# A Study of the Regulation of Expression of *dsbA* from *Salmonella enterica* serovar *Typhimurium*

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# Abstract

Correct disulfide bond formation is required for proper protein folding and stability of disulfide bond-containing proteins. For extracytoplasmic proteins, disulfide bond formation occurs in the periplasm of Gram-negative organisms, and is catalyzed by the disulfide oxidoreductase DsbA. In *E. coli*, it has been shown that *dsbA* has two promoters separated by about 1 kb. The distal promoter is regulated by the Cpx signal transduction system. The regulation of *dsbA* in *Salmonella enterica* serovar *Typhimurium* is currently unknown. This study was undertaken to examine the regulation of expression of *dsbA* in this organism. Northern analyses revealed the presence of two distinct *dsbA* mRNA transcripts differing in size by approximately 200 base pairs. This suggests that, for *S. typhimurium dsbA*, transcription may initiate from two distinct promoters. Under different conditions, the abundance of each transcript varies. The smaller transcript is more abundant when oxygen is limiting or in an *htrA* null strain. In minimal E glucose media pH 7.6 both transcripts appear more abundant compared to transcripts from an LB culture. Using a *S. typhimurium dsbA::lacZ* transcriptional fusion to examine activity of the proximal promoter, it was elucidated that expression of *dsbA* is growth phase regulated, with maximum induction of expression occurring at the onset of stationary phase. Furthermore, this phenomenon was shown not to be dependent upon RpoS or SlyA, two factors involved in the regulation of transcription during stationary phase. In a *S. typhimurium dsbA* null strain this stationary phase induction was even greater than in the wild-type, whereas in an *E. coli* strain, *dsbA* expression increased at

approximately the same rate during both logarithmic and stationary phase. Under conditions of low pH and oxygen limitation, stationary phase induction of *dsbA* expression did not occur. Western blotting showed that steady-state levels of DsbA did not vary significantly throughout the phases of growth, nor were steady-state levels different in overnight static cultures versus aerated cultures.

Clearly, this work has provided evidence that suggests that *S. typhimurium dsbA* is regulated, and this regulation is different from that seen in *E.coli*. These findings support the idea that *S. typhimurium dsbA* has two promoters, and that the proximal promoter is maximally induced by an as yet unidentified factor at the onset of stationary phase.

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# List of Abbreviations

| | |
|---|---|
| ATP | adenosine 5'-triphosphate |
| bp | base pair |
| cAMP | cyclic adenosine monophosphate |
| CSPD | Disodium 3-(4-methoxyspiro(1,2-dioxethane-3,2'-(5'chloro) tricyclo(3.3.1)decan)-4-yl)phenyl phosphate |
| CTD | carboxy terminal domain |
| Cys (C) | cysteine |
| dA | 2'-deoxyadenosine |
| dNTP | 2'-deoxynucleoside 5'- triphosphate |
| dTTP | 2'-deoxythymidine 5'- triphosphate |
| dUTP | 2'-deoxyuridine 5'- triphosphate |
| DNA | deoxyribonucleic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| F | Faraday |
| g | gram |
| His (H) | histidine |
| kb | kilobase |
| kDa | kilodalton |
| l | litre |
| LB | Luria-Bertani |
| M | molar |
| MCS | multiple cloning site |
| μ | micro |
| MOPS | 3-(N-morpholino)propanesulfonic acid |
| mRNA | messenger ribonucleic acid |
| nm | nanometre |
| NTP | nucleotide triphosphate |
| Ω | ohms |

| | |
|---|---|
| OMP | outer membrane protein |
| ONPG | *o*-nitrophenyl-β-D-galactopyranoside |
| ONP | *o*-nitrophenyl |
| ORF | open reading frame |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| Pro (P) | proline |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| rRNA | ribosomal ribonucleic acid |
| rU | ribosyluridine |
| SDS | sodium dodecyl sulfate |
| TAE | tris/sodium acetate/EDTA |
| TE | tris/EDTA |
| Thr (T) | threonine |
| $T_m$ | melting temperature |
| Tris | tris(hydroxymethyl)aminomethane |
| V | volts |
| xg | 1x gravity |
| X-gal | 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside |

# Chapter 1: Introduction

## Protein Folding in the Bacterial Periplasm

A variety of proteins produced by the bacterial cell do not remain in the cytosol, instead they are extruded into the periplasm; the region between the inner and outer membranes of the Gram-negative bacterial cell wall. Examples of these types of proteins include outer membrane (OmpA), periplasmic (alkaline phosphatase) and externally secreted (bacterial toxins) proteins.

Once in the periplasm, the protein must be folded into its proper conformation. Anfinsen, through his experiments on *in vitro* ribonuclease refolding, established that all the information required to determine the final conformation of the protein resides in the polypeptide chain itself. This means that an unfolded protein can fold into its native conformation in the absence of other proteins (Anfinsen *et al.*, 1961; Anfinsen, 1973; Gething and Sambrook, 1992). *In vitro* protein folding appears to be less efficient than *in vivo* folding, and often requires protein concentrations and physiochemical conditions very different from those occurring intracellularly (Gething and Sambrook, 1992). Considering the bacterial cell's growth rate, it is now becoming clear that some steps in *in vivo* protein folding must be catalyzed in order to occur efficiently. In addition, bacterial cells do not normally enjoy the luxury of growing in Luria Broth at a balmy $37^0$C. In the natural environment they are often in stationary phase and can encounter environmental stresses such as nutrient limitation, oxidative agents, and changes in pH, temperature or osmotic conditions. These adverse conditions can have multiple effects on the cell. For

example, in stationary phase protein turnover is slow, which means that proteins have more of a chance of becoming damaged or denatured (Visick and Clarke, 1995). In addition, the porous nature of the Gram-negative outer membrane means that changes in the external environment, such as the presence of noxious chemicals, can readily affect the periplasm. These substances can directly interfere with proteins by damaging their primary structure (eg. oxidizing agents) or indirectly by altering the redox environment of the periplasm and thereby affecting protein folding (Visick and Clarke, 1995). In any case, enzymes or mechanisms need to be present to correct or compensate for defects in the proteins in order to ensure cell survival.

Two classes of proteins have been described which deal with damaged proteins and assist the folding process. The first class is the periplasmic proteases. HtrA (also known as DegP), an ATP-independent periplasmic protease, plays a vital role in degrading damaged periplasmic proteins (Missiakas *et al.*, 1996; Strauch *et al.*, 1989). Without proteases, defective proteins would accumulate and further stress the cell. Any mechanism which a cell possesses in order to restore the activity of damaged proteins or to remove them, can spare the cell's biosynthetic systems, and can help ensure that non-functional molecules do not disrupt the cellular physiology (Visick and Clarke, 1995). The second class consists of proteins involved in protein folding, such as chaperones. Chaperones do not directly catalyze the formation of secondary or tertiary structures, rather they act by stabilizing unfolded or partially folded proteins during the folding and assembly process. In addition, they prevent the formation of inappropriate intra- and interchain interactions which could lead to protein aggregation within the periplasm (Gething and Sambrook, 1992; Bardwell and Beckwith, 1993). Classical protein

chaperones have not been identified in the periplasm, and this may be due to the fact that they require ATP, which is not available in the periplasm. However, there are a few proteins found within the periplasm, such as PapD, which are considered to have chaperone activity (Hultgren *et al.*, 1991, 1993). PapD is a periplasmic chaperone involved in the assembly of P pili. After the pilin subunits, Pap A, PapK, PapE, PapF, and PapG, are transported across the cytoplasmic membrane into the periplasm, they form individual complexes with PapD (Kuehn *et al.*, 1991; Jacob-Dubuisson *et al.*, 1993 ). This chaperone-subunit complex then targets to PapC, the outer membrane assembly protein (Dodson *et al.*, 1993). Here, PapD unloads the pilus subunit which is incorporated into a pilus fiber (Jacob-Dubuisson *et al.*, 1993).

Another group of factors involved in protein folding consists of enzymes which actually catalyze rate-limiting steps in protein folding, such as cis-trans prolyl isomerization and disulfide bond formation (Gething and Sambrook, 1992; Bardwell and Beckwith, 1993). General examples of this class include the peptidyl prolyl isomerases (PPIs) such as RotA, FkpA and SurA (Missiakas *et al.*, 1996), and the Dsb proteins which mediate disulfide bond formation (Bardwell and Beckwith, 1993).

## Disulfide Bond Formation

Certain proteins which are destined for the periplasmic or extracellular environments will require disulfide bonds in order to attain their final conformation. This is in contrast to the cytoplasm, where it is rare to find disulfide bond-containing proteins (Derman *et al.*, 1993). Disulfide bonds do not form in the cytosol due to the reducing nature of this compartment. The reducing environment is maintained by molecules such

as thioredoxin reductase and reduced glutathione (Derman *et al.*, 1993; Hwang *et al.*, 1992).

In the periplasm, which has the correct redox potential (ie. is more oxidizing) to facilitate disulfide bond formation, disulfide bridges can form in proteins requiring them (Hwang *et al.*, 1992). Disulfide bonds play an important role in determining the ultimate three-dimensional structure and stability of a protein, which in turn impacts on the protein's enzymatic activity. These bonds are formed when disulfide bonds are exchanged between a source of a disulfide and free sulfhydryl groups, such as those present in cysteine residues (Bardwell *et al.*, 1991). *In vitro*, disulfide bonds can form spontaneously in thiol/disulfide redox buffers if oxygen is present. However, this is a slow and error-prone process. *In vivo*, disulfide bond formation occurs more rapidly and accurately, and is catalyzed (Bardwell and Beckwith, 1993).

The first catalyst of disulfide bond formation was discovered in eukaryotes. Anfinsen (1961) found that the enzyme protein disulfide isomerase (PDI) catalyzed oxidative folding of proteins in the endoplasmic reticulum. In prokaryotes, it is the disulfide bond forming (dsb) system (Loferer and Hennecke, 1994) in the periplasm which is responsible for proper protein folding.

DsbA, a disulfide oxidoreductase in *E. coli*, was one of the first prokaryotic enzymes catalyzing disulfide bond formation to be discovered (Bardwell *et al.*, 1991). Now a family of proteins involved in this process has been identified. Members of this family include Por from *Haemophilus influenzae* (Tomb, 1992) and TcpG from *Vibrio cholerae* (Peek and Taylor, 1992), in addition to DsbA from *Escherichia coli* (Bardwell *et al.*, 1991) and *Salmonella enterica* serovar *Typhimurium* (herein referred to as *S.*

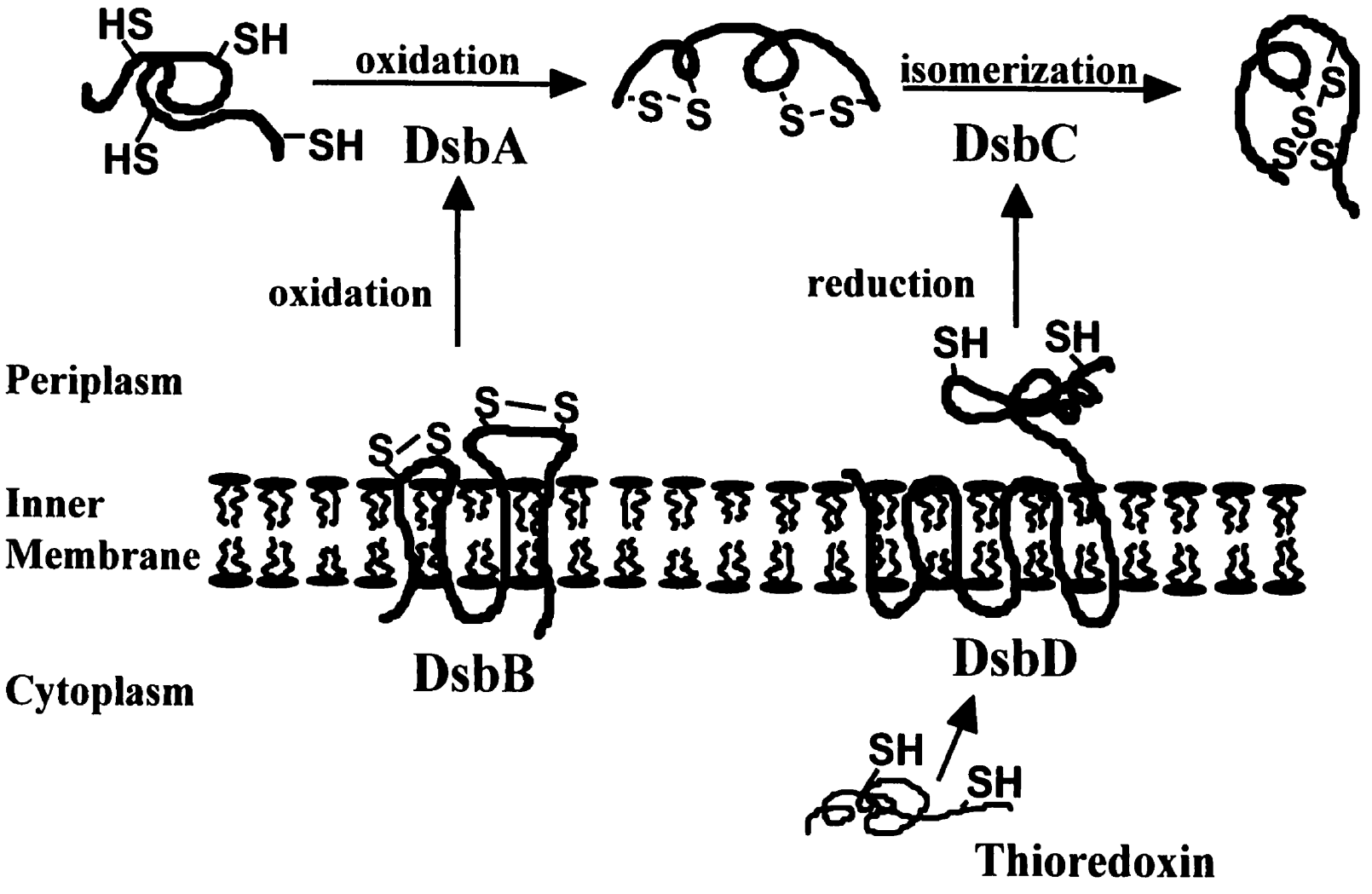
*typhimuruim*) (Turcot, 1997).

The Dsb System in *Escherichia coli*

The chromosomally-encoded dsb system in *E. coli* is composed of a number of proteins, both periplasmic and membrane bound (Figure 1). The key component, DsbA, is a very reactive periplasmic protein with strong oxidizing properties. It is a soluble, monomeric protein of 21 kD with a thioredoxin-like domain interrupted by a triple-helical domain (Bardwell and Beckwith, 1993). Thioredoxin is a small, cytoplasmic protein known to oxidize two vicinal protein-SH groups to a disulfide bond (Holmgren, 1989). The thiol oxidoreductase active site motif of DsbA is $Cys^{30}$-$Pro^{31}$-$His^{32}$-$Cys^{33}$. The two cysteines are disulfide bonded, and it is this disulfide which is donated to a reduced protein (Bardwell *et al.*, 1991). The transfer of this disulfide to a folding polypeptide occurs in two steps. First, oxidized DsbA forms a mixed disulfide with the free thiol of the protein, then a second thiol in the protein interacts with the mixed disulfide, which results in the transfer of the disulfide bond to the protein (Bardwell *et al.*, 1994). This exchange leaves DsbA in a reduced state, no longer able to catalyze disulfide bond formation. In order to resume its oxidizing activity, DsbA must be reoxidized, and the enzyme responsible for this task is DsbB (Missiakas *et al.*, 1993; Bardwell *et al.*, 1993; Jander *et al.*, 1994).

DsbB is an inner membrane protein with four cysteine residues situated on the periplasmic face of the membrane (Figure 1) (Jander *et al.*, 1994). Guilhot *et al.* (1995), and Kishigami and Ito (1996) have demonstrated that the disulfide bonded $Cys^{104}$ and $Cys^{130}$ residues of DsbB are directly involved in reoxidation of DsbA, while $Cys^{41}$ and

**Figure 1.** A model of the Dsb system in the periplasm of *Escherichia coli* (from Rietsch *et al.*, 1996). DsbA catalyzes the formation of protein disulfide bonds by oxidizing the sulfhydryl groups on cysteines. The inner membrane protein DsbB is required to reoxidize DsbA once DsbA has donated its disulfide to a reduced substrate. DsbC, another periplasmic protein, isomerizes incorrectly formed disulfide bonds to yield a stable substrate protein. DipZ (DsbD) and thioredoxin are thought to help maintain the isomerase activity of DsbC.


HS
SH
HS
SH
oxidation
DsbA
isomerization
DsbC
oxidation
reduction
Periplasm
SH
SH
Inner
Membrane
DsbB
DsbD
Cytoplasm
SH
SH
Thioredoxin

$Cys^{44}$ may facilitate the disulfide bond transfer or reoxidize $Cys^{104}$ and $Cys^{130}$. The mechanism by which DsbB is reoxidized has yet to be elucidated.

Three other components of the *E. coli* dsb system, DsbC, DsbD and DsbE have also be identified (Missiakas and Raina, 1994, 1995, 1997b). DsbC and DsbD (more commonly designated DipZ) form a disulfide bond isomerization pathway, in which DsbC is a dimeric, periplasmic thiol:disulfide isomerase and DsbD is an inner membrane thiol:disulfide reductase (Figure 1)(Rietsch *et al.*, 1996, 1997; Missiakas and Raina, 1997a). DsbC, via its isomerase activity, rearranges incorrectly formed disulfide bonds. It has been proposed that the correct disulfide bond will form after the isomerization reaction because the protein may be in a more energetically favourable conformation (Rietsch *et al.*, 1997). Since isomerization requires that DsbC be in a reduced state, the role of DsbD as reductase becomes clear. To maintain DsbD in its correct redox state, thioredoxin, the product of the *trxA* gene is necessary (Rietsch *et al.*, 1996, 1997). A second function for DsbC may also exist; in its oxidized form it may oxidize disulfide bonds by a mechanism similar to DsbA (Missiakas and Raina, 1997a). However, oxidation of disulfide bonds by DsbC may only occur when DsbC is overexpressed, or in reductive-pathway mutants (*trxA* and *dsbD*) (Rietsch *et al.*, 1997). DsbE, a fifth protein in the dsb system, is a soluble periplasmic enzyme with a Cys-Pro-Thr-Cys active site. Although little is known about DsbE, its proposed function is that of a thiol:disulfide reductase (Missiakas and Raina, 1997a).

