The transcriptional control of spx in response to oxidative stress

Montira Leelakriangsak

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THE TRANSCRIPTIONAL CONTROL OF SPX IN RESPONSE TO OXIDATIVE STRESS

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ABSTRACT

The Transcriptional Control of \textit{spx} in Response to Oxidative Stress

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Ph.D., OGI School of Science & Engineering
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Thesis Advisor: Dr. Peter Zuber

The \textit{Bacillus subtilis} \textit{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 \textit{yjbC-spx} operon and is transcribed from at least four promoters, three (P1, P2 and P3) residing upstream of \textit{yjbC} and one (PM) located in the intergenic region between \textit{yjbC} and \textit{spx}. We uncovered a second intergenic promoter, P3, from which transcription is elevated in cells treated with the thiol-specific oxidant diamide, by primer extension analysis. P3 is recognized by the $\sigma^A$ form of RNA polymerase (RNAP) in vitro without the involvement of a transcriptional activator. Deletion analysis together with point mutation analysis uncovered two negative \textit{cis}-acting control elements within the P3 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 P3 promoter utilization. PerR was previously characterized as the regulator of the inducible peroxide stress response in \textit{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 P3 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 P3 promoter DNA was impaired by addition of diamide and H2O2 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.
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 (ββ′α2ω or E) and RNAP holoenzyme (ββ′α2ωσ) (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 k1 and k-1, respectively; KB represents the binding equilibrium constant of this reaction (k1/ k-1) and the isomerization to form the open complex (RPo) can be described by forward and reverse rate constants k2 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 KB and k2 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 σ70 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 $\sigma^{70}$, 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 $17 \pm 1$ bp and 75% of defined start points were $7 \pm 1$ 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 $\alpha$-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 $\sigma$ subunit. The discriminator region, a G-C rich region downstream of -10 element, has potential to impede strand separation and also contacts the $\sigma$ subunit (Haugen et al., 2006). Further description of $\sigma$ 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* P₁ 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 *P₇₃A*, 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 *P₇₃A* promoter is enhanced by the interaction of αCTD to an UP element (Quinones et al., 2006). Host repressor LexA represses *P₇₃A* 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 (α₂ββ′ω) 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). σ⁷₀, 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 α 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 σ²⁰ (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 Eσ70 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 σ70 (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 Mg2+ (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 separating 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,
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 (Touloukhonov & 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 Mg2+ 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 (ββαω) alone is capable for transcription elongation and termination but is insufficient to initiate transcription at promoter DNA. The primary σ factor, known as σ\textsuperscript{70} in *E. coli* and σ\textsuperscript{A} in *B. subtilis* is essential for general transcription in exponentially growing cells (Helmann & Chamberlin, 1988, Paget & Helmann, 2003).

Sequence alignments of the σ\textsuperscript{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\textsuperscript{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 $\sigma^{70}$ region 3.2 from the RNA exit channel followed by displacement of $\sigma^{70}$ region 4 from the $\beta$-flap domain (Nickels et al., 2005).

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

1.1.3 Alternative $\sigma$ factors

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

E. coli contains at least six $\sigma$ factors of the $\sigma^{70}$ family, $\sigma^{70}$ (RpoD), $\sigma^S$ (RpoS), $\sigma^{32}$ (RpoH), $\sigma^F$ (FliA), $\sigma^E$ (RpoE), and $\sigma^{fecl}$ (Fecl) (Gourse et al., 2006, Gruber & Gross, 2003). The $\sigma^{54}$ (RpoN) family, a second family of $\sigma$ 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, $\sigma^S$ was first identified for its role in transcription during stationary phase. $\sigma^S$ (RpoS) is very similar to the vegetative $\sigma^{70}$ with respect to its structure, molecular function and promoter recognition properties (Gaal et al., 2001, Gruber & Gross, 2003). E$\sigma^S$ and E$\sigma^{70}$ utilize the same core promoter sequences (Hengge-Aronis, 2002b). However, the selectivity of E$\sigma^S$ 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). E$\sigma^S$ 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). $\sigma^S$ 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 $\sigma^{32}$ 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 $\sigma^{32}$ 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 $\sigma^{32}$ regulon. GroEL and DnaK also function in the regulation of both $\sigma^{32}$ activity and stability (Gruber & Gross, 2003, Guisbert et al., 2004). DnaK negatively regulates $\sigma^{32}$ activity by binding to $\sigma^{32}$ and facilitating $\sigma^{32}$ degradation (Guisbert et al., 2004). Damaged, unfolded proteins and $\sigma^{32}$ compete for DnaK binding. The DnaK-$\sigma^{32}$ interaction inhibits $\sigma^{32}$-dependent transcription (Guisbert et al., 2004). However, when the unfolded proteins are more abundant relative to DnaK, the level of free $\sigma^{32}$ increases, resulting in the induction of heat shock genes (Guisbert et al., 2004). Thus, the tight regulation exerted by DnaK through its interaction with $\sigma^{32}$ 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, $\sigma^F$ ($\sigma^{28}$), 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 ($\sigma^F$) as well as the hook-basal body proteins are transcribed from class II promoters (Chilcott & Hughes, 2000). $\sigma^F$ is required to activate the class III genes whose products function in flagellum assembly (Gruber & Gross, 2003). The negative regulator FlgM binds to $\sigma^F$ 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 $\sigma^F$ for the transcription of class III genes (Kutsukake, 1994).

