzoR2 is negatively regulated by MhqR. Expression and characterization of genes encoding azoreductase in *B. subtilis* has been reported (Sugiura *et al.*, 2006). *azoR1* and *azoR2* ORF fragments will be cloned into protein expression vector followed by protein purification. The AzoR1 and AzoR2 protein will be examined for azoreductase activity by analyzing the reducing activity against various azo dyes at different temperatures by measuring the decrease in optical density at suitable wavelengths (each dye absorbs light at a specific wavelength).

Our finding of transcriptional control by YodB in response to thiol-reactive agents could potential lead to benefit the development of a bacterial biosensor to detect those compounds that are toxic and environmental pollutants found in water and soil. The preliminary experiments will determine and compare the dissociation kinetic (K_d) for YodB at *azoR1* promoter with or without addition of compounds. The *azoR1* promoter sequence will be fused to GFP reporter gene in order to monitor the expression by microscopy. When bacterial biosensors encounter the toxic compounds (phenolic compounds in this case) in the environment, the ligand (compound) binds to the protein resulting in the derepression of the genes under its control (*azoR*1). Thus, expression of the reporter (GFP) serves as a sensitive device for detection of low levels of a pollutant.

Overall my studies regarding transcriptional control of *spx* and *azoR1* in response to oxidative stress provide understanding for transcriptional regulation mechanisms controlling processes that allow bacteria to cope with various stresses. Because bacteria play important roles in the environment including recycling many chemical compounds in nature. Understanding them in molecular level will help us develop many applications, such as the biosensor described above. Additionally, the ability of bacteria to detect and to degrade toxic compounds plays a crucial role in development of microbial-based methods of bioremediation.

Figure. 5.1 Structure of OhrR and YodB.
(A) Reduced OhrR is shown as a ribbon diagram with the N and C termini of both subunits and secondary structural elements of one subunit colored and labeled. The dyadic mate is colored in pink. One wing is labeled with a "W" (Hong et al., 2005).
(B) Sequence alignment of structurally determined of OhrR (Hong et al., 2005) and YodB prepared by Clustal W. The secondary structure elements of OhrR and DNA bound OhrR are indicated above the sequence by arrows (β strands) and solid rectangles (α helices). The conserved residues are indicated as asterisks and dots. DNA contacting residues of the wing are denoted by red asterisks. The conserved cysteine residues are in red box.
(C) The predicted secondary structure of YodB using PredictProtein. Helix: H, Sheet: E, Blank: other or loop.

Figure.5.2 Sequence alignment of YodB and its homolog of DUF24/MarR family proteins prepared by Clustal W. Residues that are identical in all homologs are denoted as asterisk (*). The amino acids that similar are indicated by : and · respectively. The Arg residue involving in DNA contacting of OhrR is denoted by green asterisk. The predicted secondary structure elements of YodB using PredicProtein are indicated above the sequence by red rectangles (α helices) and blue rectangles (β strands).

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BIOGRAPHICAL SKETCH

Montira Leelakriangsak was born in Yala, Thailand on November 23, 1974. She received her B.S. in Aquatic Science from Prince of Songkla University in 1996. She received a grant from Prince of Songkla University to conduct an undergraduate project on "Formaldehyde residue in marine fish at Hat Yai municipal market". During being employed as research assistant at Department of Oral Biology and Occlusion, Faculty of Dentistry, Prince of Songkla University, she is awarded the fellowship from WHO as a short-term visiting acholar at Unit of Mechnisms of carcinogenesis, IARC, Lyon, France. In 2000, she received full support scholarship by the Royal Thai Government with a scholarship for graduate level study on Molecular Genetics. She pursued her M.S. in Biochemistry and Molecular Biology at the Oregon Health and Science University during the fall of 2001 then she continued her Ph.D. degree at Biochemistry and Molecular Biology at the Oregon Health and Science University under the supervision and research of Dr. Peter Zuber. Following the successful defense of her dissertation, Montira will return to Thailand and be a part of faculty staff of Prince of Songkla University, Pattani campus.

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