## Disulfide Oxidoreductases in *Salmonella typhimurium*

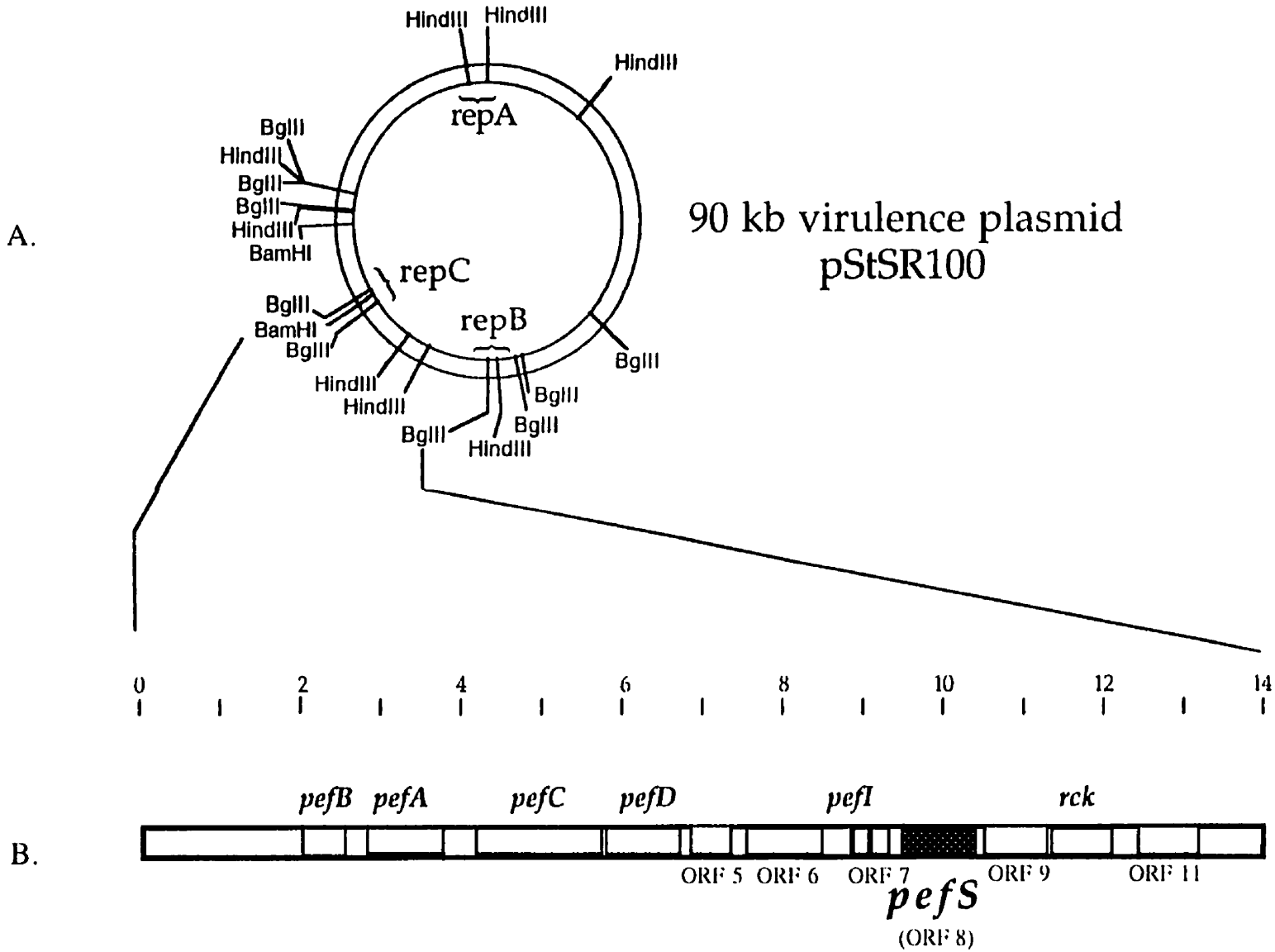
Recently, *dsbA* was cloned and characterized in *S. typhimurium* (Turcot, 1997).

At the amino acid level, it was found that *S. typhimurium* DsbA had 87% identical, 5.8% conserved and 7.2% unconserved residues when compared to *E. coli* DsbA. In addition, the Cys-Pro-His-Cys active site was identical. Functional assays demonstrated that the activity of *S. typhimurium* DsbA compared to *E. coli* DsbA varied depending on the substrate used. For example, in an *E. coli dsbA* null background strain, *S. typhimurium* DsbA oxidizes the disulfide bonds of alkaline phosphatase and the MalF-LacZ λ102 fusion protein more efficiently, however it is less efficient in restoring motility in comparison to *E. coli* DsbA (Turcot, 1997).

*S. typhimurium* possesses a 90 kb virulence plasmid which enhances its ability to invade the mesenteric lymph nodes, spleen and liver after initial infection of the intestinal epithelium (Gulig, 1990). A 13.9 kb region of this plasmid was sequenced by Friedrich *et al.* (1993), and a 7 kb segment was found to encode the genes for a novel type of fimbriae designated *pef* for plasmid-encoded fimbriae. Five of these coding regions are similar in sequence to those encoding the *E. coli* fimbrial biosynthetic *pap* operon, and appear to have similar roles in fimbrial assembly (Friedrich *et al.*, 1993). As illustrated in Figure 2, two open reading frames (*orf5* and *orf6*) are located between *pefD* and *pefI* which have also been shown to be important in fimbrial production, followed by *orf7* and *orf8*, renamed *pefS*, which is 33% identical at the protein level to *E. coli* DsbA. Complementation analysis demonstrated that in multicopy, *pefS* was able to substitute for *dsbA*, and that PefS required DsbB for its activity (Martin *et al.*, 1998). From this data, it is evident that *S. typhimurium* carries two genes which code for disulfide oxidoreductases.

Upon closer examination of PefS at the protein level, obvious differences become

**Figure 2.** The 90 kb virulence plasmid of *Salmonella typhimurium* (A) and the 13.9 kb region of the virulence plasmid containing the *pef* operon and neighbouring open reading frames (from Friedrich *et al.*, 1993).


A.
90 kb virulence plasmid
pStSR100
HindIII
HindIII
HindIII
repA
BglII
HindIII
BglII
BglII
HindIII
BamHI
repC
BglII
BamHI
BglII
repB
BglII
HindIII
HindIII
BglII
BglII
BglII
HindIII
0
2
4
6
8
10
12
14
B.
pefB
pefA
pefC
pefD
pefI
rck
ORF 5
ORF 6
ORF 7
ORF 9
ORF 11
pefS
(ORF 8)

apparent. There is only 53.2% overall amino acid similarity between PefS and *S. typhimurium* DsbA. Furthermore, the active sites differ; the histidine residue in the DsbA active site is replaced by a proline in the PefS active site. Low amino acid similarity (around 50%) in the putative peptide-binding regions suggests that these two disulfide oxidoreductases may have different functions or specificity for different substrates *in vivo*. Functional assays provided more insight into the differences between PefS and DsbA. In an *E. coli dsbA*$^-$ background strain, PefS was inefficient in oxidizing 1) the FlgI disulfide bond (Dailey and Berg, 1993), 2) alkaline phosphatase and 3) the MalF-LacZ fusion protein disulfide bonds, nor could it restore motility. These differences in oxidizing capabilities could well be related to the differences in the active sites. Graushopf *et al.* (1995) have shown that the CPHC active site of DsbA has a higher oxidative potential than the CPPC active site of PefS.

Prokaryotic Transcription

To study the regulation of transcription, it is necessary to understand what transcription is and what the key elements involved are. Transcription is a process whereby one strand of deoxyribonucleic acid (DNA) is used as a template for the synthesis of a complementary ribonucleic acid (RNA). Transcription is the first event in the expression of the gene product encoded by the DNA. Once a gene has been transcribed, the RNA is then translated, and this results in the synthesis of a protein. Many factors play a role in the events leading to the synthesis of a protein from the original DNA message. The only factors which will be discussed here are those involved in transcription.

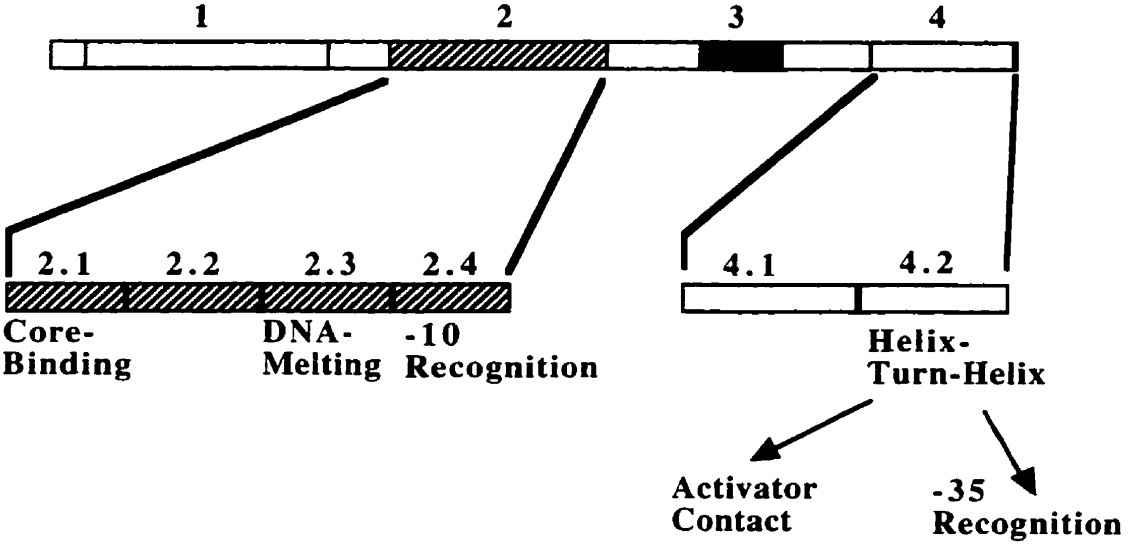
Transcription Initiation

Two variables which affect gene expression are the rate of transcription and mRNA stability. These variables, in turn, are affected by the rate of productive transcription initiation.

The key enzyme in transcription is the DNA-dependent RNA polymerase. It is a five subunit enzyme. The four polypeptide chains comprising the core polymerase (E) are two α, one β and one β', and they are encoded by the *rpoA*, *rpoB* and *rpoC* genes, respectively. To form the holoenzyme (Eσ), the fifth "specificity" subunit is required, and this is the sigma (σ) factor (Burgess *et al.*, 1987). A core polymerase can initiate transcription from ends, nicks and open regions of DNA, however the holoenzyme containing the σ factor is required for promoter-specific initiation. Several σ factors are usually encoded by the genome of an organism, for example, *E. coli* encodes at least six, and each directs the RNA polymerase to a different set of promoter sequences. The three that are relevant to my work are $\sigma^{70}$, $\sigma^{E}$ (also called $\sigma^{24}$) and $\sigma^{S}$ (also called $\sigma^{38}$). Both $\sigma^{70}$ and $\sigma^{E}$ belong to the $\sigma^{70}$ family of sigma factors and have four highly conserved regions (Figure 3) (Gross *et al.*, 1992; Helmann, J.D., 1994) $\sigma^{70}$ is required for transcription of genes involved in basic cell functions such as metabolism and biosynthesis, while $\sigma^{E}$, $\sigma^{S}$, $\sigma^{32}$and other σ factors coordinate transcription of functionally related sets of coregulated genes. For example, $\sigma^{32}$ and $\sigma^{E}$ regulate genes involved in the heat shock response (Gross *et al.*, 1992; Lonetto and Gross, 1996).

Transcription initiation is a multistep process involving: 1) promoter location and recognition by the RNA polymerase holoenzyme, 2) formation of the closed promoter-holoenzyme complex, 3) isomerization to form the open-promoter-holoenzyme complex,

**Figure 3.** The proposed structure-functon map of $\sigma^{70}$ containing four conserved regions. The functional characteristics of regions 2 and 4 are also indicated (from Gross *et al.*, 1992).


1
2
3
4
2.1
2.2
2.3
2.4
4.1
4.2
Core-
Binding
DNA-
Melting
-10
Recognition
Helix-
Turn-Helix
Activator
Contact
-35
Recognition

4) initiation of RNA synthesis and 5) release of the sigma factor and formation of the elongation complex (Record *et al.*, 1996).

Before transcription begins, the RNA polymerase holoenzyme scans the DNA until it finds a promoter region. Once it locates a promoter region, the enzyme makes strong contacts with the DNA and is not easily removed. The promoter region which lies just upstream of the transcription start site contains specific consensus sequences which appear to be recognized by the σ factor of the holoenzyme. For $E\sigma^{70}$, $E\sigma^{32}$ and $E\sigma^{E}$ these sequences are at positions -10 and -35, with respect to the transcription start site which is designated +1 (there is no zero) (Harley and Reynolds, 1987; Lisser and Margalit, 1993).

For some promoters, the α subunits of the RNA polymerase can play a role in promoter recognition by interacting with sequences upstream of the -35 region. In addition, the amino-terminal two-thirds of the α subunits are involved in holoenzyme assembly (Record *et al.*, 1996). The β and β' subunits of the *E. coli* RNA polymerase form the major structural backbone of the enzyme. The β subunit not only binds to the DNA, but also contains the binding site for the initial nucleotide substrate and is involved in the initial polymerization steps leading to transcript elongation (described below). The function of β' is not as well understood, however it appears to bind non-specifically to DNA and seems to interact with the nontemplate strand of the promoter DNA (Record *et al.*, 1996).

The first step in transcription initiation is reversible, specific binding of the holoenzyme to the promoter region on the DNA and results in the formation of an initial closed complex. It is called a closed complex because the promoter DNA remains entirely double-stranded. At this stage the RNA polymerase is interacting with the DNA

both upstream and downstream of the transcription start site. Next, the open complex forms. In the open complex, the DNA strands are separated from the -10 region to the region of the transcription start site +1. This open complex is ready to bind the first ribonucleotide thereby initiating transcription. Transcription commences at the transcription start site which is located 6 to 8 bp downstream of the -10 region, and is usually a purine (Record *et al.*, 1996).

Once the first ribonucleotide is in place, the next ribonucleotide, as specified by the DNA template, can then bind covalently to the previous ribonucleotide. At this point one of two events can occur, transcription continues normally or the initiated complex is released leading to abortive initiation. However, after a 7- to 15-ribonucleotide-long transcript has been synthesized, abortive initiation can no longer occur and synthesis of the full transcript continues (Carpousis and Gralla, 1985; Grachev and Zaychikov, 1980; Krummel and Chamberlin, 1989; Munson and Reznikoff, 1981; Straney and Crothers, 1987).

## Regulation of Transcription Initiation

In bacterial systems, the frequency of transcription initiation can vary greatly. Some genes may be transcribed once per second while others are only transcribed once per generation (Hahn *et al.*, 1977; McClure, 1985). Most of this variation is due to the strength of the promoters. The strength of the promoter is related to how closely it matches the consensus sequence. The higher the identity between the promoter and the consensus sequence, the stronger the promoter (Lodish *et al.*, 1995). Additional factors, such as activators and repressors can also act on promoter regions thereby inducing

positive or negative regulation, respectively. Although many regulatory systems which modulate transcription initiation exist, only positive regulation will be described as it has been shown to play a role in *dsbA* regulation.

Positive regulation can increase the basal strength of a promoter by one of several mechanisms. Activator molecules play a role in increasing transcription from a specific promoter. In this respect, at least two separate targets have been identified in the DNA-binding domains of RNA polymerase with which transcription activator proteins may bind. Blatter *et al.* (1994) have shown that the C-terminal 85 amino acids (αCTD) of the α RNA polymerase subunit is an independently folded domain capable of dimerization and DNA binding. It is connected to the remainder of the α subunit by a 13-20 residue linker, and it may be this linker which gives αCTD the flexibility to make different interactions at different promoters. An example of an activator which could target this domain of the RNA polymerase is the catabolite gene activator protein (CAP) which activates transcription from the *lac* promoter. A model has been presented in which CAP recruits αCTD to the DNA region immediately upstream of the -35 box, thereby enhancing the affinity of the RNA polymerase for the promoter and activating transcription (Bushby and Ebright, 1994). αCTD may also interact with specific sequences in the DNA itself. For example, the seven ribosomal RNA genes (*rrn*) in *E. coli* have exceptionally strong promoters (discussed in Bushby and Ebright, 1994). These genes have a ~20 bp AT-rich region upstream of the -35 hexamer, called the upstream (UP) element, which interacts with the RNA polymerase. It has been proposed that αCTD interacts with the UP element to enhance transcription from these promoters (Bushby and Ebright, 1994).

A second target in RNA polymerase for activators is located in region 4 of $\sigma^{70}$. Evidence for this was put forth by Li *et al.* (1994). They demonstrated that the bacteriophage λ cI protein (λcI) interacts with $\sigma^{70}$ region 4 to activate transcription from $\lambda P_{RM}$ (promoter of repressor maintenance). The key residue in this interaction is R596 of $\sigma^{70}$. Through substitution of this amino acid, activation of transcription by the transcription factors AraC and MalT was also found to be affected (Hu and Gross, 1985).

Clearly, αCTD and $\sigma^{70}$ region 4 through their interaction with specific activators have been shown to enhance transcription of the aforementioned genes. Other activators most likely exist which can interact with these two targets in RNA polymerase. In addition, synergistic activitation of these two targets by activators is also possible (Joung *et al.*, 1994; Bushby and Ebright, 1994). In the case of *S. typhimurium dsbA*, it will be interesting to determine whether or not positive regulation plays a role in *dsbA* expression and if so, in what form.

Transcription Elongation and Termination

Once the holoenzyme has cleared the promoter, which occurs after 7- to 15-ribonucleotides have been transcribed, the σ factor is released and the core polymerase begins transcript elongation. This process involves the sequential addition of nucleotides to the growing transcript by the core polymerase which uses the bases in one strand of DNA as a template to direct the selection of the specific nucleotide to be added. On average, elongation occurs at a rate of 43 nucleotides per second (Richardson, 1993).

During elongation, the RNA polymerase transcription elongation complex covers approximately 24 to 40 bp. The upstream- and downstream-most ends of the polymerase

contact the DNA tightly, whilst the middle region contains a single-stranded DNA bubble of 14 to 18 nucleotides, which reanneals before leaving the complex. Addition of ribonucleotides occurs in the active site of the polymerase. Upon leaving the active site, the nascent transcript appears to remain paired to the DNA template for a short period of time. In fact, it seems that 8 to 12 bp of nascent transcript form an RNA:DNA hybrid. As elongation continues, the 5' end of the nascent transcript is released from the DNA template and leaves the complex through a transcript exit channel (Chan and Landick, 1994).