The $\sigma^E$ 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). $\sigma^E$ activity is negatively regulated by RseB through RseA (Missiakas et al., 1997). RseA, a membrane-bound anti-sigma-factor, with help of RseB inactivates $\sigma^E$ 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 $\sigma^E$-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 $\sigma^{70}$, there are six other $\sigma$ factors, and each recognizes a different set of promoters having a primary structure that is recognized by a specific sigma subunit. $\sigma^S$ plays a crucial role in stationary phase or under stress conditions. $\sigma^{32}$ controls heat shock promoters. $\sigma^F$ is the master regulator of the flagellar genes. $\sigma^E$ is induced by extracytoplasmic stresses and $\sigma^{fecI}$ 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 \( \text{sigM} \) is positively autoregulated with transcription initiating from two promoters, \( P_M \) (\( \sigma^M \)-dependent promoter) and \( P_A \) (\( \sigma^A \)-dependent promoter) (Horsburgh & Moir, 1999). Sequence comparisons of the \( \text{sigM} \), \( \text{sigW} \) and \( \text{sigX} \) promoter sites recognized by \( \sigma^M \), \( \sigma^W \) and \( \sigma^X \) 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 \( \sigma^W \) and \( \sigma^X \) respectively (Horsburgh & Moir, 1999, Huang et al., 1998a, Jervis et al., 2007).

The \( \sigma^W \), encoded by \( \text{sigW} \), is modulated by a specific anti-sigma factor RsiW, encoded by \( \text{rsiW} \) (\( ybbM \)), which resides immediately downstream of \( \text{sigW} \) (Schobel et al., 2004, Yoshimura et al., 2004). The \( \sigma^W \)-controlled genes encode products that function in detoxification and protection against antimicrobials (Cao et al., 2002b, Butcher & Helmann, 2006). The activity of \( \sigma^W \) is controlled by three proteolytic steps of RsiW degradation resulting in the increase of free \( \sigma^W \) concentration and activation of the \( \sigma^W \) 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 \( \sigma^W \) and transcription of \( \sigma^W \)-control genes (Frees et al., 2007, Zellmeier et al., 2006). The mechanism of the \( B. \ subtilis \) cell wall stress response mediated by \( \sigma^W / \text{RsiW} \) is similar to the mechanism of the \( E. \ coli \) extracytoplasmic stress response mediated by \( \sigma^E / \text{RseA} \), 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 \( \sigma^W \)-controlled transcription in \( B. \ subtilis \) is in contrast to FecR, in which the cytoplasmic domain activates \( \sigma^{fecI} \)-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 ($\text{O}_2\text{•}^-$), hydrogen peroxide ($\text{H}_2\text{O}_2$) and hydroxyl radical ($\text{HO}_\text{•}$) (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 $\text{H}_2\text{O}_2$ and $\text{O}_2$, whereas, $\text{H}_2\text{O}_2$ 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 & Buttnner, 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 ($O_2^{-}$) 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 ($HO•$) 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 $HO•$ via Fenton reaction (Keyer et al., 1995, Keyer & Imlay, 1996). $H_2O_2$ is formed by spontaneous or enzyme-catalyzed reactions of $HO•$ (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 $O_2^{-}$-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-dimethlylamine)], 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$_2$O$_2$ with cysteinyl thiolates, which are major targets of H$_2$O$_2$, 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-nitrothiol (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 *yjhA* (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$_2$O$_2$, 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 $\alpha$-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$_2$O$_2$ (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$_2$O$_2$, 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 $\sigma^B$, 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\textsubscript{232}XC\textsubscript{234} and C\textsubscript{265}XXC\textsubscript{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\textsubscript{232} with C\textsubscript{234} and C\textsubscript{265} with C\textsubscript{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 intramolecular 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 \( \sigma^R - \text{RsrA} \). 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 \textit{Streptomyces coelicolor} (Paget \textit{et al.}, 1998, Zeller & Klug, 2006). Transcription of \( \text{trxBA} \), encoding thioredoxin reductase and thioredoxin, is initiated from two promoters, \( \text{trxBp}1 \) and \( \text{trxBp}2 \), which are induced by diamide treatment (Paget \textit{et al.}, 1998). The level of \( \text{trxBp}1 \) transcript is induced under disulfide stress in a \( \sigma^R \)-dependent manner (Paget \textit{et al.}, 1998). \( \sigma^R \) is encoded by \( \text{sigR} \) gene, which is transcribed from two promoters, \( \text{sigRp}1 \) and \( \text{sigRp}2 \) (Paget \textit{et al.}, 1998). \( \sigma^R \) itself contains no cysteines and so does not function alone in redox sensing during disulfide stress (Paget \textit{et al.}, 1998).

RsrA (regulator of \( \text{sigR} \)) encoded by the \( \text{rsrA} \) gene lying immediately downstream of \( \text{sigR} \), regulates \( \sigma^R \)-directed transcription in response to disulfide stress (Kang \textit{et al.}, 1999). RsrA is an anti-sigma factor that binds directly to \( \sigma^R \) only in the reduced form. RsrA activity is modulated by oxidation and reduction of cysteine residues in the RsrA protein (Kang \textit{et al.}, 1999). RsrA contains seven cysteine residues and C11, C41 and C44 are essential for anti-sigma activity (Paget \textit{et al.}, 2001). Although C11 is not absolutely conserved, His37, Cys41 and Cys44 are conserved and form a HisXXXCysXXCys motif (Kang \textit{et al.}, 1999, Paget \textit{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 \textit{et al.}, 2001).

Zn(II) coordinated to three cysteine residues (C3, C41 and C44) and one histidine residue (H7) was proposed by Bae \textit{et al.} in their model for the single zinc-binding site in RsrA (Bae \textit{et al.}, 2004). However, the model proposed by Li \textit{et al.}, which was confirmed by Zdanowski \textit{et al.}, predicts the single zinc atom in RsrA is coordinated by C11, H37, C41 and C44 (Li \textit{et al.}, 2003, Zdanowski \textit{et al.}, 2006). During oxidation, zinc is released and a disulfide bond is formed, which results in RsrA dissociation from \( \sigma^R \) leading to activation of its regulon (Kang \textit{et al.}, 1999, Li \textit{et al.}, 2003, Paget \textit{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 \textit{et al.}, 1999, Li \textit{et al.}, 2003).
In conclusion, the thiol disulfide status of *S. coelicolor* is monitored by a regulatory system consisting of a $\sigma^R$ and anti-sigma factor RsrA. In the reducing environment, RsrA binds a single zinc atom and forms a complex with $\sigma^R$ that represses transcriptional activity of $\sigma^R$. 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 $\sigma^R$. The free $\sigma^R$ then is free to interact with core RNAP and activate transcription of its target genes, including *trxBA*, in response to disulfide stress.

1.2.2.3 *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 $\sigma^A$-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$_2$O$_2$ (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$_2$O$_2$ whereas ahpC and hemA promoters are weakly induced by H$_2$O$_2$, 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$_2$O$_2$, while the manganese form of PerR is relatively insensitive to H$_2$O$_2$ (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$_4$Zn), which is important to stabilize the dimeric form of the protein (Traore et al., 2006, Herbig & Helmann, 2001). The Cys$_4$Zn site is not involved in sensing low levels of H$_2$O$_2$, however, intramolecular disulfide bonds between cysteine residues in the two CXXC motifs occur under severe oxidative stress conditions (10 mM H$_2$O$_2$) 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\(_4\)Zn), 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\(_4\)Zn 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.,...
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<sup>cxS-16</sup>, 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<sup>cxS-16</sup> 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<sup>cxS-16</sup>) 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<sup>cxS-16</sup>. Mutant RpoA<sup>cxS-1</sup>, 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 α1 of 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*<sup>DD</sup> cells, but not in the IPTG-treated *spx*<sup>DD rpoA<sup>cys1</sup></sup> cells (Nakano et al., 2003a). *spx*<sup>DD</sup> 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<sup>DD</sup>-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<sup>DD</sup>-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<sup>DD</sup>-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 \textit{trxA} and \textit{trxB} and transcriptional repression of \textit{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 \( \alpha \)CTD and exerts both positive and negative control over transcription initiation of multiple genes in response to disulfide stress.

\textbf{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 \textit{trxA} and \textit{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 \( \alpha \), to position RNAP optimally for its transcriptional activation under oxidizing conditions (Newberry et al., 2005). Preliminary data suggests that Spx also interacts with the \( \beta \) subunit of RNA polymerase in the vicinity of the \( \beta \)-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$_2$O$_2$ 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 addition to trxA and trxB, Spx-controlled genes include those that function in organosulfur metabolism: 1) genes such as ytmI, yxel 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-dimensional 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 (multiple antibiotic resistance regulator) family of transcriptional regulators control resistance to multiple 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

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 inserted 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 $\alpha$ helices and three $\beta$ 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 $\beta_1, \alpha_3, \alpha_4, \beta_2$ and $\beta_3$ (Hong et al., 2005). Cys15 is located at the N terminus of helix $\alpha_1$ 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 $\alpha_2$ 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 promoter 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$_2$O$_2$) and superoxide (O$_2$•-) are generated endogenously by autooxidation via electron transport chain reaction. Superoxide is rapidly converted to H$_2$O$_2$ by superoxide dismutase (SOD). H$_2$O$_2$ 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 radical (•OH) in the Fenton reaction (indicated by green box) which cause DNA damage. H$_2$O$_2$ 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 sulfenic acid (SO$_2$H) and sulfonic acid (-SO$_3$H) 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 P₁, P₂ and P₃ and two promoters located within the intergenic region of yjbC-spx, P₃ and P₄. 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