The movement of the transcription complex along the DNA is discontinuous; multiple parts move, yet their individual movements are somehow coupled. The active site moves along the DNA in single-nucleotide steps, the downstream edge of the transcript bubble moves in steps of 4 nucleotides, the product binding site in steps of 8 nucleotides and the downstream edge of the polymerase may move in steps of 10 nucleotides or more (Chan and Landick, 1994). As the transcription complex travels along the DNA, it can encounter three types of elongation control sites: pause sites, arrest sites and termination sites.

Pausing occurs when the RNA polymerase hesitates during transcription but then spontaneously resumes elongation. Pausing may have a regulatory function, as is the case with histidine biosynthesis. The RNA polymerase pauses to allow ribosomal binding, thereby coordinating transcription and translation. Pausing may also occur due to the variations in binding constants for the substrate nucleoside trisphosphates at different template positions (Chan and Landick, 1994). Transcription arrest, an *in vitro* phenomenon, occurs when the RNA polymerase stops transcription without releasing the

template or transcript, and elongation does not resume spontaneously (Chan and Landick, 1994).

Finally, there are transcription termination sites. Termination occurs when a nascent RNA is released from its complex with the RNA polymerase and DNA template. Two types of transcription termination events have been identified, Rho-dependent and Rho-independent.

Rho-dependent termination requires the activity of an RNA-dependent ATPase and helicase called Rho. Each subunit of this homohexameric protein has three distinct structural domains connected by trypsin-sensitive linkers. The amino domain contains the primary polynucleotide binding site, while the middle domain contains the ATP-binding site. At present, the role of the third domain has not been identified, though it does contain some of the conserved sequence segments of NTP binding sites (Dolan *et al.*, 1990; Richardson, 1990). It is the amino-terminal domain which is probably responsible for the binding of Rho to the *rut* (rho utilization) region on the RNA transcript upstream of the 3' endpoint. Features of the *rut* site include an unstructured region of 70-80 nucleotide residues within a few hundred residues of the 3' endpoint (Morgan *et al.*, 1985) and what Alifano *et al.* (1991) call a C>G bubble. The events following binding of Rho to the *rut* sites on the RNA are not fully understood. One model proposes that the binding of Rho to the *rut* region leads to NTP (usually ATP) hydrolysis and enables Rho to move in the 3' direction toward the RNA polymerase transcription complex. Brennan *et al.* (1987) propose that upon encountering the transcription complex, Rho's ATPase activity helps unwind the RNA-DNA duplex in the transcription bubble, thereby facilitating the release of the transcript from the

transcription complex. They also suggest that a pause by the RNA polymerase must occur simultaneously with helicase activity, since unwinding alone will not release the nascent RNA transcript if the RNA-DNA duplex is continually being extended.

Two other proteins, NusG and NusA, are required for Rho-dependent transcription termination. Sullivan and Gottesman (1992) have identified a 21 kDa NusG protein which acts as a cofactor, stimulating termination by direct interaction with Rho. In cells depleted of NusG, no Rho-dependent termination occurred. Li *et al.* (1992) suggest that NusG acts as a bridge between RNA polymerase and Rho when Rho arrives at the transcription complex. They have also shown the importance of NusG in antitermination of transcription during λ-phage gene expression, however this is beyond the scope of this work.

NusA, identified by Ishii *et al.* (1984), is an elongation factor. This protein binds reversibly to core RNA polymerase, but will not bind to the holoenzyme. It replaces the sigma factor during elongation, and may even occupy the same binding site (Richardson, 1993; Greenblatt and Li, 1981; Gill *et al.*, 1991). Its function is to modulate transcription by prolonging pausing at natural pause sites. By retarding transcription at certain sites, NusA keeps translation of the mRNA closely coupled to transcription. When NusA interacts with Rho and the RNA polymerase, it alters the interaction between the 3' end of the RNA and the β and β' subunits of the core polymerase (Liu and Hanna, 1995). It may be this feature which is responsible for its positive influence on transcripition termination. Like NusG, NusA also plays an essential role in mediating antitermination (Richardson, 1993).

The second mechanism of transcription termination is Rho-independent

termination. As its name indicates, Rho-independent termination does not require a Rho factor. Instead, two other components are required, a G+C rich sequence capable of forming a stem-loop secondary structure followed by a sequence of 8 bp containing a run of dA residues on the DNA template strand. The formation of the stem-loop structure appears to slow elongation (Arndt and Chamberlin, 1990) , while the pairing of dAs to rUs results in an unstable hybrid at the 3' end of the nascent transcript, and increases the ability of the DNA and RNA to dissociate (Martin and Tinoco, 1980). One other factor which affects Rho-independent termination is the sequence downstream of the RNA polymerase at the stop point. It may assist termination by influencing the unwinding of the DNA or the progression of the enzyme, or through sequence variation could decrease the ability of the polymerase to bind to the DNA (Richardson, 1990; Lee *et al.*, 1990; Richarson and Greenblatt, 1996).

It is clear that the unstable dA-rU hydrid can readily dissociate, releasing the nascent RNA transcript from the transcription complex (Martin and Tinoco, 1980). However, complete transcription termination would also require the release of the RNA polymerase. To this end, Arndt and Chamberlin (1988) have demonstrated that addition of excess sigma factor results in an increased rate of polymerase recycling, thereby lending support to the theory that sigma catalyzes the release of the core polymerase after the dissociation of the RNA and DNA. Therefore, in addition to its role in transcription initiation, sigma is also a termination factor (Richarson and Greenblatt, 1996).

Mechanisms which affect gene regulation in *Escherichia coli*

As mentioned earlier, a number of proteins have been identified in the bacterial

periplasm which catalyze different steps in the folding pathway of secreted and outer membrane proteins. The importance of an efficient protein folding system becomes apparent when one realizes that not only proteins in the cell envelope, but also those in the periplasm, can be affected by a range environmental stresses, perhaps even more so than cytoplasmic proteins. These stresses, such as extremes of temperature and pH, and osmotic variation, can damage proteins rendering them non-functional (Visick and Clarke, 1995). An accumulation of misfolded proteins in the periplasm or a weak cell wall are of course very detrimental to the cell. Thus, maintenance and repair of these periplasmic and outer membrane proteins is imperative to cell survival.

## Heat Shock Response

The classical heat shock response is one of the most highly conserved regulatory responses among all organisms (Connolly *et al.*, 1997). Upon exposure to stressors such as heat, ethanol, or aggregated proteins, cells undergo transcriptional induction of a set of conserved genes called heat shock genes. These gene products, which are largely chaperones or proteases, are required by the cell in order to cope with the heat stress. They help fold, prevent aggregation of, or degrade misfolded proteins.

This "classical" heat shock response, elucidated by the laboratories of T. Yura, F.C. Neidhardt and C. Gross, is essentially a cytoplasmic response, and is under the transcriptional regulation of $\sigma^{32}$, the product of the *rpoH* gene, which complexes with RNA polymerase (RNAP) core (E) to form the $E\sigma^{32}$ holoenzyme. $\sigma^{32}$ recognizes the specific promoters of the genes comprising the heat shock regulon. Some of products of these genes include the chaperones DnaK, DnaJ and GrpE, and the proteases Lon and the

Clp family. Both the chaperones and proteases are instrumental in preventing protein aggregation, disposing of damaged proteins and maintaining proteins in a competent state (Missiakas *et al.*, 1996b).

## Extracytoplasmic Stress Response

Recently, a response system was discovered in *E. coli* which deals with protein misfolding, aggregation and imbalance in the extracytoplasmic compartment (Erickson and Gross, 1989; Rouviere *et al.*, 1995). Although a full understanding of this system has yet to be gained, at least two signal transduction systems appear to play a role in its regulation. These are the $\sigma^E$-mediated and the Cpx signal transduction systems.

## $\sigma^E$-Mediated Response

$\sigma^E$, encoded by *rpoE*, was discovered independently by Erickson and Gross (1989) and Wang and Kaguni (1989). The latter group had designated it $\sigma^{24}$ because it is a 24 kDa protein. In both cases it was identified while the researchers were investigating the regulation of $\sigma^{32}$.

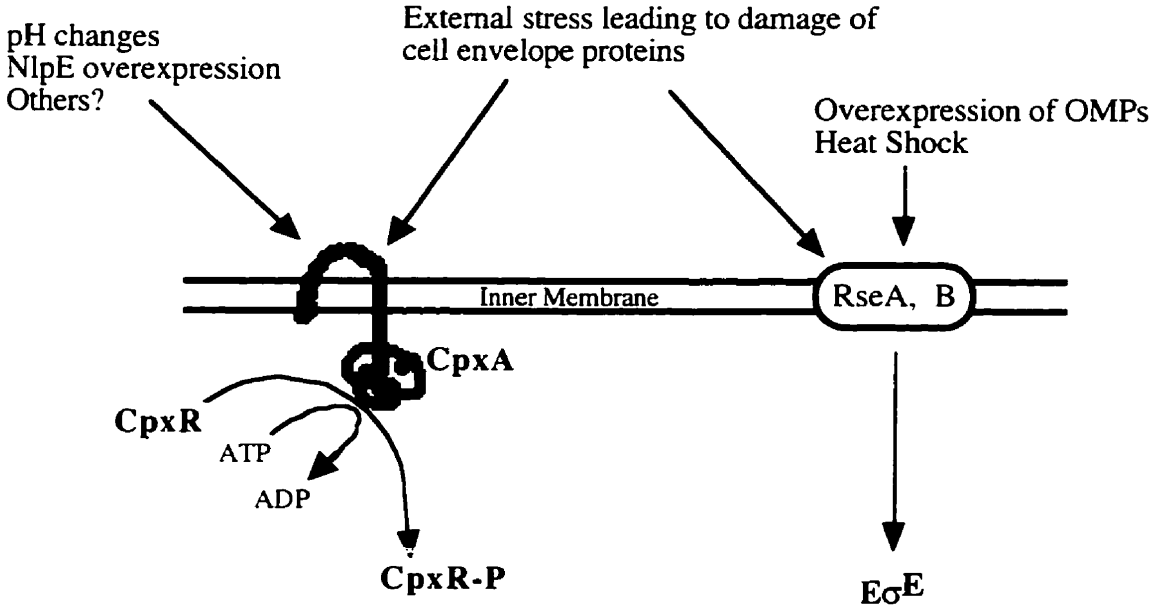
The *rpoH* gene encoding $\sigma^{32}$ can be transcribed from up to 5 promoters. Under normal growth conditions transcription occurs from P1, P3 and P4, however transcription is turned off from P1 and P4 after a temperature shift to 50$^0$C. At 50$^0$C, transcription occurs solely from P3. It was known that that promoters P1 and P4 were $\sigma^{70}$ dependent, but P3 was not recognized by either $\sigma^{70}$ or $\sigma^{32}$. The outcome then of both groups' research efforts was the discovery of a novel sigma factor. In addition, a second gene has been shown to be transcriptionally regulated by $\sigma^E$. HtrA codes for a periplasmic

endopeptidase which is indispensible for *E. coli* growth at elevated temperatures (Lipinska *et al.*, 1990). Thus, it appears as though a second stress response regulon exists in *E. coli*.

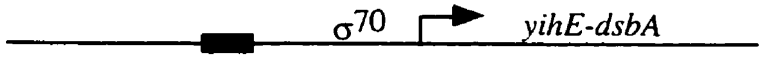
Because of its close relatedness to a putative extracytoplasmic transcription factor AlgU from *Pseudomonas aeruginosa*, $\sigma^E$ is proposed to be a member of the subgroup of sigma factors whose members (including AlgU) regulate extracytoplasmic functions (ECF). Support for this proposal comes from the observation that induction of the $\sigma^E$-dependent stress response seems to occur when misfolded or immature outer membrane proteins accumulate in the extracytoplasmic space (Mecsas *et al.*, 1993). Further studies by Raina *et al.* (1995) provided more evidence that events ultimately leading to an accumulation of misfolded or unfolded proteins induce the $\sigma^E$-dependent stress response. Using *dsbA*, *dsbC*, and *htrA* null mutants which affect the proper folding of disulfide bond-containing proteins, they found that *dsbA* and *dsbC* null mutations lead to increased $\sigma^E$ activity. In addition, combined *dsbA-htrA* mutations caused a six-fold increase in $\sigma^E$ activity; this was higher than with a *dsbA* mutant alone. When DsbA and HtrA are lacking, unfolded proteins may accumulate which might have otherwise been proteolyzed by HtrA. In addition, they found that *rpoE* null mutants were temperature sensitive above $40^0$C, and this phenomenon was more severe than in *htrA* null mutants. This finding indicated that $\sigma^E$ regulates additional genes whose products are necessary for bacterial growth at high temperatures. Therefore, they suggest that transcription of *rpoE* is sustained at elevated temperatures ($50^0$C) or under other conditions which induce protein misfolding (Raina *et al.*, 1995).

Promoter analysis of *rpoE* illustrated the presence of two promoters. Based on high sequence homology with the -35 and -10 boxes of *rpoH*P3 and *htrA*, it appears that the proximal promoter, *rpoE*P2, is $\sigma^E$-dependent. Additionally, transcription from this promoter was sustained upon a temperature shift from $30^0$C to $50^0$C (Raina *et al.*,1995; Rouvière *et al.*, 1995). This appears to be a mechanism by which $\sigma^E$ can positively regulate its own expression. The distal promoter *rpoE*P1, on the other hand, is not transcribed by $E\sigma^{70}$, $E\sigma^{32}$ or $E\sigma^E$ holoenzymes, and its transcription wanes with increasing temperature (Raina *et al.*, 1995; Rouvière *et al.*, 1995). $\sigma^E$ is also subject to negative regulation by putative anti-sigma factors. Just downstream of *rpoE* lie three genes, *rseABC* (regulator of sigma E) which are co-expressed with *rpoE*. The first two genes, *rseA*, an inner membrane protein and, *rseB*, a periplasmic protein are negative modulators of $\sigma^E$ activity. When *E. coli* is growing under normal conditions, RseA binds directly to the sigma factor inhibiting its transcriptional activity, while RseB binds directly to RseA thereby enhancing RseA's anti-sigma factor activity. When damaged proteins accumulate, RseA is displaced from $\sigma^E$, allowing $\sigma^E$ to induce transcription of the genes under its control. The exact mechanism by which RseAB sense the presence of misfolded proteins is not known, however it is likely that they possess "receiver" domains which perform this function. In contrast, RseC, also an inner membrane protein, is a positive modulator of $\sigma^E$, ensuring a positive feedback control on $\sigma^E$ activity (Missiakas *et al.*, 1996b; de las Peñas *et al.*, 1997; Missiakas and Raina, 1997c). Clearly, in this stress response system, $\sigma^E$ responds to extracytoplasmic stresses via RseA and induces transcription of the periplasmic protease *htrA* (Figure 4). In turn, *htrA* degrades damaged proteins.

**Figure 4.** A model illustrating the relationship between the Cpx two-component regulatory system and the $\sigma^E$ pathway in *E. coli*. When the inner membrane sensor kinase CpxA is stimulated by changes in pH or overexpression of NlpE it undergoes autophosphorylation on specific histidine residues. Phosphorylated CpxA can then phosphorylate CpxR , the cytoplasmic response regulator. Phosphorylated CpxR recognizes and binds to specific sequences in the DNA (black boxes) of certain promoters, such as the *E. coli yihE- dsbA* or *htrA* promoters, to enhance transcription. Other stress signals such as the overexpression of outer membrane proteins (OMPs) are communicated to $\sigma^E$ by the Rse proteins. $\sigma^E$ then activates transcription of certain genes, such as *htrA* (adapted from Pogliano *et al.*, 1997).




Regulation by CpxR:




Regulation by CpxR and $\sigma^E$:

Interestingly, another system has been elucidated which also induces *htrA* transcription, but does not appear to alter the levels of $\sigma^E$. Studies on this system led to the discovery that it also plays a part in regulating *E. coli dsbA* expression (Raina *et al.*, 1995). This system, designated Cpx, is based on the premise that in order for periplasmic protein folding catalysts to be regulated, a mechanism must exist which senses cell envelope protein folding defects or conditions that could cause folding defects, and then transmits this information across the inner membrane into the cytoplasm where the DNA is located. The ideal candidate for such a communication circuit would be a two-component signal transduction system (Pogliano *et al.*, 1997).

The Cpx Two-Component Regulatory System in *Escherichia coli*

A two-component signal transduction system is comprised of two protein components, an inner membrane sensor and a cytoplasmic response regulator which communicate with one another through phosphorylation and dephosphorylation reactions (Parkinson, 1993). The sensor monitors an environmental parameter, and upon stimulation undergoes autophosphorylation. During autophosphorylation, the sensor's autokinase activity attaches phosphoryl groups from ATP to a histidine residue in a reversible process (Hess *et al.*, 1988; Ninfa and Bennett, 1991). The phosphohistidine, a high energy intermediate in this process, then transfers the phosphoryl group to an aspartate residue in the response regulator (Sanders *et al.*, 1989, 1992). The phosphorylated cytoplasmic response regulator can then mediate an adaptive response, such as a change in gene expression (Parkinson, 1993).