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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: P1 recognized by the σA form of RNAP, P2 and P3 utilized by the σW and σB RNAP holoenzymes, respectively. In the yjbC-spx intergenic region, the P4 promoter was identified (Thackray & Moir, 2003), which is utilized by the σM form of RNAP. The σB RNAP holoenzyme is a crucial component of the general energy and environmental stress responses (Hecker & Volker, 1998). The σW 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, P3, is utilized by the σA form of RNAP and is associated with two negative control elements. The accompanying paper describes the two repressors that target the spx P3 promoter region.
2.2 RESULTS

2.2.1 Identification of P3 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 σW (P2) and σB (P3) 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 σM 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 P3 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 P3 does not require spx

Transcription of spx from P3 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 \( rpoA^{cxr-1} \) mutation (Fig. 2.2B), which renders RNAP \( \alpha \) 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 \( rpoA^{cxr-1} \) 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 \( \beta \)-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 \( \sigma^W \) and \( \sigma^B \) 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 P3

The result of Fig. 2.1D showed that the P3 promoter can be utilized by purified $\sigma^A$ RNAP in vitro without the presence of a positive control factor, suggesting that the diamide induction of P3 transcription might be caused by a reversal of negative control. To identify cis-acting control sequences associated with the P3 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 P3 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 P3 transcriptional start-site resulted in higher basal level expression.

2.2.5 A negative control element resides downstream of the P3 promoter sequence

As summarized in Fig. 2.4, deletions of the 5′ end had little effect on the diamide-dependent induction of P3. 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 P3 promoter, does not change the induction pattern of P3 transcription (lanes 1-3), which is also observed when the endogenous spx P3 transcript is examined (lanes 4-6). Deletion of the DNA 3′ of the P3 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 P3 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 P2 and Pb promoter transcripts can be detected in RNA from untreated cells, while diamide treatment has no effect on the level of P2 transcript but results in loss of the Pb transcript. At present, we do not know if this repression of Pb is operon specific or due to reduction of σB activity upon disulfide stress.

### 2.2.6 Point mutations suggest the presence of a second negative control site within the P3 promoter

Experiments were conducted to uncover mutations in trans-acting loci, with hopes of identifying the putative repressor controlling transcription from P3. 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. 2.5S2). 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 P3 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. 2.5S3). 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 \textit{spx}-directed BgaB activity (Fig. 2.6B), and by primer extension analysis (Fig. 2.6C). Of the seven, five showed elevated basal \textit{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 \textit{cis}-acting, negative control region within the P3 promoter region.

\section*{2.3 DISCUSSION}

The transcription of \textit{spx} is driven by five promoters of the \textit{yjbC-spx} operon, although not all of the promoters are active under the culture growth conditions used in the study described herein. The promoters \textit{P}_M and \textit{P}_3 reside in the intergenic region of \textit{yjbC-spx}, but only transcription from \textit{P}_3 is observed in the primer extension analysis of RNA from cells grown in minimal TSS medium, before and after diamide treatment. The \textit{P}_3 promoter contains a consensus -10 sequence that is utilized by the major, \textit{σ}^\text{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 \textit{P}_3 increase 10 min after diamide treatment. Transcription from \textit{P}_3 is catalyzed by \textit{σ}^\text{A} holoenzyme in vitro without the requirement for an additional transcription factor, which suggested that transcription from \textit{P}_3 is under negative control. The intergenic region contributes significantly to \textit{spx} transcription as shown in experiments using \textit{yjbC-spx}- and \textit{spx-bgaB} fusions, and by complementation of a \textit{spx} null mutation with respect to diamide sensitivity using a single copy of the \textit{spx} gene with the accompanying \textit{yjbC-spx} intergenic region. Spx concentration increases after diamide treatment, which was shown to be due in part to post-transcriptional control of \textit{spx} expression (Nakano et al., 2003a), perhaps by down-regulation of ClpXP-dependent proteolysis of Spx. However, the observation that \textit{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 P3 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 P3 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 P3 transcriptional start-site and three upstream and within the P3 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 P3 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 P3 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 P3 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 *P₃* 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 P₃ 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 P₃ 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 OD₆₀₀ ∼ 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 P₃ 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 P₃ 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 (1 mM) 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, 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 μCi α-³²P-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\textsuperscript{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\textsubscript{2}, 0.3 mM MnCl\textsubscript{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\textsuperscript{+} 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.
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<sup>a</sup> wt, wild-type.
### Table 2.2 Oligonucleotides used

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</tr>
<tr>
<td>oML02-51</td>
<td>5′-TAAATTGAgATTACTCTAAAAAG-3′</td>
</tr>
</tbody>
</table>

*Bases in lowercase type are mutagenic*
Table 2.S1 Percentage of auxotrophic by UV mutagenesis

<table>
<thead>
<tr>
<th>Irradiation time (min)</th>
<th>Auxotrophic (%)</th>
</tr>
</thead>
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<tr>
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<td>1</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>25 (2 auxotrophic out of 8 colonies)</td>
</tr>
</tbody>
</table>
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 \( \sigma^M \) 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 \( \mu \)M 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<sup>ex-1</sup>) 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 Miller 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 P3 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 P₃ 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 P₃ 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 OD₆₀₀ 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 (σᴬ, σᴮ, σᴹ, ...
and σW). 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 P3 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 P3 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 P3 promoter and its activation during disulfide stress are described. P3 resides in the intergenic region between yjbC and spx, 79 bp upstream from the spx ATG start codon. It is utilized by the σA 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 P3-directed transcription initiation, one downstream of the transcriptional start site and the other within the P3 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 P3 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 P3 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 P3 transcript levels (Fig 3.3, lanes 1-3). An increase in P3 transcript was also observed in the diamide-treated yodB (lanes 4-6) and perR (lanes 7-9) mutant cells. Importantly, P3 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 \textit{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 \textit{yodB} or \textit{perR} single mutant. Unexpectedly, the double mutant, when treated with diamide, reproducibly showed sharply reduced \textit{spx} P3 transcript (lane 12).

3.2.2 \textbf{Purified PerR and YodB proteins repress transcription from \textit{spx P3} in vitro}

YodB and PerR protein was obtained from \textit{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 $\sigma^A$ from \textit{B. subtilis} and linear \textit{spx} P3 DNA fragments generated by PCR. Template DNA was also obtained by PCR amplification of mutant P3 promoter DNA that was isolated as described in the accompanying paper. The \textit{spx} mutations T24C, T-26A, T-20G, T-19G, and A-14T caused \textit{spx} transcription from P3 to be higher than that observed in wild-type cells (Leelakriangsak & Zuber, 2007). Deletions of the region 3’ to the P3 transcription start site also resulted in derepression of transcription from P3. Addition of YodB to the run-off transcription reactions showed that YodB could repress transcription from P3 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 P3 DNA. YodB could repress transcription of a template made from T24C mutant DNA or from DNA of the deletion mutant $\Delta +5$ (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 \textit{spx} P3 DNA, but not from $\Delta +5$ or from T24C. Neither repressor significantly affected transcription from the control \textit{rpsD} (ribosomal protein S4) promoter DNA. These results suggested that sequences in the \textit{spx} P3 promoter were required for YodB-dependent repression, whereas sequences 3’ of the \textit{spx} P3 start site were necessary for PerR-
dependent repression. As predicted from work described in the accompanying paper, the \textit{in vitro} transcription analysis uncovered two \textit{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 \textit{spx} transcription initiation and the respective \textit{cis}-acting elements was obtained by examining the \textit{in vivo} basal level of \textit{spx} expression using the \textit{spx-bgaB} fusion construct. Fig. 3.5 shows that expression of mutant \textit{spx-bgaB} fusions bearing mutations in the regulatory \textit{cis}-acting sites in wild-type cells and \textit{perR} (Fig. 3.5A) or \textit{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 \textit{perR} vs. wild-type cells (Fig. 3.5A), while the upstream mutations residing in the P3 promoter affected the ratio of \textit{yodB} vs. wild-type expression. These data confirm that YodB acts on the upstream \textit{cis} regulatory sequence, while PerR acts on the negative control element located 3’ to the transcription start site.

\textbf{3.2.3 YodB and PerR proteins interact with two distinct regions of the \textit{spx} P3 promoter DNA}

DNase I footprinting analysis was performed to confirm that the regions of \textit{spx} P3 DNA required for transcriptional repression \textit{in vitro} were sites of YodB and PerR interaction. End-labeled P3 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 P3 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 \textit{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 \textit{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 consistent with the in vivo and in vitro phenotype of the spx P3 mutations and the yodB and perR insertion mutations.

### 3.2.4 Diamide and hydrogen peroxide reduce binding of YodB and PerR proteins to spx P3 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 H2O2. Using the DNase I protection assay, we determined if binding of PerR and YodB to P3 was reduced if diamide or H2O2 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 P3 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 P3 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 H2O2 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 spxP3-directed β-galactosidase activity in spx-lacZ fusion bearing cells after treatment with 100 μM H2O2. Untreated cells showed activity of 68.4±1.8 and treated cells had activity of 98.7±0.8 after 30 min. H2O2 treatment.
3.3 DISCUSSION

The \textit{yjbC-spx} operon is under complex transcriptional control involving the activities of four RNAP holoenzyme forms utilizing promoters in the \textit{yjbC} promoter regions and in the intergenic region of \textit{yjbC} and \textit{spx}. Under the growth conditions used in the study reported here and the accompanying paper (Leelakriangsak & Zuber, 2007), transcription of \textit{spx} was observed to be initiated from an intergenic promoter, P3, that is recognized by the major \(\sigma^A\) form of RNAP. Although a promoter recognized by \(\sigma^M\) 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 P3 promoter.