The two-component signal transduction system which plays a role in regulating

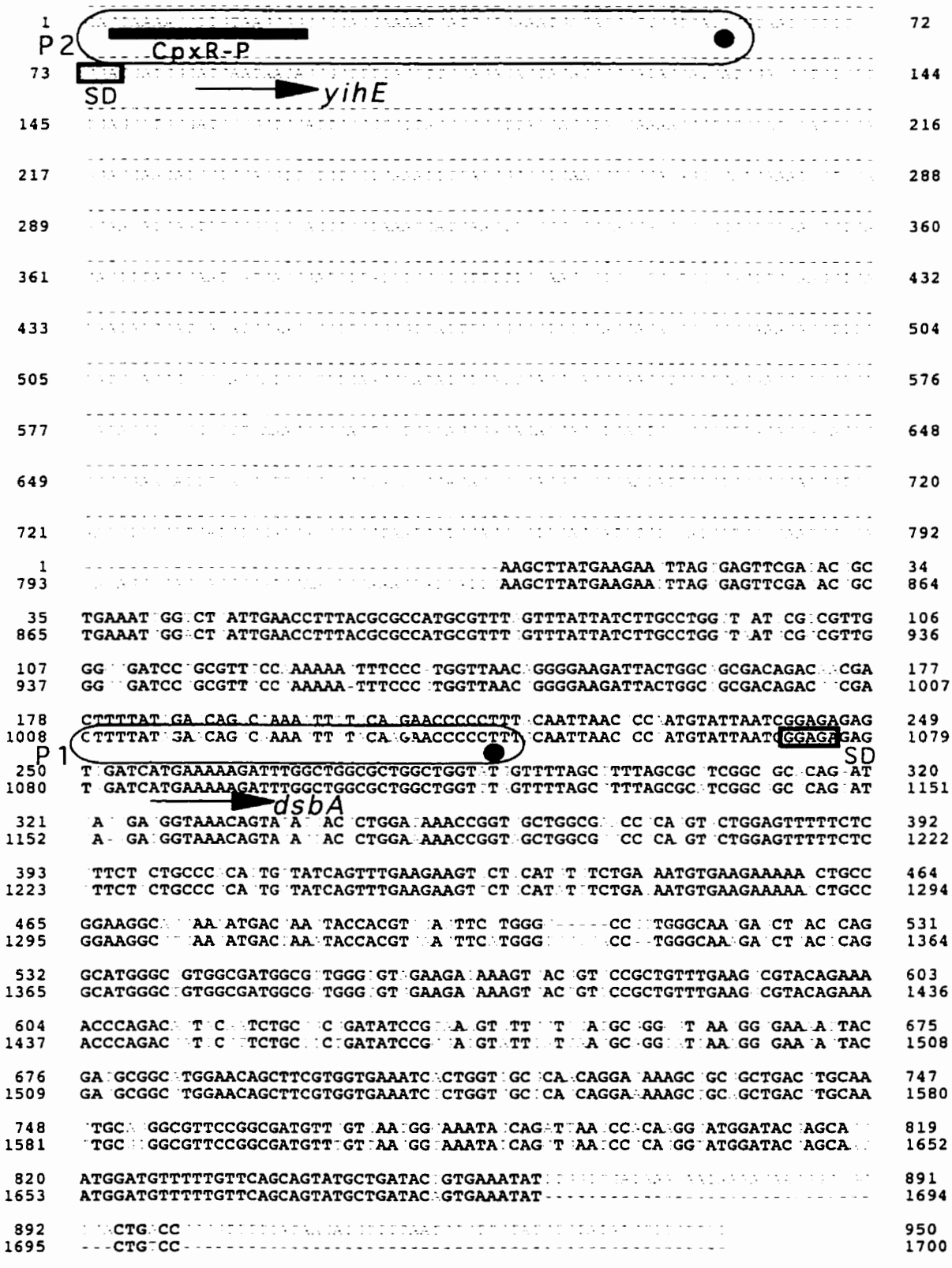
*dsbA* expression in *E. coli*, is called the Cpx pathway (Figure 4)(Pogliano *et al.*, 1997; Danese and Silhavy, 1997). The sensor kinase in this pathway is CpxA, and upon stimulation it activates the cytoplasmic response regulator CpxR by phosphorylation (Dong *et al.*, 1993). CpxR-P can then bind to a specific site on the DNA upstream of the transcription start site. The putative consensus sequence of the CpxR-P binding site is 5'-GTTAAAN$_{(5-6)}$GTAAA-3' . Binding of CpxR-P to the DNA helps $\sigma^{70}$ recognize the promoter region of a specific gene, thereby facilitating transcription initiation (Pogliano *et al.*, 1997).

One gene whose expression is regulated in part by the Cpx system is *htrA* (also called *degP)*. Interestingly, the *htrA* promoter is the only $\sigma^{E}$-dependent promoter on which CpxR-P is known to act. The finding that a gain-of-function mutation in *cpxA* (*cpxA**) induced *htrA* expression, and a null mutation of *cpxA* decreased *htrA* expression, clearly demonstrated the involvement of the Cpx system in *htrA* transcriptional regulation (Danese *et al.*, 1995). Additional support came from the discovery that an extracytoplasmic stimulus, the overexpression of the envelope lipoprotein NlpE, which stimulates wild-type CpxA also increased transcription of *htrA* approximately 10-fold (Danese *et al.*, 1995). In the cases where CpxA activity is increased, phosphorylation of CpxR is increased making more CpxR-P available to bind to its consensus site. Acting in concert with E$\sigma^{E}$, CpxR-P can then drive transcription of *htrA* (Danese *et al.*, 1995). The opposite holds true in cases where activation of CpxA is decreased. Since $\sigma^{E}$ seems to function as an extracytoplasmic stress response pathway, and $\sigma^{E}$ in conjunction with CpxAR control *htrA* transcriptional expression, Cosma *et al.* (1995) have suggested that the Cpx system constitutes part of a stress response pathway involved in monitoring

periplasmic protein folding. Along this line of thought, it seemed reasonable to propose that CpxA may be a sensor of cell envelope defects and that other genes involved in the maturation of extracytoplasmic proteins may therefore be regulated by the Cpx system (Pogliano *et al.*, 1997).

Studies were undertaken to determine if *cpxA** mutants, which constitutively express HtrA, increase the expression of other periplasmic protein folding catalysts such as DsbA. Indeed, Pogliano *et al.* (1997) observed that DsbA expression was induced 4 to 8-fold by *cpxA** mutations, which was comparable to the level of induction of HtrA in the same experiment. Further evidence that DsbA is regulated by the Cpx system came from studies which demonstrated that overexpression of NlpE induced DsbA expression in the presence of wild-type CpxA, but not when the *cpx* operon was deleted (Pogliano *et al.*, 1997). This group also elucidated that it is transcription from the distal promoter of *E. coli dsbA* which is affected by Cpx. *E. coli dsbA* has two promoters, a proximal promoter (P1) located directly uptream of *dsbA* and a distal promoter (P2) located upstream of *yihE* which precedes *dsbA* (Figure 5) (Belin and Boquet, 1994). Currently, the function of YihE is unknown, however it appears to be unimportant for growth and disulfide bond formation under normal laboratory conditions (Pogliano *et al.*, 1997; Belin and Boquet, 1994). Belin and Boquet (1994) also revealed that P1 had 66% identity with the $\sigma^{70}$-dependent promoter consensus sequence, and the same held true for the -10 box of P2, however no typical, correctly spaced -35 box was identified for P2. DNase I footprinting analysis with $His_6$-CpxR-P by Pogliano *et al.* (1997) showed that for *dsbA*, the CpxR-P binding site was in the region of P2 where the -35 box would normally be located. Thus CpxR-P may be acting in place of a normal -35 box to direct the binding of $\sigma^{70}$ to P2.

**Figure 5.** Alignment of the *Salmonella typhimurium* and *Escherichia coli dsbA* genes and upstream regions. The upper sequence is from *S. typhimurium* and the lower sequence is from *E. coli*. All non-identical bases are shown in red. The two promoter regions for *E. coli dsbA*, P1 and P2, are circled in light blue (Belin and Boquet, 1994). Their corresponding transcriptional start sites and putative Shine-Dalgarno (SD) sequences are indicated by brown solid circles and green open boxes, respectively (Belin and Boquet, 1994). The proposed $\sigma^{70}$ consensus sequences are circled in yellow, and the CpxR-P binding site is underlined with a dark blue box (Belin and Boquet, 1994; Pogliano *et al.*, 1997).


P2
CpxR-P
SD
yihE
P1
SD
dsbA

Factors affecting transcription from P1 have yet to be elucidated. One possibility is that since the normal housekeeping sigma factor, $\sigma^{70}$, may act on P1, transcription from P1 may be constitutive. Therefore it follows that transcription from P2 may only be induced under times of stress when demand for accessory foldases is greater.

Thus, studies in *E. coli* have provided valuable information as to how transcription of *dsbA* is regulated in this organism. It will now be interesting to determine how expression of DsbA is regulated in *S. typhimurium*, an organism similar to *E. coli*, yet which possesses two disulfide oxidoreductases. Furthermore, the region upstream of *dsbA* in *S. typhimurium* has not been sequenced, nor have Cpx-like proteins been identified.

## $\sigma^S$-Mediated Response

Changes in the regulation of gene expression for a number of genes accompanies entry into and survival in stationary phase. These changes are, for the most part, mediated by $\sigma^S$, the stationary phase sigma factor (Lange and Hengge-Aronis, 1991; Loewen, 1994). $\sigma^S$ or RpoS, encoded by *rpoS*, shows a high level of protein sequence similarity to $\sigma^{70}$, however it acts on a different set of genes (Mulvey and Loewen, 1989). RpoS regulates the stationary-phase expression of a wide variety of genes in response to nutrient-limiting conditions (Lange and Hengge-Aronis, 1991; McCann *et al.*, 1991). Many of these genes encode stress response proteins which play vital roles in increasing the organism's resistance to various environmental stresses such as nutrient deprivation, heat-, oxidative- and osmotic shock. It may seem a waste of precious cellular energy and resources to induce all *rpoS*-dependent systems upon entry into stationary phase when a

cell may not encounter all of these stressful conditions at one time. However, environmental conditions can fluctuate rapidly, thus induction of *rpoS*-dependent genes upon onset of stationary phase increases the cell's chance of survival under starvation conditions, when rapid *de novo* protein synthesis may not be possible (Hengge-Aronis, 1993). These stressful conditions may affect protein folding in the cell, and some of the products of the genes regulated by RpoS may contain disulfide bonds, thereby causing an increased need for protein folding catalysts such as DsbA.

*Salmonella typhimurium*

*Salmonella* species are facultative intracellular pathogens. These flagellated, Gram-negative, non-spore forming rods present a global health concern. The major infections associated with *Salmonella* are typhoid fever and Salmonellosis (*Salmonella* gastroenteritis). The former is caused by several serovars of *Salmonella typhi*, while the latter is caused by over 2000 *Salmonella* serovars, with *S. typhimurium* being the organism which most frequently infects humans (Prescott *et al.*, 1993; Cotran *et al.*, 1994).

Based on mouse and tissue culture models, *Salmonella* infections arise after oral ingestion of contaminated food or water. When the organism reaches the small intestine, it penetrates the intestinal epithelium and is often found to associate with M cells in the Peyer's patches (Finlay and Falkow, 1989). After passing through the intestinal epithelial barrier, the organism is ingested but not killed by macrophages. The macrophages then act as a wonderful, "bullet-proof" taxi service, transporting the organism to regional lymph nodes, the spleen, and the liver. In these organs, the bacterium can multiply and

disseminate throughout the body via the circulatory system (Finlay and Falkow, 1989). To go into greater detail about the mechanism of *Salmonella* invasion is beyond the scope of this work, however, it is important to realize that *Salmonella* encodes the factors necessary for invasion and survival within the host on its chromosome and 90 kb virulence plasmid. For example, encoded by the *inv*, *spa* and *prg* chromosomal loci is an invasion-associated secretion system which secretes specific proteins required by the organism for invasion of eukaryotic cells (Galàn, 1996). The PhoP-PhoQ system, which regulates survival in macrophages, is also encoded on the chromosome (Fields *et al.*, 1989), while some of the factors responsible for invasion beyond the Peyer's patches and resistance to complement killing are encoded on the virulence plasmid (Caldwell and Gulig, 1991; Heffernan *et al.*, 1992).

Another key player in the virulence of *Salmonella typhimurium* is the previously described stationary phase sigma factor, RpoS. Evidence for the vital role of RpoS in *S. typhimurium* virulence was clearly presented by Wilmes-Riesenberg *et al.* (1997). They demonstrated that the altered *rpoS* allele of *S. typhimurium* LT2, which contains a rare UUG start codon (Lee *et al.*, 1995), contributes to the attenuation of this laboratory strain. Furthermore, Fang *et al.* (1992) have shown that RpoS regulates the *Salmonella* virulence plasmid-encoded *spv* genes which are required for systemic infection, in addition to regulating other chromosomally encoded virulence genes. Nickerson and Curtiss (1997) also report that RpoS plays a role in the colonization of murine Peyer's patches in the early stages of infection and it regulates the expression of a variety of genes involved in resistance to osmotic, oxidative and acid stress (Hengge-Aronis, 1996; Loewen, 1994; Loewen and Triggs, 1984; Lee *et al.*, 1995). Clearly, this sigma factor is important not

only in the context of stationary phase growth, but also in the virulence properties of *S. typhimurium*.

Objectives

The aim of this study is to gain insight into the regulation of expression of *Salmonella typhimurium dsbA*. It is hypothesized that the expression of *S. typhimurium dsbA* is regulated and that *dsbA* expression changes in response to environmental factors. Several approaches will be used to meet this objective. Sequencing of the upstream *dsbA* region in *S. typhimurium* will help to characterize the *dsbA* promoter region in this organism. Given the high homology between *E. coli* and *S. typhimurium*, two promoters may exist for *S. typhimurium dsbA*. Northern analysis will be used to determine the number and size of the *S. typhimurium dsbA* mRNA transcripts. To gain a better understanding of the factors influencing the expression of *dsbA*, a *lacZ* transcriptional fusion with a putative proximal *dsbA* promoter region will be constructed, and activity from this promoter will be measured during all phases of growth under physiologically relevant conditions. This will indicate if the rate of transcription varies with growth phase, or if any other condition affects transcription rate, thereby providing evidence of regulation.

# Chapter 2: Materials and Methods

## 1. Bacteriological Techniques

### 1.1 Bacteriological strains

All bacterial strains and plasmids used in this research are listed and described in Tables 1, 2 and 3.

**Table 1.** *Escherichia coli* strains used in this study.

| **Strain (alternate name)** | **Description** | **Source/Reference** |
|---|---|---|
| NLM 264 (DH5α) | *supE*44 Δ*lac*U169 (ϕ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1 | Hanahan, 1983 |
| NLM 166 (MC4100) | *araD*193 Δ(*argF-lac*)U169 *rpsL*150 *relA*1 *flbB*5301 *deoC*1 *ptsF*25 *rbsR* | Silhavy *et al.*, 1984 |

**Table 2.** *Salmonella typhimurium* strains used in this study.

| **Strain (alternate name)** | **Description** | **Source/Reference** |
|---|---|---|
| NLM 144 (SL1344) | *his* $Str^R$ | Wray and Sojka, 1978 |
| NLM 267 | SL1344 *dsbA*::kan | Turcot *et al.*, 1998 |
| NLM 2154 (ATCC14028s) | wild-type | F. Fang |
| NLM 2156 | NLM 2154 *htrA*::cat | F. Fang |
| NLM 2157 (SF1005) | NLM 2154 *rpoS*::pRR10 (Δ*trfA*)$Pen^R$ | F. Fang |

| | | |
|---|---|---|
| NLM 2158 (SL2236) | NLM 2154 *slyA*::pRR10 (Δ*trfA*)Pen$^R$ | F. Fang |

**Table 3.** Plasmids used in this study.

| Plasmid | Description | Source/Reference |
|---|---|---|
| pLAFR2-31 | cosmid clone containing *dsbA* from *S. typhimurium* library | Turcot *et al.*, 1998 |
| pIT1 | pBAD18 containing a 1.7 kb insert encoding *S. typhimurium dsbA* | Turcot *et al.*, 1998 |
| pBAD24 | 4.5 kb pBR322 derivative with MCS downstream of the pBAD promoter | Guzman *et al.*, 1995 |
| pUC19 | 2.6 kb cloning vector | Sambrook *et al.*, 1987 |
| pMP190 | 15 kb transcriptional fusion vector containing a promoterless *lacZ* | Spaink *et al.*, 1987 |
| pMEG1 | pBAD24 with a 6.9 kb fragment from pLAFR2-31 containing *dsbA* | This study |
| pMEG2 | pMP190 with a 258 bp fragment from immediately upstream of the *dsbA* start site | This study |

1.2 Media and growth conditions

Strains were generally grown overnight at 37$^0$C in Luria Bertani (LB) broth (1% NaCl, 1% tryptone, 0.5% yeast extract), and were subcultured the following morning. The cultures were aerated using either a G10 gyrotory shaker (New Brunswick Scientific Co.) set at approximately 195 rpm or a tube rotator (Glas-col) set at approximately 60

rpm.

When cultures were to be grown on solid media, 1.5% (w/v) agar was added to the LB broth. Cultures on plates were also grown at 37$^0$C overnight and then stored at 4$^0$C.

For certain experiments, static cultures were required. These cultures were grown in a test tube containing 10 ml LB broth at 37$^0$C but without aeration.

When cells grown under low pH conditions were needed, an overnight culture was started in minimal E glucose media (per 100ml 50x stock solution: 1 g $MgSO_4 \cdot 7H_2O$, 10 g citric acid$\cdot H_2O$, 50 g $K_2HPO_4$, 17.5 g $NaNH_5PO_4 \cdot 4H_2O$) pH 7.6 (Vogel and Bonner, 1956) plus 0.4% glucose (from a 20% (w/v) stock solution) and 1x amino acids. The next day the cells were subcultured 1 in 100 or 1 in 1000 in minimal E glucose media pH 4.6 plus 0.4% glucose and 1x amino acids. The pH of aliquots of the media was monitored using pH strips (EM-Reagents) to ensure that the pH did not change significantly as the cells grew.

When antibiotics were required, the media was supplemented with the appropriate antibiotic at the following concentrations: chloramphenicol 30 μg/ml, ampicillin 200 μg/ml, kanamycin 40 μg/ml, tetracycline 40 μg/ml (Sigma Chemical Co.), streptomycin (Amersham International) 100 μg/ml.

The amino acids were prepared as follows. For a 50x stock solution of hydrophobic amino acids (isoleucine, phenylalanine, tryptophan, tyrosine), 250 mg of each amino acid were dissolved in 100 ml of distilled water, the pH was adjusted to 8.0 with $Na_2CO_3$ and the solution was filter sterilized. The 100x stock solution of hydrophilic amino acids (all remaining amino acids excluding cysteine) consisted of 250

mg of each amino acid 50 ml dissolved in distilled water and filter sterilized.

Screening for blue/white colonies required the addition of X-gal (Fisher Biotech). Two ml/L of an X-gal stock solution (20 mg/ml in dimethyl formamide) was added to the media before the plates were poured.

**2. RNA Techniques**

2.1 RNA Isolation

2.1.1 Alkaline lysis

RNA was isolated according to the protocol described by T. Karunakaran and H. Kuramitsu (1996), two changes are stated below. Bacterial cells were harvested as described or cells were harvested when they had reached the desired $OD_{600}$. Pelleting of the cells was performed at room temperature, at 11 000 rpm for 5 minutes.

2.1.2 Hot Phenol

RNA was isolated using a protocol in Warner *et al.* (1966) which had been modified by A. Stintzi (personal communication). 1.5 ml of culture were pelleted, resuspended in 200 µl lysis solution (20 mM sodium acetate, 0.5% (w/v) sodium dodecyl sulfate (SDS), 1 mM EDTA, pH 8.0) and placed on ice for 5 minutes. 200 µl of hot phenol ($65^0$C) were added, and after mixing by inversion, the tubes were placed in a hot ($65^0$C) water bath for 5 minutes. To separate the aqueous and organic phases, the tubes were centrifuged at 11 000 rpm for 10 minutes at room temperature. The aqueous phase was extracted into a clean tube and 1 volume of chloroform was added. After mixing the contents of the tube by inversion, the tubes were centrifuged at 11 000 rpm for 10 minutes at room temperature. The aqueous layer was extracted . To precipitate the RNA,

0.1 volumes of sodium acetate and 3 volumes of 100% ethanol were added and the tubes were keep at $4^0$C for 2 hours. The tubes were then centrifuged at 11 000 rpm for 10 minutes at $4^0$C. The pellet was washed in 100 µl of 70% ethanol, dried and resuspended in DEPC(diethyl pyrocarbonate)-treated water.

2.1.3 Trizol™ (GibcoBRL, Life Technologies)

The protocol accompanying the TRIZOL reagent was followed. Depending on the $OD_{600}$, 1-2 ml of bacterial cells were used. The final RNA pellet was resuspended in formamide and stored at $-20^0$C.

2.1.4 Quantitation of RNA

RNA was quantitated using the Beckman DU-600 spectrophotometer. The $OD_{260}$ reading was used in the following equation to calculate the amount of RNA:

Concentration of RNA (in µg/ml) = $OD_{260}$ x 40 µg/ml x dilution factor

where 40 µg/ml = the concentration of RNA at an $OD_{260}$ of 1.

2.2 Electrophoresis

The samples and gel were prepared according to the protocol from Fourney *et al.* (1988) with a few modifications. The samples were prepared by adding 5 µl (20-30 µg) of RNA dissolved in formamide, 1 µl ethidium bromide and 25 µl of sample buffer [0.75 ml deionized formamide, 0.15 ml 10x MOPS (see below), 0.24 ml formaldehyde, 0.1 ml DEPC-treated distilled water, 0.1 ml glycerol, 0.08 ml 10% (w/v) bromophenol blue] to a microfuge tube. The samples were heated at $65^0$C for 15 minutes and then loaded on the gel.

To prepare the agarose-formaldehyde gel, 1.0-1.5 g of agarose and 87 ml of DEPC-treated water were added to an RNase-free flask or beaker and heated in a microwave to dissolve the agarose. The melted agarose was cooled to approximately $50^0$C, then 10 ml 10x MOPS (see below) and 5.1 ml of formaldehyde were added. This recipe was double or tripled depending of the gel size. After the gel was poured, it was allowed to solidify in the fume hood for approximately one hour. Before loading the samples, the wells were flushed with the 1x MOPS/EDTA running buffer {0.2 M Mops[3-(N-morpholino)propanesulfonic acid], 50 mM sodium acetate, 10 mM EDTA, pH 7.0}. Once the samples were loaded on the gel, the gel was electrophoresed at approximately 95 V for two and a half hours. After electrophoresis, the RNA was visualized on a transilluminator emitting ultra-violet light.

## 2.3 Northern Analysis

### 2.3.1 RNA Transfer

Prior to transferring the RNA to a nylon membrane, the gel was soaked with gentle shaking, for two 20-minute periods at room temperature in 10x SSPE (from a 20x stock solution: 3 M NaCl, 0.2 M $NaH_2PO_4 \cdot H_2O$, 0.02 M EDTA, pH 7.4). Meanwhile, the nylon membrane (Amersham Hybond-N or Boehringer Mannheim positively charged nylon membrane) was soaked in distilled water for 5 minutes, and then in 10x SSPE for 5 minutes. Capillary action in 10x SSPE was used to transfer the RNA to the membrane. After approximately 22 hours, the transfer was stopped and the membrane was baked at $80^0$C for 2 hours. To determine if the transfer had been successful, the gel was again visualized under ultra-violet light.

2.3.2 Preparation of probe

The DNA probe was prepared by amplifying the *dsbA* ORF from the plasmid pIT1 using polymerase chain reaction (PCR). The primers used, were NM7 (5'GGAATTCACCATGAAAAAGATTTGGCTCC 3') which primes from the start codon (underlined) and NM9 (5'AGTCTAGATCAGTGACCGGCGTTC3'). The resulting PCR product is a 680 bp fragment. This fragment was purified from an agarose gel using the QIAquick gel extraction kit (QIAGEN). The probe was random primed labelled with digoxigenin (DIG) according to the protocol outlined in the Genius™ Nonradioactive Nucleic Acid Labeling and Detection System.

2.3.3 Northern hybridization

All steps were performed as outlined in the Genius™ Nonradioactive Nucleic Acid Labeling and Detection System User's Guide, hereafter referred to as the DIG Guide (Boehringer Mannheim). Changes are described in each subsection.

The membrane was incubated for at least one hour at 50-55$^0$C in high SDS buffer {5X SSC (from a 20X stock: 0.3 M sodium citrate, 3 M NaCl) , 2.0% (w/v) Blocking reagent [from 100 mM maleic acid; 150 mM NaCl, pH 7.5, 10% (w/v) Blocking reagent (Boehringer Mannheim)], 50 mM sodium phosphate (pH 7.0), 0.1% (w/v) sarkosyl, 7% (w/v) SDS, 50% (v/v) deionized formamide}. The DIG-labelled DNA probe was boiled for 10 minutes and then diluted to a concentration of approximately 20 ng/ml in high SDS buffer; this is the hybridization solution. The hybridization solution was reused a few times, between uses it was stored at -20$^0$C. When this solution was reused, the probe was denatured by heating at 68$^0$C for 10 minutes. The membrane was incubated in the hybridization solution overnight at at 50-55$^0$C.

After hybridization, the membrane was washed twice, 15 minutes per wash in 2X wash solution [2X SSC (from a 20X stock), 0.1% (w/v) SDS] at room temperature, and then 2 times, 15 minutes per wash in 0.5X wash solution [0.5X SSC (from a 20X stock), 0.1% (w/v) SDS] at 68$^0$C.

Chemiluminescent detection was used to detect the DIG-labelled DNA probe. The detection steps were performed according to the DIG Guide. The chemiluminescent substrate used was CSPD (Boehringer Mannheim). Prior to exposing the film, the membrane was incubated in 1/1000 dilution of CSPD in detection buffer (100 mM Tris-HCl, 100 mM NaCl, pH 9.5) for 5 minutes. The membrane was then placed between two pieces of plastic wrap and incubated at 37$^0$C for 15 minutes. To detect the chemiluminescent signal, the membrane was exposed to X-Omat film from Kodak for varying lengths of time, from 1 hour to overnight.

2.3.4 Membrane stripping

Method I in the DIG Guide was followed to strip the probe from the northern blot.

## 3. Molecular Biology Techniques

3.1 Plasmid extraction

Two techniques were routinely used. Most plasmids were extracted using alkaline lysis (Sambrook *et al.*, 1989) Modifications which were made include: one chloroform extraction after a 1:1 phenol:chloroform extraction, two chloroform extractions for plasmids to be sequenced, and 2 to 3 70% ethanol washes.

The 15 kb plasmids, pMP190 and derivatives, were extracted using the special

applications QIAGEN plasmid preparation (QIAGEN Handbook, February 1995). A 500 ml overnight culture and a QIAGEN-tip 100 were used.

3.2 Rapid lysis preparation

This technique is used to screen for a plasmids containing inserts during cloning procedures. Five hundred microlitres of an overnight culture were pelleted and resuspended in 40µl of 0.2 N NaOH/0.5% (w/v) SDS/20% (w/v) sucrose. After vortexing for 20 seconds, the samples were incubated at 70$^0$C for 10 minutes, then cooled to room temperature. Twenty microlitres of 4 M KCl and 10 µl of loading buffer [5% (v/v) glycerol, 0.04% (w/v) bromophenol blue, 0.04% (w/v) xylene cyanol FF] were added and the samples were chilled on ice for 5 minutes. Before the samples were loaded onto an ethidium bromide containing agarose gel, they were centrifuged for 5 minutes at 12 000 xg. This method was adapted from Sambrook *et al.*, 1989.

3.3 Polymerase chain reactions (PCR)

PCR was performed in the Perkin Elmer GeneAmp PCR System 2400 using 2 units Vent polymerase (New England Biolabs) in a 100 µl reaction mixture with 1X of the accompanying buffer, 200 µM of each dNTP, primer, 2 mM of $MgSO_4$ for extension longer than 2 kb, and variable amounts of template.

The DNA was first denatured at 94$^0$C for 2 minutes before the Vent polymerase was added. When the primers NM22 (5'ACAAGATCTATTAATACATTGGCGTT3') and NM24 (5'CCCCTCGAGAAGCTTATGAAGAAGTT3') or NM7

(5'GGAATTCACCATGAAAAAGATTTGGCTCC3') were used a two-step reaction was run. The underlined portions represent the homologous regions. Since these primers contain unique restriction sites which are not homologous to the template, the annealing temperature for these first three cycles was 2 degrees below the $T_m$ of the homologous region. The parameters for the first three cycles were 1 min at 94°C for denaturation, 1.5 min at the annealing temperature and 1 min/kb at 74°C for extension. The parameters for the second step consisting of 22 cycles were 1 min at 94°C for denaturation, 1.5 min at annealing temperature and 1min/kb at 74°C for extension. For the amplification of *S. typhimurium dsbA* from pIT1 using primers NM7 and NM9, the annealing temperatures were 50°C (step 1) and 65°C (step 2). While annealing temperatures of 42°C (step 1) and 62°C (step 2) were used for amplifying the *S. typhimurium dsbA* promoter region from pIT1 (NM22 and NM24).

## 3.4 DNA Manipulations

### 3.4.1 Quantitation of DNA

DNA was quantitated using the Beckman DU-600 spectrophotometer. The $OD_{260}$ reading was used in the following equation to calculate the concentration of DNA:

Concentration of DNA (in μg/ml) = $OD_{260}$ x 50 μg/ml x dilution factor

where 50 μg/ml = the concentration of double-stranded DNA at an $OD_{260}$ of 1. The $OD_{260}/OD_{280}$ ratio was used to assess the purity of the sample.

3.4.2 Enzymatic reactions performed on DNA

Restriction digests, ligations and vector dephosphorylations were performed according to the manufacturer's instructions. Restriction enzymes were purchased from either New England Biolabs or GibcoBRL (Life Technologies Inc.), T4 DNA ligase was from New England Biolabs or Boehringer Mannheim, while the calf intestinal phosphatase was purchased from Promega.

3.4.3 Gel purification of DNA

1X loading buffer [5% (v/v) glycerol, 0.04% (w/v) xylene cyanol FF and 0.04% (w/v) bromophenol blue] was added to the DNA samples, which were then loaded onto an agarose gel (ICN or FMC Bioproducts) immeresed in 1X TAE running buffer (40mM Tris-acetate, 1mM EDTA pH 8.0). To determine the size of the DNA fragments, λDNA/*Eco*I301/*Mlu*1 ladder from MBI Fermentas was used. To allow visualization of the DNA under ultra-violet light, 1μg/ml of ethidium bromide was added to the molten agarose. The samples were run according to standard protocols (Sambrook *et al.*, 1989). After electrophoresis the desired band(s) were cut from the gel and one of two gel purification procedures was used to remove the DNA from the gel.

For fragments shorter than 600 bp the QIAGEN gel purification protocol was followed (QIAquick Handbook, 09/95). The only change to the protocol was that instead of doing one 50 μl elution with TE, two 25 μl elutions were done. To concentrate the DNA, it was precipitated with 1 μl glycogen (20mg/ml, Boehringer Mannheim), 0.1 volume 4M LiCl and 3 volumes of cold 99% ethanol at -20$^0$C overnight. Thereafter, it was centrifuged at 4$^0$C for 30 minutes at 11 000 xg, washed with 70% ethanol, dried and resuspended in a smaller volume of sterile, distilled water or TE.

The second method involved using the Prep-A-Gene® DNA Purification System from Bio-Rad. The DNA isolation from agarose gel slices protocol was followed.

## 3.5 Electroporation

### 3.5.1 Preparation of electrocompetent cells

To prepare electrocompetent cells, an overnight LB culture was diluted 1/50 in 500 ml of LB and the cells were grown at 37°C with vigorous shaking until they reached an $OD_{600}$ between 0.6 and 0.9. The culture was chilled on ice for 30 min before being centrifuged at 4000 xg for 15 min at 4°C. All subsequent steps were carried out at 4°C and on ice. After centrifugation, the cells were washed in 1 volume of cold, sterile, distilled water and pelleted again under the same conditions. Two more washes were carried out, the first in 0.5 volume of cold, sterile, distilled water and the second in 1/50 volume of cold 10% (v/v) glycerol. Centrifugation conditions after each step were as previously described. Finally, the cells were resuspended in 1/500 volume of cold 10% (v/v) glycerol, and 50-100 µl aliquots were first frozen in ice-cold ethanol, then stored at -70°C.

### 3.5.2 Electroporation

40 µl of electrocompetent cells and 1-2 µl of DNA were mixed and put into a chilled 0.2 cm electroporation cuvette (Bio-Rad). Electroporations using the Gene Pulser electroporator from Bio-Rad were performed under the following conditions: 200 Ω, 25 µF and 2.5 kV. Immediately after electroporation 1 ml of SOC media (per 100 ml: 2 g tryptone, 0.5 g yeast extract, 0.06 g NaCl, 0.36 g dextrose, 0.02 g $MgCl_2$ and 0.25 g $MgSO_4$) was added, and the cells were allowed to recover for 1 hour at 37°C. Afterwards

the cells were plated on LB plates containing the appropriate antibiotics.

3.6 DNA sequencing and primer synthesis

When DNA sequencing was required, the DNA sample and appropriate primer(s) were couriered to B. Cooney in the Lab Services Division of the University of Guelph.

Primers were prepared by CORTEC DNA Services Lab Inc. at Queen's University, Kingston, ON. Primers used to sequence pMEG1 include NM23 (5'TCACTAACTTCTTCATA3'), NM40 (5'GCGCGTAAAGGTTCAATGAGAC3'), NM 22 (see Section 3.3) and $pBAD_{down}$ (5'GGCTGAAAATCTTCTCT3'). Primers used to sequence pMEG2 were NM22 and NM24 (see Section 3.3).

3.7 Southern hybridization

All steps were performed as outlined in the Genius™ Nonradioactive Nucleic Acid Labeling and Detection System User's Guide (Boehringer Mannheim). Changes are described in each subsection.

3.7.1 DNA transfer

After electrophoresis, the agarose gel was submerged and shaken in 250 mM HCl for 8 minutes to depurinate the DNA, and then rinsed in distilled water. To denature the DNA, the agarose gel was submerged and gently shaken in denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 45 minutes. Finally, the agarose gel was neutralized in neutralization solution (1.0 M Tris-HCl, pH 8.0, 1.5 M NaCl) for 45 minutes. The DNA was transferred to a nylon membrane (Amersham Inc.) by vacuum

transfer (Tyler Research Instruments)with 20X SSC. After the transfer the membrane was rinsed in 5X SSC and baked at 120$^0$C for 30 minutes.

3.7.2 Preparation of probe

The DIG-labelled *dsbA* DNA probe used for the Southern blots in which *dsbA* needed to be detected was previously prepared by I. Turcot (1997).

The probe for the detecting the *dsbA* promoter insert in pMP190 was created by labelling this 258 base pair insert with digoxigenin-11-dUTP during PCR. The protocol in the DIG guide for labelling a probe during PCR was followed. The dUTP:dTTP ratio was 1:23. Reactions parameters for the two primers NM22 and NM24 are described in Section 3.3, and the template was the plasmid pIT1.

3.7.3 Southern hybridization

The membrane was incubated for 2 hours at the desired temperature in standard buffer (5X SSC, 1.0% (w/v) Blocking reagent [from 100 mM maleic acid; 150 mM NaCl, pH 7.5, 10% (w/v) Blocking reagent {Boehringer Mannheim}], 0.1% (w/v) sarkosyl and 0.2% (w/v) SDS). The membrane was then incubated with the hybrization solution (standard buffer with 10 - 25 ng/ml probe) overnight at the same temperature. This probe was stored at -20$^0$C and reused.

The post-hybridization washes and chemiluminescent detection were performed according to the DIG Guide, and as previously described in Section 2.3.3.

3.7.4 Membrane stripping

When a membrane needed to be re-probed, the membrane was stripped according to Method I for removal of probe from a Southern blot in the DIG Guide.

## 4. Protein Techniques

### 4.1 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Whole cell lysates were prepared by pelleting 500 µl - 1 ml of an overnight culture and resuspending the pellet in 100-200 µl of SDS-sample buffer (130 mM Tris-HCl, pH 6.8, 30% (w/v) glycerol, 2% (w/v) SDS and 0.001% (w/v) bromophenol blue). Before the samples were loaded onto the polyacrylamide gel, they were boiled for 10 minutes.

The samples were run on 12% polyacrylamide gels at 125 V for approximately 2 hours. The running buffer consisted of 0.3% (w/v) Tris, 1.4% (w/v) glycine and 0.1% (w/v) SDS, pH 8.5. After electrophoresis, the gels were stained for 2 hours in Coomassie blue stain (25% (v/v) methanol, 10% (v/v) acetic acid, and 0.25% (w/v) Coomassie blue R-250), and destained overnight in a destain solution of 5% (v/v) methanol and 7% (v/v) acetic acid. Once the gels were sufficiently destained (faint blue background), the gels were placed between wet cellophane sheets and dried for 40 minutes at $80^0$C in a Bio-Rad Gel Dryer 543.

### 4.2 Quantitation of proteins

The BCA Protein Assay Reagent Kit from Pierce was used to quantiate the amount of protein loaded for some of the protein gels and westerns. The microtiter plate protocol was followed. The absorbance at 550 nm was used to measure the colour response.

4.3 Western blotting

After the proteins were electrophoresed, they were transferred to a 0.2 μm nitrocellulose membrane (Schleicther and Schuell) with a Bio-Rad transfer apparatus for western blots. The transfer was performed in cold transfer buffer [27 mM Tris, 192 mM glycine and 20% (v/v) methanol] for one hour at 100 V.