Studies described in the accompanying paper uncovered the P3 promoter and two putative \textit{cis}-acting negative control elements, mutations in which caused derepression of \textit{spx} transcription \textit{in vivo} (Leelakriangsak & Zuber, 2007). As shown herein, the two \textit{cis}-acting sites are operators for two repressor proteins PerR and YodB. The \textit{perR} and \textit{yodB} null mutations also lead to derepression of \textit{spx} transcription from P3 and the products of the two genes interact directly with \textit{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 \textit{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 \textit{spx} as a stress-induced gene. Proteomic and transcriptomic studies have shown that \textit{spx} transcript levels increase in response to ethanol stress and phosphate limitation (Antelmann et al., 2000, Thackray & Moir, 2003). Mutations of \textit{yjbC} and \textit{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 \textit{spx} gene as being induced by reactive oxygen species, but microarray analysis of the PerR regulon showed that \textit{spx} transcript levels increased in a \textit{perR} mutant (Hayashi et al., 2005). Additionally, treatment of cells with the thiol-specific oxidant, diamide was reported to increase \textit{spx}
transcript levels (Leichert et al., 2003). The accompanying paper provides data that diamide induction involves accelerated transcription from the P₃ 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 P₃ 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* P₃ 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* P₃.

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 H₂O₂ 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* HxIR (Yurimoto et al., 2005) is the only member of known regulatory function. HxIR activates the *hxlAB* operon encoding the ribulose monophosphate pathway of formaldehyde fixation. HxIR 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 \textit{yodB} gene resides adjacent to \textit{yodC}, which encodes a putative nitroreductase. \textit{yodC} and \textit{yodB} are in divergent orientation with respect to one another and preliminary analysis of \textit{yodC-lacZ} expression has suggested that YodB is a repressor of \textit{yodC} transcription (M.L. and P. Z., unpublished). The expression of the \textit{yodB} and \textit{yodC} genes are induced by catechol treatment (Tam et al., 2006b), conditions that also induce the \textit{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 \textit{B. subtilis} through its control of \textit{yodC} and \textit{spx}.

A curious observation is the reduction of \textit{spx} transcript in the \textit{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 \textit{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 \textit{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 P3 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 *P₃*) 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 ORB4563 replacing Cm' cassette to Spc' 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 P3 promoter region (-330 to +50 relative to the P3 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 (spxP3Δ+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 (spxP3Δ+50(wt)-bgaB, yodB::cat). Chromosomal DNA of ORB6267 (perR::kan) was used to transform cells of strain ORB5058 to generate ORB6268 (spxP3Δ+50(wt)-bgaB, perR::kan). The yodB perR double mutant was constructed by transformation of ORB6288 with chromosomal DNA from ORB6267 to generate ORB6594 (spxP3Δ+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 (spxP3Δ+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 SPβc2del::Tn917::pML8-15 (spxP3-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 NaH2PO4, 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 NaH2PO4, 300 mM NaCl, 20 mM imidazole). His6-PerR or His6-YodB was eluted with elution buffer C (50 mM NaH2PO4, 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™ (Invitrogen) at 30°C for 4 hr to remove the N-terminal His6 tag. The AcTEV™ 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 μcui α-32P-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-32P]-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 MgCl2, 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 H2O2 used were 25 mM, 50 mM and 100 mM to detect the effect of H2O2 on the DNA-binding. Proteins were incubated with 100,000 cpm 32P 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

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Plasmids

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**Table 3.2 Oligonucleotides used**

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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 OD₆₀₀ 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’).
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 P3 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 H$_2$O$_2$ on the DNA-binding. PerR and YodB were incubated with DNA as follows: Lane 1-2, DNA alone control; lane 3-6, 11, 12, 1 µM PerR; lane 7-12, 2 µM YodB. The concentrations of H$_2$O$_2$ used were: 25 mM, 50 mM and 100 mM.
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 P₃ 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 P₃ 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 
$\Delta mhqR$ 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 $\Delta yodB$ 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 $\Delta yodB$ 
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 $\Delta yodB$ 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 $\Delta yodB$ 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 TACTWTTTTgWWAGTA, 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 P3 promoter DNA was impaired in the presence of diamide and H2O2 (Leelakriangsak et al., 2007). We further determined whether the binding activity of YodB on the azoR1 promoter is reversed by the addition of diamide, H2O2 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, H2O2 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 H2O2 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, H₂O₂ 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 ΔyodB 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 (ΔyodB/yodBC101A), ORB6716 (ΔyodB/yodBC108A) and ORB6777 (ΔyodB/yodBC101,108A) was similar to the wild-type (ORB6284). However, the expression of spx was derepressed in the ORB6599 (ΔyodB/yodBC6A) strain. The activity observed in the ΔyodB 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 spxP₃ 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 spxP3 and azoR1 transcripts increased after diamide treatment (Fig. 4.5B, lane 3). The transcript concentration of azoR1 and spxP3 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 spxP3 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 spxP3 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 sensitivity, 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 ΔazoR2 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 ΔazoR1 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 ΔazoR2ΔazoR1 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 ΔazoR2ΔazoR1 double mutant was observed to be more sensitive to catechol and 2-MHQ stress compared to the ΔazoR2 single mutant (Fig. 4.7C). Moreover, ΔazoR2ΔazoR1 mutant cells showed a growth defect in the presence of 1 mM diamide compared to wild-type cells whereas the ΔyodBΔmhqR double mutant grew faster in the presence of 1 mM 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 ΔazoR2ΔazoR1 double mutant towards thiol-reactive compounds compared to the ΔazoR2 single mutant suggests that AzoR2 could complement AzoR1 deficiency with respect to detoxification.

To verify that the resistance of the ΔyodB mutant to catechol and MHQ was attributed to azoR1 derepression, the ΔazoR1 mutation was introduced into the ΔyodB
mutant. Notably, the ΔyodBΔazoR1 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 ΔyodB 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-benzoquinone imine and 1,4 naphthoquinone 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-benzoquinone
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 S₂Sn 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-galactosidase 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*azoR1ΔazoR2* (ST9) and Δ*azoR1ΔyodB* (ST10) double mutants, respectively. The Δ*mhqR* mutant (TF176) was transformed with chromosomal DNA of the *yodB::pMutin4* (ST5) mutant to generate the
\( \Delta yodB\Delta mhqR \) 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 \( \mu g/ml \)), chloramphenicol (5 \( \mu g/ml \)), erythromycin plus lincomycin (1 and 25 \( \mu g/ml \), respectively), and tetracycline (12.5 \( \mu g/ml \)).

### 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 (≈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 erythromycin-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 (ΔyodB::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 His6 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-[³⁵S]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-[³⁵S]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 performed 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, catechol, diamide and nitrofurantoin stress in the wild-type (wt) and derepression in the ΔyodB mutant\(^a\) by Antelmann group.

<table>
<thead>
<tr>
<th>Operon</th>
<th>Gene</th>
<th>Transcriptome (wt)</th>
<th>Proteome (wt)</th>
<th>Transcriptome ΔyodB/wt</th>
<th>Proteome ΔyodB/wt</th>
<th>Function or similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Catechol 2-MHQ Dia</td>
<td>Catechol 2-MHQ Diamide NT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>azoR1-barB</td>
<td>azoR1(yocJ)</td>
<td>36.7 42.1 106 66.3 38.4</td>
<td>18 19.6 11.9 19.3 8.66 7.42 5.26 8.475</td>
<td>70.9 82.4</td>
<td>19.6 21.3</td>
<td>FMN-dependent NADH-azoreductase 1</td>
</tr>
<tr>
<td></td>
<td>yodC</td>
<td>5.1 9.2 13.6 10.1 12.4</td>
<td>12.6 3.7 3.8 2.8</td>
<td>3.9 3.3</td>
<td>3.5 3.7</td>
<td>NAD(P)H nitroreductase</td>
</tr>
<tr>
<td></td>
<td>tsp</td>
<td>4.1 5.6 3.4 6.7 7.6</td>
<td>3.9 3.1</td>
<td>2.2 3.7</td>
<td>regulator of thiol-specific oxidative stress response</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ribD</td>
<td>4.5 3.3</td>
<td>3.7</td>
<td>1.9 5.2</td>
<td>riboflavin-specific deaminase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ribE</td>
<td>6.3 3.3</td>
<td>3.3</td>
<td>2.2 5.4</td>
<td>riboflavin synthase (alpha subunit)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ribA</td>
<td>6.2 3.3</td>
<td>3.3</td>
<td>2.2 4.8</td>
<td>GTP cyclohydrolase II</td>
<td></td>
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<tr>
<td></td>
<td>ribH</td>
<td>4.8 3.6</td>
<td>3.6</td>
<td>2.2 4.8</td>
<td>riboflavin synthase (beta subunit)</td>
<td></td>
</tr>
</tbody>
</table>