Once the transfer was finished, the membrane was incubated in a blocking solution containing 1x PBS (140 mM NaCl, 27 mM KCl, 43 mM $Na_2HPO_4$) and 3% skim milk (Carnation) for 20 minutes. The antibody, polyclonal rabbit anti-*E. coli* DsbA antisera was diluted 1/3000 in 1x PBS/1% (w/v) skim milk. The membrane was incubated overnight at room temperature in the diluted antibody. The next day, the membrane was rinsed in 1x PBS and incubated with a 1/5000 dilution of secondary antibody, peroxidase conjugated goat anti-rabbit IgG (Kirkegaard and Perry Laboratories Inc.), in 1x PBS/1% (w/v) skim milk for 1 1/4 hours at room temperature. Thereafter, the membrane was washed in 1x PBS for 15 minutes and in 1 M Tris-HCl, pH 9.6 for 20 minutes. The LumiGLO chemiluminescent detection kit or TMB membrane peroxidase substrate system both from Kirkegaard and Perry Laboratories Inc. were used to detect the proteins. With the chemiluminescent system, proteins were visualized on X-Omat film (Kodak).

**5. β-galactosidase Assays**

5.1 Construction of the *dsbA::lacZ* transcriptional fusion

To assay possible promoter activity from the region upstream of the *dsbA* coding

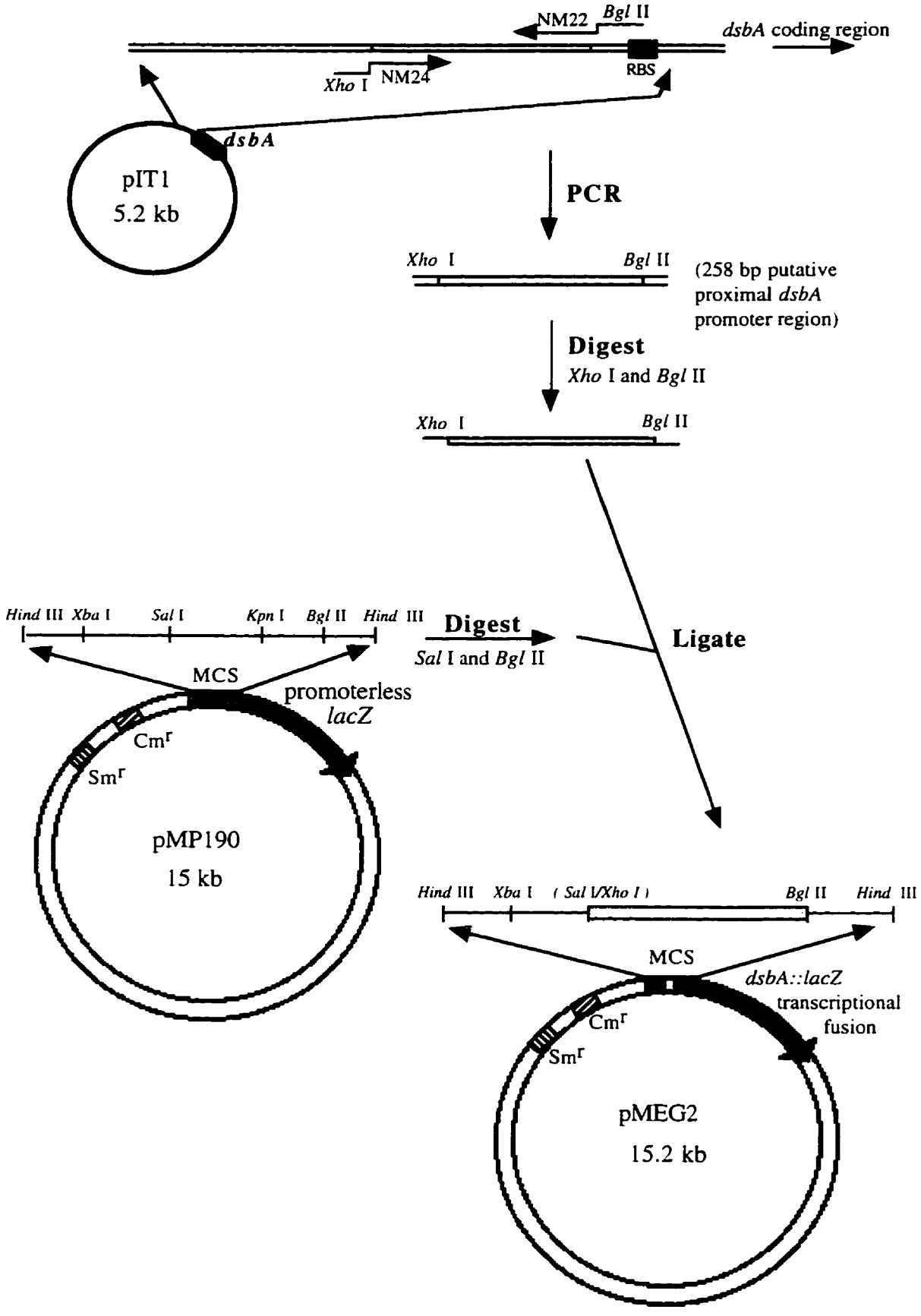
region, a transcriptional fusion was constructed between this region and a promoterless *lacZ* gene. The 15 Kb plasmid pMP190 containing a promoterless *lacZ* gene was digested with *Sal*I and *Bgl*II (both from New England Biolabs)(Figure 6), and the digested plasmid was purified from an agarose gel using Prep-A-Gene®. A 258 bp region upstream of the *S. typhimurium dsbA* transcriptional start site was amplified by PCR. The primers NM 22 containing the *Bgl*II restriction site and NM 24 containing a *Xho*I restriction site (compatible to *Sal*I) were used (Figure 6). The PCR product was digested with the appropriate enzymes and gel purified using the QIAGEN method. The putative promoter region and the digested vector pMP190 were ligated using T4 DNA ligase from New England Biolabs, afterwards, 1.5 µl of the ligation mixture were electroporated into *E. coli* DH5α cells.

Plasmids were isolated using a standard alkaline lysis procedure from colonies which were blue and grew on chloramphenicol. The plasmids were digested with *Hind*III to screen for those containing inserts. This restriction digest would release an approximately 280 bp fragment containing the multiple cloning site and putative *dsbA* promoter region, and the 15 kb vector. Southern hybridization at 85°C - 90°C with a *S. typhimurium* DNA probe homologous to the 258 base pair insert (Section 3.7.2) was used to identify the correct construct. Sequencing was used as definitive verification of the construct. The construct, designated pMEG2, was then isolated from *E. coli* and electroporated into various *S. typhimurium* strains.

## 5.2 Assaying *dsbA* expression

Transcriptional activity could be measured by assaying β-galactosidase activity.

**Figure 6.** Cloning strategy for the construction of pMEG2, the *dsbA:lacZ* transcriptional fusion vector. A description of the cloning procedure is given in the text. NM22 and NM24 are the PCR primers used to amplify the 258 bp putative proximal *dsbA* promoter region from the *dsbA*-containing plasmid pIT1. Restriction sites in brackets were lost after cloning. $Cm^r$ and $Sm^r$ refer to chloramphenicol and streptomycin resistance, respectively.


NM22
Bgl II
dsbA coding region
RBS
Xho I
NM24
dsbA
pIT1
5.2 kb
PCR
Xho I
Bgl II
(258 bp putative proximal dsbA promoter region)
Digest
Xho I and Bgl II
Xho I
Bgl II
Hind III
Xba I
Sal I
Kpn I
Bgl II
Hind III
Digest
Sal I and Bgl II
Ligate
MCS
promoterless lacZ
Cmr
Smr
pMP190
15 kb
Hind III
Xba I
( Sal I/Xho I )
Bgl II
Hind III
MCS
dsbA::lacZ transcriptional fusion
Cmr
Smr
pMEG2
15.2 kb

β-galactosidase assays were performed as described by Miller (1972). Overnight cultures were diluted 1/100 or 1/1000 into fresh media. Prior to addition to the fresh media, the cells were pelleted and resuspended in a small volume of fresh media. Cultures were grown to the desired $OD_{600}$ and an aliquot was removed to be assayed. The succeeding steps and solutions were as described by Miller (1972). The pH of the assay in minimal E glucose media pH 4.5 was approximately 7. Once the colour change had occurred, the samples were centrifuged and the $OD_{420}$ and $OD_{550}$ were measured using a Beckman DU-600 spectrophotometer.

β-galactosidase activity was calculated based on the following equation:

$$\text{Activity (in Miller Units)} = \frac{OD_{420} - 1.75(OD_{550})}{t \times v \times OD_{600}}$$

Where: $t$ = reaction time in minutes

$v$ = volume of culture used in the assay in millilitres

$OD_{420}$ measures ONP produced

$OD_{550}$ measures cell debris

$OD_{600}$ measures cell density before the reaction

# Chapter 3: Results

1. Cloning and sequencing the region upstream of *Salmonella typhimurium dsbA*

Previous studies by Belin and Boquet (1994) determined that *E. coli dsbA* has two promoters. It would therefore be interesting to determine if *S. typhimurium* also has two. Initially, to find out if a promoter exists in a region of DNA, one can examine the DNA sequence and look for the presence of consensus sequences, such as the -35 and -10 boxes for $\sigma^{70}$ promoters. However, in the case of *S. typhimurium dsbA*, if a second promoter was to exist in the same location as in *E. coli* (ie. approximately 1 kb upstream of the *dsbA* transcription start site), insufficient upstream *dsbA* sequence is available in *S. typhimurium* in order to determine the possible existence of the distal promoter. Thus, part of this project included subcloning and sequencing this region.

Initially, a restriction map of the region surrounding *S. typhimurium dsbA* was generated. The *S. typhimurium dsbA*-containing 51 kb cosmid pLAFR2-31 was isolated using a standard alkaline lysis protocol, and was digested with five different enzymes, alone and in combination. The restriction digest fragments were separated on an agarose gel and transferred to a nylon membrane. A Southern hybridization was performed at $65^0$C using a 680 bp *S. typhimurium dsbA* DNA probe.

Information obtained from this Southern blot was added to the information provided by a restriction map of this region previously prepared by I. Turcot (1997). From this new restriction map, it was determined that the approximately 7 kb *Nco*I fragment should contain enough sequence upstream of *dsbA* to include a distal *dsbA*

promoter if it exists.

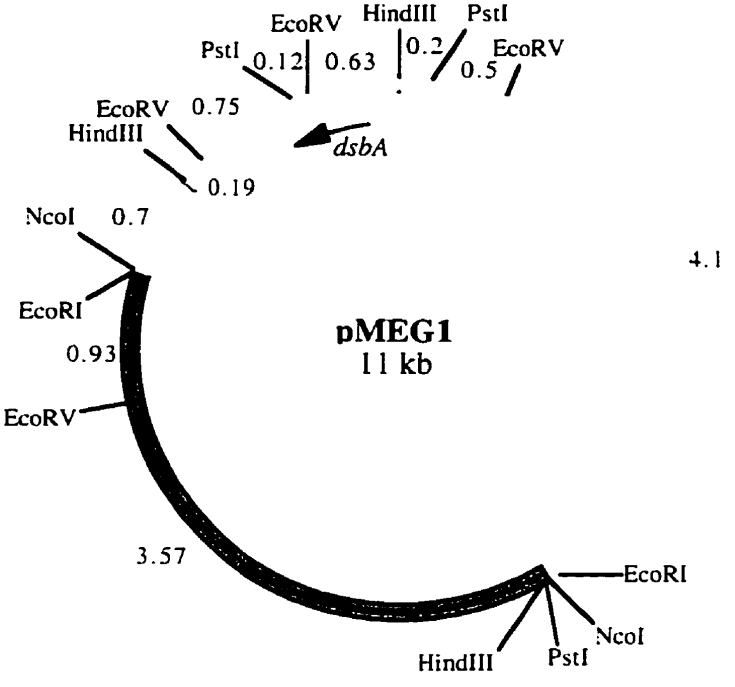
The *Nco*I fragment from the cosmid was purified from an agarose gel and cloned into the *Nco*I site of the dephosphorylated vector pBAD24 (Guzman *et al.*, 1995). The ligation product was electroporated into *E. coli* DH5α (NLM 264). Ampicillin resistance was used to screen for colonies containing plasmid. Colonies which grew were restreaked onto ampicillin plates to ensure purity. A rapid lysis plasmid preparation was used to screen for plasmids containing inserts. Potential clones were mini-prepped and digested. One clone had the correct restriction pattern and was designated pMEG1.

To confirm that pMEG1 contained the correct insert and to restriction map this insert, pMEG1 was digested with several different enzymes, the restriction fragments were separated on an agarose gel and transferred to a nylon membrane (Amersham Inc.). A Southern hybridization was performed at $80^0$C using a 680 bp *S. typhimurium dsbA* DNA probe. The positive control for the Southern blot was a *Hind*III digested *S.typhimurium dsbA*-containing plasmid pIT1. This Southern hybridization confirmed the presence of *dsbA* in pMEG1 (data not shown), and a restriction map of pMEG1 was also created (Figure 7).

A primer, NM23, was designed for sequencing the upstream *S. typhimurium dsbA* region. After several failed sequencing attempts, it was discovered that the melting temperature of the primer was too low. A new primer, NM40, was designed, however, no sequence was obtained using this primer either. Two other primers, $pBAD_{down}$ and NM22, which had previously been successful for sequencing from other plasmids were also used to sequence pMEG1, unfortunately to no avail.

Work is currently underway to subclone a 1.3 kb *Eco*RV fragment containing the

**Figure 7.** A restriction map of the construct pMEG1. The vector pBAD24 is represented by the black segment and the approximately 7 kb insert containing *S. typhimurium dsbA* (arrow) is represented by the the grey segment. The values on the figure represent the approximate fragment sizes in kb.


EcoRV
HindIII
PstI
PstI
0.12
0.63
0.2
0.5
EcoRV
EcoRV
0.75
HindIII
dsbA
0.19
NcoI
0.7
4.1
EcoRI
pMEG1
11 kb
0.93
EcoRV
3.57
EcoRI
NcoI
HindIII
PstI

N-terminal region of *dsbA* from pMEG1 into the vector pUC19 for sequencing and promoter mapping analysis. For more information, please see Appendix I.

2. Northern analyses of the *Salmonella typhimurium dsbA* transcript

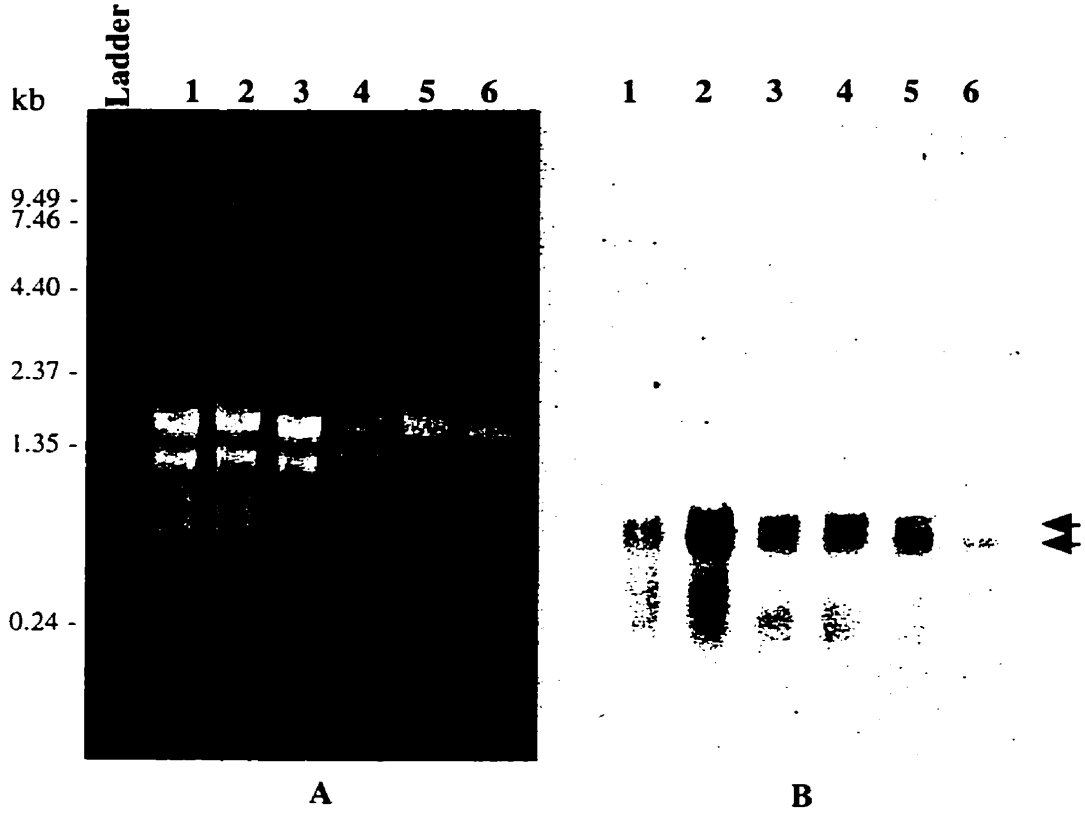
Northern analyses were done for a number of reasons: 1) to determine the number of differently sized *dsbA* transcripts, 2) to determine the size of the *S. typhimurium dsbA* transcript(s), and 3) to determine if conditions such as decreases in oxygen or pH affect transcription. These two conditions have previously been shown to affect the expression of certain genes in *S. typhimurium* (Ernst *et al.*, 1990; Miller *et al.*, 1989; Lee *et al*, 1995).

Three total RNA isolation protocols were tried before Trizol™ became the method of choice. The two other protocols include one based on the principle of alkaline lysis (Karunakaran and Kuramitsu, 1996) and a hot phenol protocol (Warner *et al.*, 1966).

RNA was isolated using Trizol™ from *S. typhimurium* cultures grown at 37°C under different conditions: 1) an aerated LB culture, 2) a static overnight LB culture, and 3) minimal E glucose media pH 7.6 and 4.5 cultures. In addition, RNA was isolated from an *htrA* null *S. typhimurium* strain. In all cases except the overnight static culture, the cells were harvested when they reached an $OD_{600}$ between 0.8 and 1.0. After electrophoresis of the RNA on a formaldehyde-agarose gel (Figure 8A), the RNA was transferred to a nylon membrane. Northern hybridization was performed at 55°C with a 680 bp *S. typhimurium dsbA* DNA probe in high SDS buffer.

The resulting Northern blots gave very interesting results. From all conditions analyzed, two transcripts of approximately 0.85 and 1 kb were detected (Figure 8B).

**Figure 8.** An agarose-formaldehyde gel of *S. typhimurium* RNA isolated from cells grown under different conditions (A). The RNA was transferred to a nylon membrane and probed with a 680 bp *dsbA* DNA digoxigenin-labelled probe. The corresponding Northern blot is illustrated in panel B. Lane 1: LB aerated culture (30.5 μg), 2: E media pH 7.6 (32.5 μg), 3: E media pH 4.5 subcultured 1/1000 from an overnight E media pH 7.6 culture (31.7 μg), 4: E media pH 4.5 subcultured 1/1000 from an overnight LB culture (35.5 μg), 5: LB static overnight culture ($OD_{600}$=0.25) (27 μg) and 6: *htrA* null strain (32.5 μg). The numbers in brackets refer to the amount of RNA loaded. Unless otherwise stated, an overnight culture was subcultured 1/100 and grown to an $OD_{600}$ between 0.82 and 1.0. The arrows indicate the two *dsbA* transcripts.


Ladder
1 2 3 4 5 6
kb
9.49
7.46
4.40
2.37
1.35
0.24
A
1 2 3 4 5 6
B

After taking into consideration the differences in the amount of RNA loaded for each sample, the two transcripts are more abundant in cells grown in minimal E glucose media pH 7.6 than under the other conditions. Furthermore, in cells grown in a static LB culture and in the *htrA* null mutant strain, the two transcripts were detected, however the 1 kb transcript was less abundant than the 0.85 kb transcript. In addition, the 0.85 kb transcript was more abundant in the static culture cells than in the aerated culture cells. Northern analyses were performed at least twice for each condition. The results shown in Figure 8 are representative of those obtained from other Northern blots.

Total RNA from *E. coli* was also isolated and attempts were made to detect *dsbA* mRNA on a Northern blot using the *S. typhimurium dsbA* probe. However, no *E. coli dsbA* mRNA was detectable using this probe (data not shown). An interesting feature of *E. coli* RNA is that only two bands corresponding to the 23S and 16S rRNA are visible after electrophoresis. In contrast, in *S. typhimurium* four bands are visible on an agarose-formaldehyde gel, none of which migrate like 23S rRNA, one band migrates like 16S rRNA (Figure 8).

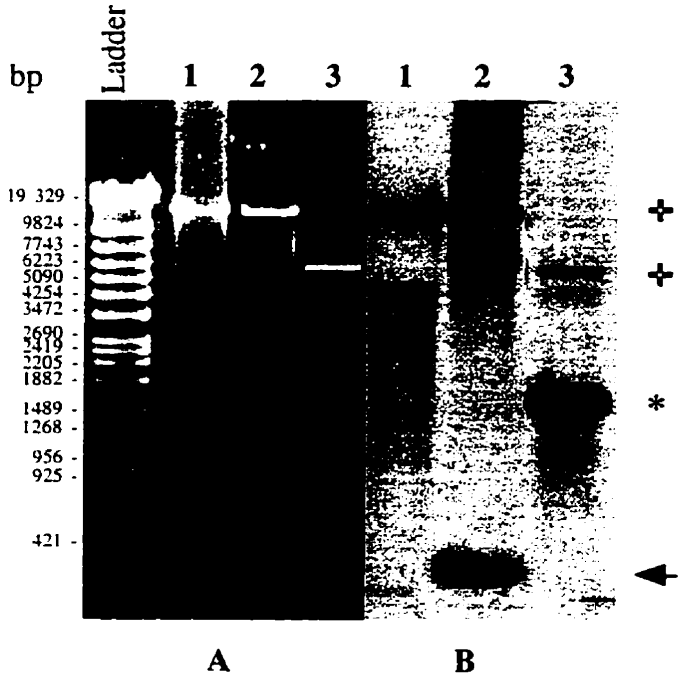
3. Expression from the *Salmonella typhimurium dsbA* proximal promoter region

To determine whether or not the region immediately upstream of the *S. typhimurium dsbA* coding region contained an active promoter, a transcriptional fusion between a putative *dsbA* proximal promoter region and a promoterless *lacZ* gene on a plasmid was constructed. Measuring β-galactosidase activity under different growth conditions should provide 1) evidence that the region immediately upstream of the *dsbA* coding area contains a promoter, and 2) insight into factors which may play a role in

regulating expression of *S. typhimurium dsbA* from this promoter, if indeed it is regulated.

The construction of the *dsbA*::*lacZ* transcriptional fusion is described below and a schematic is presented in Figure 6. A 258 bp region immediately upstream of the *S. typhimurium dsbA* transcription start site was amplified by PCR from the plasmid pIT1 and inserted into the vector pMP190, creating pMEG2. pMP190 is a 15 kb, chloramphenicol resistant plasmid which contains a promoterless *lacZ* gene adjacent to the multiple cloning site (MCS)(Spaink *et al.*, 1987). To confirm that this fusion was correctly constructed, restriction digests were done. Enzymes present in the MCS of pMP190 were chosen since the backbone of pMP190 has not been sequenced. *Hind*III was chosen because it would release a 280 bp fragment containing the insert DNA. The DNA was transferred to a nylon membrane and a Southern hybridization was done at 85-90$^0$C using a 258 bp *S. typhimurium* DNA probe which was 100% homologous to the insert cloned into pMP190. The Southern blot revealed that the small, 280 bp *Hind*III fragment was present in the pMEG2 construct (Figure 9B, arrow). The probe also reacted strongly with the vectors (Figure 9B, crosses), this is most likely due to the presence of a large amount of DNA which nonspecifically sequestered the probe. Lane 3 of Figure 9 contains the positive control, this being the *S. typhimurium dsbA*-carrying plasmid pIT1, which, when digested with *Hind*III, releases a 1.7 kb fragment containing *dsbA*. This band was also detected on the Southern blot (Figure 9B, asterisk). Subsequent bi-directional sequencing of pMEG2 has confirmed the presence of the putative promoter region in this plasmid. In addition, the sequencing results indicated that no mutations had

**Figure 9.** An agarose gel with *Hind*III digests of pMP190 (Lane 1; negative control), pMEG2 (Lane 2) and pIT1 (Lane 3; positive control) (A). The DNA was transferred to a nylon membrane and probed with a 258 bp DNA digoxigenin-labelled probe homologous to the putative proximal *dsbA* promoter region. The corresponding Southern blot is illustrated in panel B. The arrow indicates the 258 bp putative proximal *dsbA* promoter region which was cloned into the transcriptional fusion vector pMP190. The asterisk (*) indicates the 1.7 kb fragment from pIT1 containing *dsbA*. The crosses indicate the vector backbones.


bp
Ladder
1
2
3
1
2
3
19 329
9824
7743
6223
5090
4254
3472
2690
2419
2205
1882
1489
1268
956
925
421
*
A
B

been introduced into the 258 bp putative proximal *dsbA* promoter region (Figure 10).

Once it was clear that the desired construct, pMEG2, had been successfully created, both the vector pMP190 and pMEG2 were electroporated separately into *S. typhimurium* SL1344 (NLM144). To determine if this 258 bp region upstream of *S. typhimurium dsbA* contained a promoter, and which environmental factors, if any, affected it, β-galactosidase assays were performed following the standard protocol (Miller, 1972). For all β-galactosidase assays performed in this work, unless otherwise stated, an overnight culture was diluted 1 in 100, and samples were taken throughout log phase and for several hours during stationary phase. To set up cultures for the assays, a sample of the overnight culture was spun down, the supernatant was removed, and the pellet was resuspended in a equal volume of the appropriate fresh media. This was done to try to eliminate the possibility that something present in the media, such as a secretory product from the organism itself, was affecting regulation of *dsbA*.

Two controls were performed. First, since chloramphenicol is known to increase plasmid copy number (Sambrook *et al.*, 1989), and chloramphenicol is needed in the media to maintain the plasmids (pMP190 and pMEG2), the assay was performed with and without chloramphenicol to determine if the presence of chloramphenicol increased copy number, which in turn would exaggerate the measured activity. From Figure 11, it is clear that chloramphenicol does not have a detectable effect on plasmid copy number, as the activity from cultures grown with and without chloramphenicol was virtually the same.

Second, in the preliminary β-galactosidase assays performed in LB, the maximum activity attained was 1400-1600 Miller units, and the cleavage of ONPG occurred in 2-3

**Figure 10.** The sequence of the 258 bp proximal promoter region of *S. typhimurium dsbA* (blue) which has been cloned upstream of the promoterless *lacZ* gene (pink) in the vector pMP190 (black) to create a *dsbA*::*lacZ* transcriptional fusion. The orange sequence represents the N-terminal region of the chloramphenicol acetyltransferase (*cat*) gene which contains a ribosome binding site.

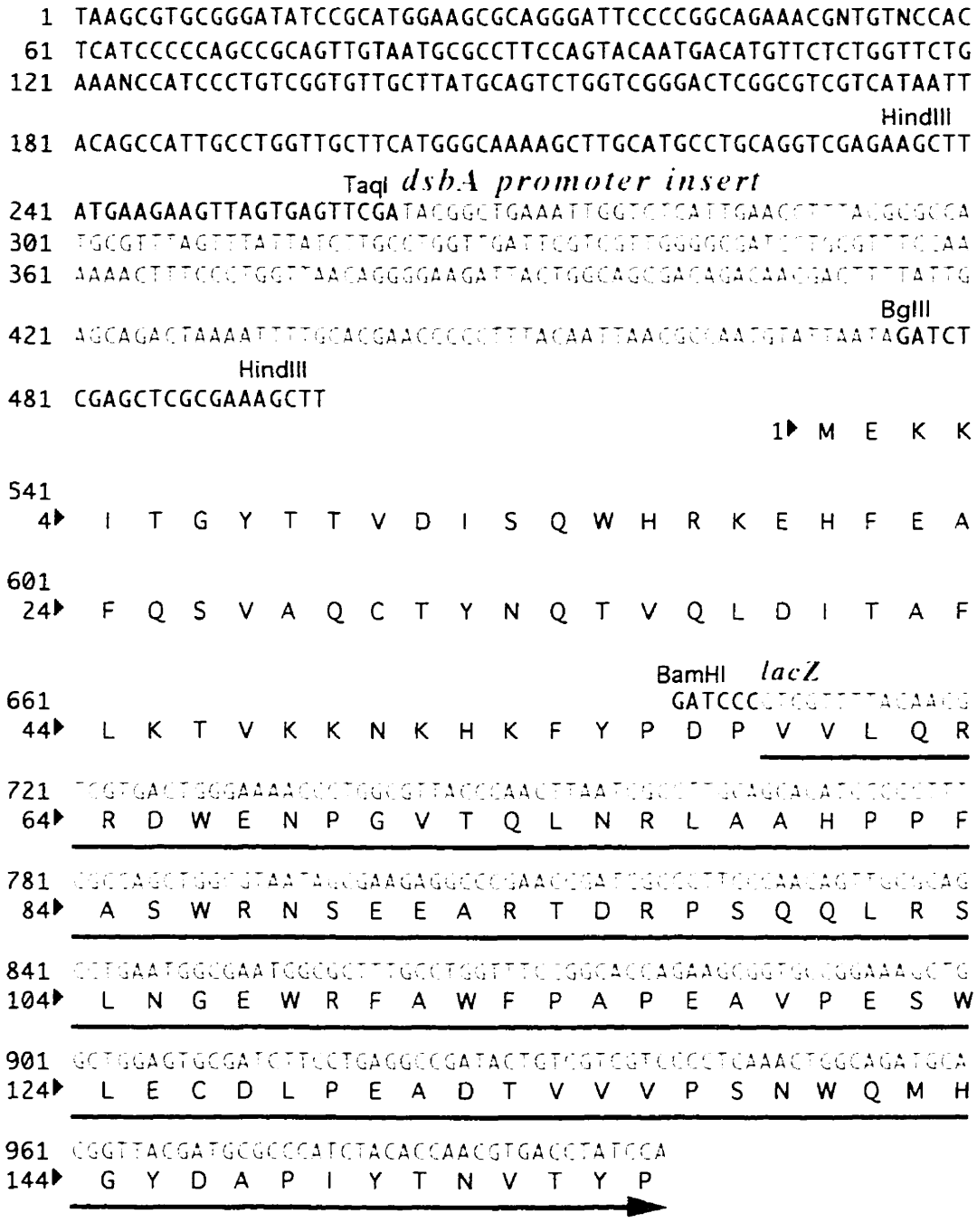
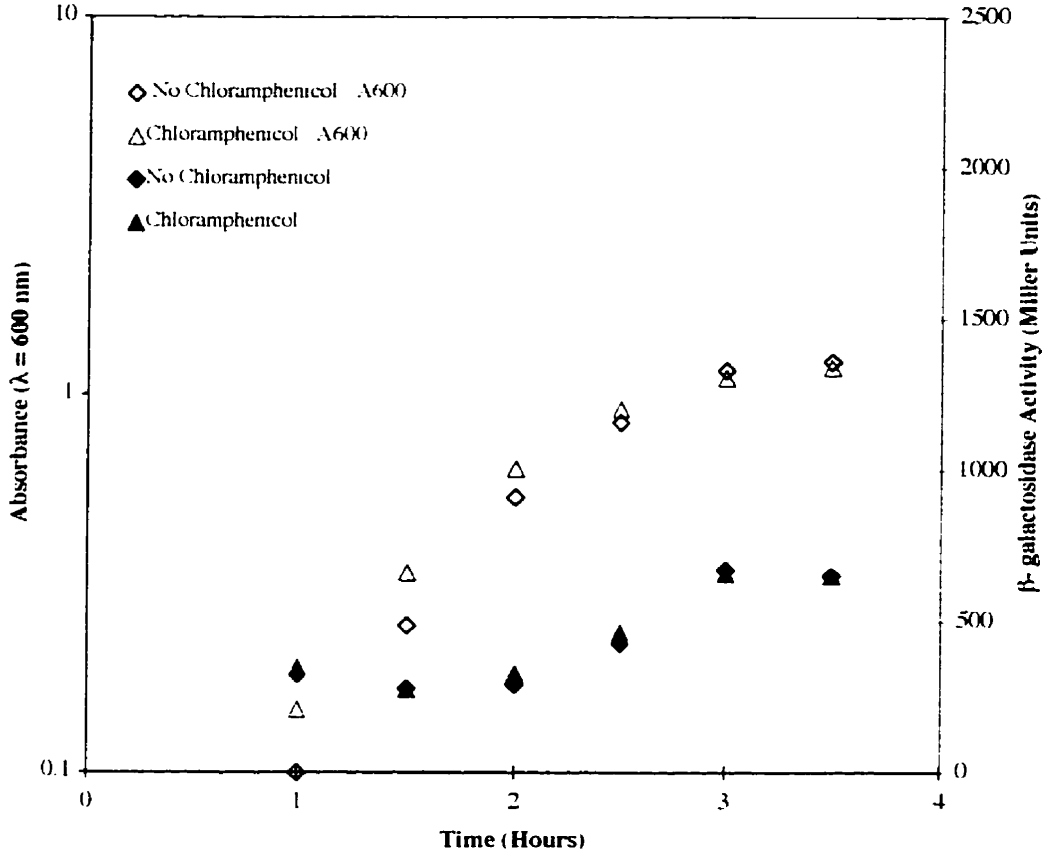

1 TAAGCGTGCGGGATATCCGCATGGAAGCGCAGGGATTCCCCGGCAGAAACGNTGTNCCAC
61 TCATCCCCCAGCCGCAGTTGTAATGCGCCTTCCAGTACAATGACATGTTCTCTGGTTCTG
121 AAANCCATCCCTGTCGGTGTTGCTTATGCAGTCTGGTCGGGACTCGGCGTCGTCATAATT
HindIII
181 ACAGCCATTGCCTGGTTGCTTCATGGGCAAAAGCTTGCATGCCTGCAGGTCGAGAAGCTT
TaqI dsbA promoter insert
241 ATGAAGAAGTTAGTGAGTTCGA
BglII
421 AGCAGACTAAAATTTTGCACGAACCCCGTTTACAATTAACGGCAATGTATTAATAGATCT
HindIII
481 CGAGCTCGCGAAAGCTT
1 M E K K
541
4 I T G Y T T V D I S Q W H R K E H F E A
601
24 F Q S V A Q C T Y N Q T V Q L D I T A F
BamHI lacZ
661
GATCCC
44 L K T V K K N K H K F Y P D P V V L Q R
721
64 R D W E N P G V T Q L N R L A A H P P F
781
84 A S W R N S E E A R T D R P S Q Q L R S
841
104 L N G E W R F A W F P A P E A V P E S W
901
124 L E C D L P E A D T V V V P S N W Q M H
961
144 G Y D A P I Y T N V T Y P

**Figure 11.** Expression of a *dsbA::lacZ* transcriptional fusion in *S. typhimurium* SL1344 grown with (red) and without chloramphenicol (blue). This is a control to determine whether chloramphenicol increases plasmid copy-number, thereby affecting the results of the β-galactosidase assay. The open symbols represent the growth curve, solid symbols represent β-galactosidase activity. This is representative of one trial.


10
1
0.1
2500
2000
1500
1000
500
0
0
1
2
3
4
No Chloramphenicol A600
Chloramphenicol A600
No Chloramphenicol
Chloramphenicol
Absorbance (λ = 600 nm)
β- galactosidase Activity (Miller Units)
Time (Hours)

minutes. To ensure that the substrate, ONPG, was not limiting, the assay was performed using a 1 in 10 dilution of the culture. As is evident from Figure 12, the ONPG was not limiting. If it had been, then the amount of activity from the diluted sample assays should have been the same as or possibly more than the activity from the standard assay.

β-galactosidase assays were first carried out at 37$^0$C in LB with aeration to determine what the level of activity from the putative *dsbA* promoter region was under standard laboratory growth conditions. In Figure 13 (and in all other figures of this nature), each point on a β-galactosidase activity curve is the average of two samples from a single culture, and each curve is representative of assays performed on different days. From Figure 13, it is clear that there is little variation among assays from different days. This figure also illustrates that the β-galactosidase activity from the vector pMP190 alone was always less than 20 units. Under all other conditions assayed (described below), background β-galactosidase activity was also very low (data not shown). Given that there is virtually no β-galactosidase activity produced by pMP190, the β-galactosidase activity produced by pMEG2 must come from the 258 bp insert, thus the insert must contain a promoter. Expression from this promoter is very interesting. Half an hour after subculturing, the activity is quite high, approximately 900 units, which is equivalent to the activity seen during early stationary phase. The activity then drops to approximately 150 units at one hour after subculturing. Thereafter, it increases and reaches a maximum during stationary phase. It remains at this level for several hours; even after 24 hours, activity only decreases to 865 units (Table 4) .