\(a\)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 nitrofurantoin (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>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype</th>
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<tr>
<td>JH642</td>
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<td>J.A. Hoch</td>
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<td>168</td>
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<tr>
<td>TF176</td>
<td>trpC2 mhqR::cat</td>
<td>(Töwe et al., 2007)</td>
</tr>
<tr>
<td>TF277</td>
<td>trpC2 yodB::cat</td>
<td>(Leelakriangsak et al., 2007)</td>
</tr>
<tr>
<td>ST5</td>
<td>trpC2 yodB::Erm (yodB::pMutin4)</td>
<td>(Duy N.V., 2007)</td>
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<tr>
<td>ST6</td>
<td>trpC2 yodB::Erm ykvE::cat</td>
<td>This study</td>
</tr>
<tr>
<td>ST7</td>
<td>trpC2 azoR1::cat</td>
<td>This study</td>
</tr>
<tr>
<td>ST8</td>
<td>trpC2 azoR2::Erm (azoR2::pMutin4)</td>
<td>(Töwe et al., 2007)</td>
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<tr>
<td>ST9</td>
<td>trpC2 azoR1::cat azoR2::Erm (azoR2::pMutin4)</td>
<td>This study</td>
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<tr>
<td>ST10</td>
<td>trpC2 yodB::Erm azoR1::cat</td>
<td>This study</td>
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<tr>
<td>ORB6208</td>
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<td>ORB6734</td>
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<td>ORB6775</td>
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<td>ORB6777</td>
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</tr>
<tr>
<td>pML60</td>
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<td>pML74</td>
<td>YodBC6A expression plasmid in pPROEX-1</td>
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Table 4.3 Oligonucleotide primers used

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<td>TCCTCTAATTAGTAGGATGAACAT</td>
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<td>yocJ_for</td>
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<td>This study</td>
</tr>
<tr>
<td>yocJ_rev</td>
<td>CTAATCGACTCACTATAGGGAGAAGTGGT</td>
<td>This study</td>
</tr>
<tr>
<td>bsrB_for</td>
<td>CAGATTGGTCAGGTATTGG</td>
<td>This study</td>
</tr>
<tr>
<td>bsrB_rev</td>
<td>CTAATCGACTCACTATAGGGAGACGGCCGCTTCATTTCACAC</td>
<td>This study</td>
</tr>
</tbody>
</table>
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 ΔyodB mutant and repression in the Δmhqr (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 10 min after 2-MHQ, catechol, diamide and nitrofurantoin challenge or in the ΔyodB and Δmhqr mutant at control conditions (red image). Proteome 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. DNase I 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 binding 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 ΔyodB mutant is resistant (A), the ΔyodBΔmhqR double mutant is hyperresistant (B) and the ΔazoR2ΔazoR1 double mutant is hypersensitive (C) to catechol and MHQ stress. *B. subtilis* wild type (wt), ΔyodB, ΔyodBΔmhqR, ΔazoR2 and ΔazoR2ΔazoR1 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 ΔazoR2ΔazoR1 double mutant is more sensitive and the ΔyodBΔmhqR double mutant is more resistant to 1mM diamide compared to the wild-type. *B. subtilis* wild-type, ΔazoR2ΔazoR1 and ΔyodBΔmhqR 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 ΔyodBΔazoR1 double mutant is not resistant to catechol and 2-MHQ. B. subtilis wild-type and ΔyodBΔazoR1 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 ΔyodBΔazoR1 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 P₃ promoter which is induced by the thiol-specific oxidant diamide

The transcription start site of the newly identified spx P₃ promoter was determined by primer extension analysis. Transcription from the spx P₃ promoter initiated from an A nucleotide 79 bp upstream of the spx start codon. The spx promoter region contains candidate recognition elements for σ⁴⁵ RNAP. The transcription of spx from the P₃ 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^ccs-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 P₃ promoter sequence

Deletion analyses were performed to identify cis-acting elements associated with the P₃ promoter. Deletions of the sequence either 5’ or 3’ relative to P₃ were constructed and inserted in to bgaB reporter gene fusion construct. Levels of BgaB activity and the P₃ 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 P3 was detected in 3’ deletion mutants, as was loss of diamide induction. The analysis of spx P3 point mutations obtained by UV and random PCR mutagenesis suggested the existence of a second cis-acting negative control region within the P3 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 P3 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 P3 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 P3 promoter.

5.1.3 PerR and YodB negatively control spx P3 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 spxP3 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 P3 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 \textit{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 \textit{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 \textit{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).

\textbf{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 H₂O₂ 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 H₂O₂. 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-through 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 P3 promoter was impaired upon addition of diamide and H$_2$O$_2$. As expected, in the presence of diamide, H$_2$O$_2$ or MHQ, reduced binding of YodB to the azoR1 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: α1, α2, β1, α3, α4, β2, β3, α5 and α6 (Hong et al., 2005). OhrR is homodimer, each subunit consists of two functional domains: α1, α2, α5 and α6 which involve in dimerization between N and C termini and β1, α3, α4, β2 and β3 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 (Kd) 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 (*azoR1*). 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.

**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 \cdot 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).


Fuangthong, M., A. F. Herbig, N. Bsat & J. D. Helmann, (2002) Regulation of the *Bacillus subtilis fur* and *perR* genes by PerR: not all members of the PerR regulon are peroxide inducible. *J. Bacteriol.* **184**: 3276-3286.


complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature* **390**: 249-256.


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 scholar at Unit of Mechanisms 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.

Publications


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**Abstracts**