**Figure 12.** Expression of a *dsbA::lacZ* transcriptional fusion in *S. typhimurium* SL1344. To determine whether or not the substrate ONPG was limiting, the standard β-galactosidase assay was performed according to Miller (1972) (blue), and a 1/10 dilution (red) of the culture was also assayed. The open symbols represent the growth curve, solid symbols represent β-galactosidase activity.

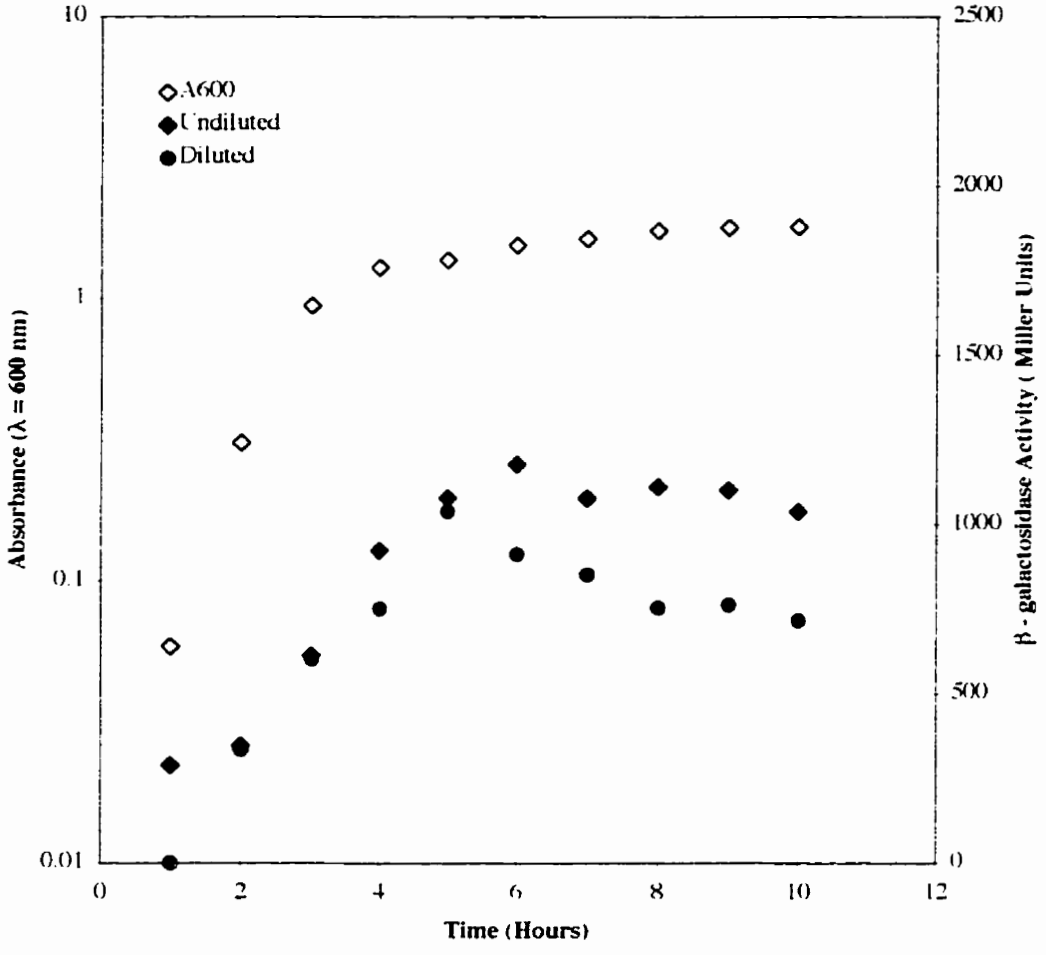

10
1
0.1
0.01
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
A600
Undiluted
Diluted
Absorbance (λ = 600 nm)
β - galactosidase Activity ( Miller Units)
Time (Hours)

**Figure 13.** Expression of a *dsbA::lacZ* transcriptional fusion (pMEG2) in *S. typhimurium* SL1344 during growth in an aerated LB culture. pMP190 (blue) contains a promoterless *lacZ* gene and was used to create the *dsbA* proximal promoter transcriptional fusion (pMEG2). The open symbols represent the growth curve, solid symbols represent β-galactosidase activity. All β-galactosidase activity curves are representative of at least three trials performed in duplicate.

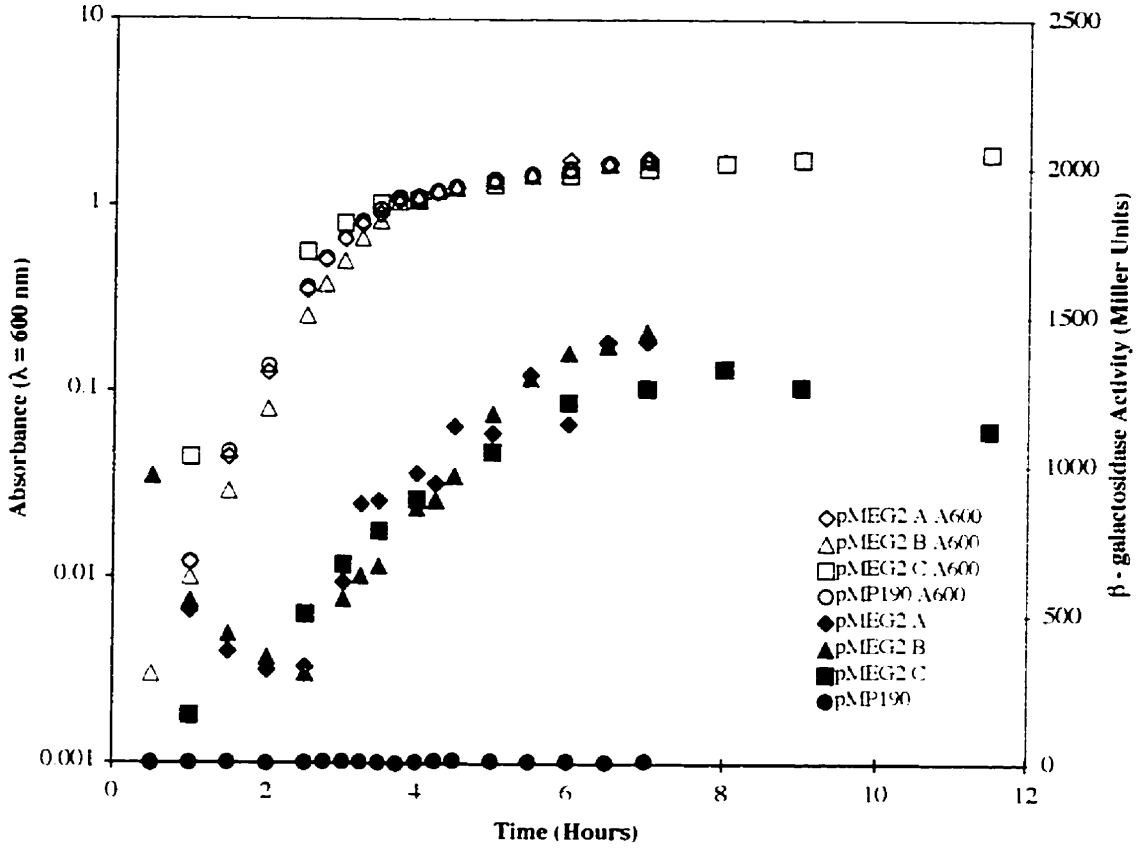

10
1
0.1
0.01
0.001
0
2
4
6
8
10
12
2500
2000
1500
1000
500
0
Absorbance (λ = 600 nm)
β - galactosidase Activity (Miller Units)
Time (Hours)
pMEG2 A A600
pMEG2 B A600
pMEG2 C A600
pMP190 A600
pMEG2 A
pMEG2 B
pMEG2 C
pMP190

**Table 4.** Average expression from the *dsbA*::*lacZ* transcriptional fusion after 24 hours as measured by β-galactosidase activity.

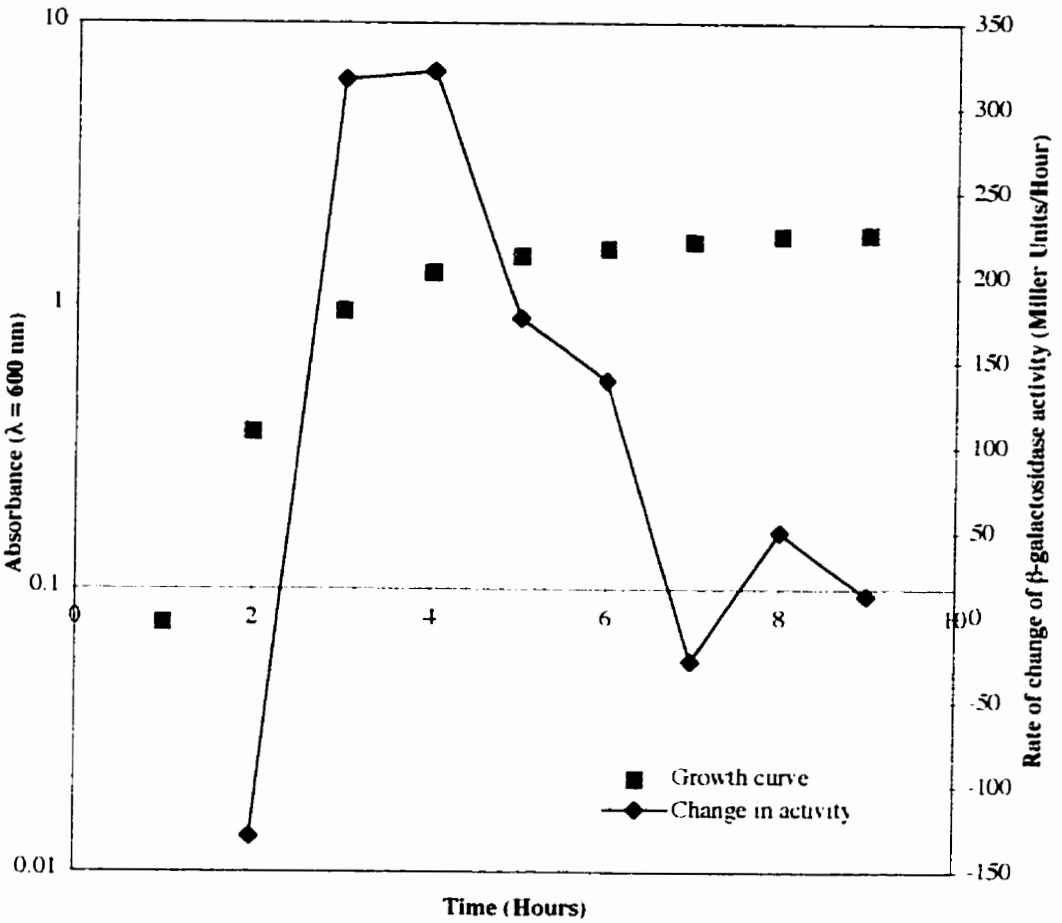
| Condition | β-galactosidase Activity (Miller Units)* |
|---|---|
| Vector only (pMP190) | 6 |
| Aerated LB | 865 |
| Static LB | 484 |
| *dsbA* null | 1357 |
| *E. coli* MC4100 | 749 |

* each value is the average β-galactosidase activity of 2 - 5 separate 24 hour cultures

Figure 14 illustrates this stationary phase induction more clearly. The data on the change in β-galactosidase activity curve represents the average of several trials. The standard deviations range form 17 to 100. In early log phase, between the first and second hour of growth, activity was decreasing as is illustrated by the first point at -129. By late log phase or entry into stationary phase, activity has increased dramatically. Between the second and third hour of growth, activity increases approximately 445 units, and this is followed by another large increase in activity (316 units) between the third and fourth hours of growth. Once in stationary phase, much smaller increases in activity are seen. After seven hours of growth, activity did not increase or decrease significantly.

The discovery that *dsbA* expression from this promoter is growth phase regulated, and that maximal induction occurs during stationary phase suggests that perhaps *rpoS*, the stationary phase sigma factor, may play a role in regulating expression of this *dsbA* promoter. To test this hypothesis pMP190 and pMEG2 were electroporated, separately, into the *rpoS* null *S. typhimurium* 14028s strain (NLM 2157). Since this *rpoS* null mutant is in a different *S. typhimurium* strain from that originally tested, the plasmids were also

**Figure 14.** Growth of *S. typhimurium* at 37$^0$C in an aerated LB culture and the rate of change of β-galactosidase activity per hour elapsed. The peak of the change in β-galactosidase activity curve corresponds to early stationary phase growth. Each point on the change in activity curve (red) represents the difference in activity between the time of the plotted point and that preceeding it (eg. the first point represents the change in activity between the first and second hour). This curve is an average of five trials, and the standard deviations range between 17 and 100.


10
1
0.1
0.01
Absorbance (λ = 600 nm)
350
300
250
200
150
100
50
0
-50
-100
-150
Rate of change of β-galactosidase activity (Miller Units/Hour)
0
2
4
6
8
10
Time (Hours)
Growth curve
Change in activity

electroporated into a *S. typhimurium* 14028s wild-type strain. Activity in the wild type *S. typhimurium* 14028s strain is very similar to activity in wild type *S. typhimurium* SL1344, only after 8 hours of growth does there appear to be a difference (compare Figures 13 and 15). *S. typhimurium* 14028s has approximately 300 units more activity, however this falls within the range of activity seen from *S. typhimurium* SL1344 in stationary phase, and therefore is not significant. When the activity from the *rpoS* null mutant is compared to the wild type (Figure 15), it is evident that expression is similar in both strains. Again, the approximately 400 unit difference occurring after 8 hours of growth falls within the range of trial to trial variation.

In addition to the *rpoS* null strain, a *S. typhimurium slyA* mutant was also available. SlyA is a transcriptional regulator which is expressed during stationary phase and during infection of macrophages. Its expression is not *rpoS* -dependent. SlyA regulates genes necessary for adaptation to environmental stimuli encountered while infecting a host (Buchmeier *et al.*, 1997). *dsbA* expression was therefore analyzed in the *slyA* null strain which was also created in *S. typhimurium* 14028s. Lack of SlyA does not affect transcription from this *dsbA* promoter region (Figure 16). The curves are representative of two trials performed in duplicate and again, the differences in activity are not significant.

To determine whether the lack of *dsbA* would affect the regulation of *dsbA* expression from this promoter region, pMP190 and pMEG2 were electroporated into a *S. typhimurium dsbA* null strain (NLM267 ). As shown by Figure 17, the same growth-phase dependent induction of *dsbA* expression occurs as in the wild type strain. However, the maximum activity is approximately 700 units greater in the *dsbA* mutant

**Figure 15.** Expression of a *dsbA::lacZ* transcriptional fusion in wild type (blue) and *rpoS* null (red) *S. typhimurium* 14028s during growth in an aerated LB culture. The open symbols represent the growth curve, solid symbols represent β-galactosidase activity.

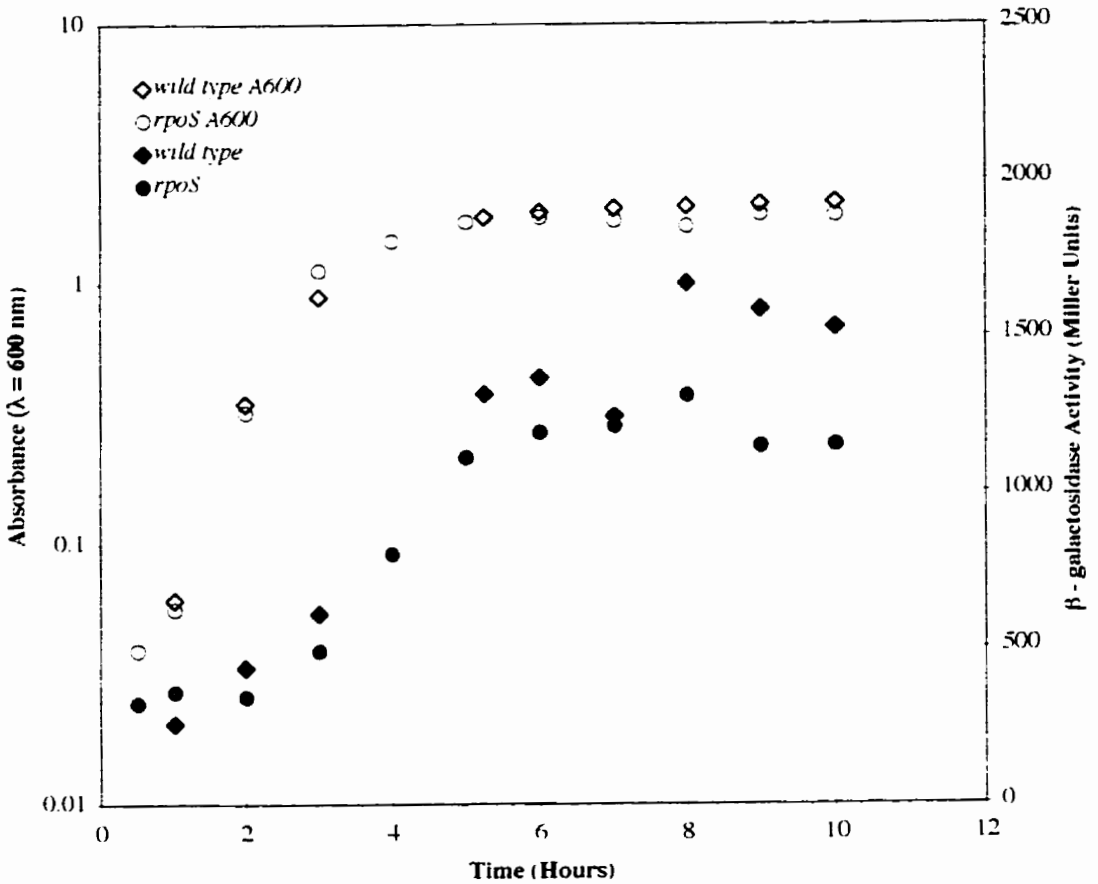

wild type A600
rpoS A600
wild type
rpoS
Absorbance (λ = 600 nm)
β - galactosidase Activity (Miller Units)
Time (Hours)
10
1
0.1
0.01
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12

**Figure 16.** Expression of a *dsbA::lacZ* transcriptional fusion in wild type (blue) and *slyA* null (red) *S. typhimurium* 14028s during growth in an aerated LB culture. The open symbols represent the growth curve, solid symbols represent β-galactosidase activity. The data for the *slyA* null mutant is representative of two trials performed in duplicate.

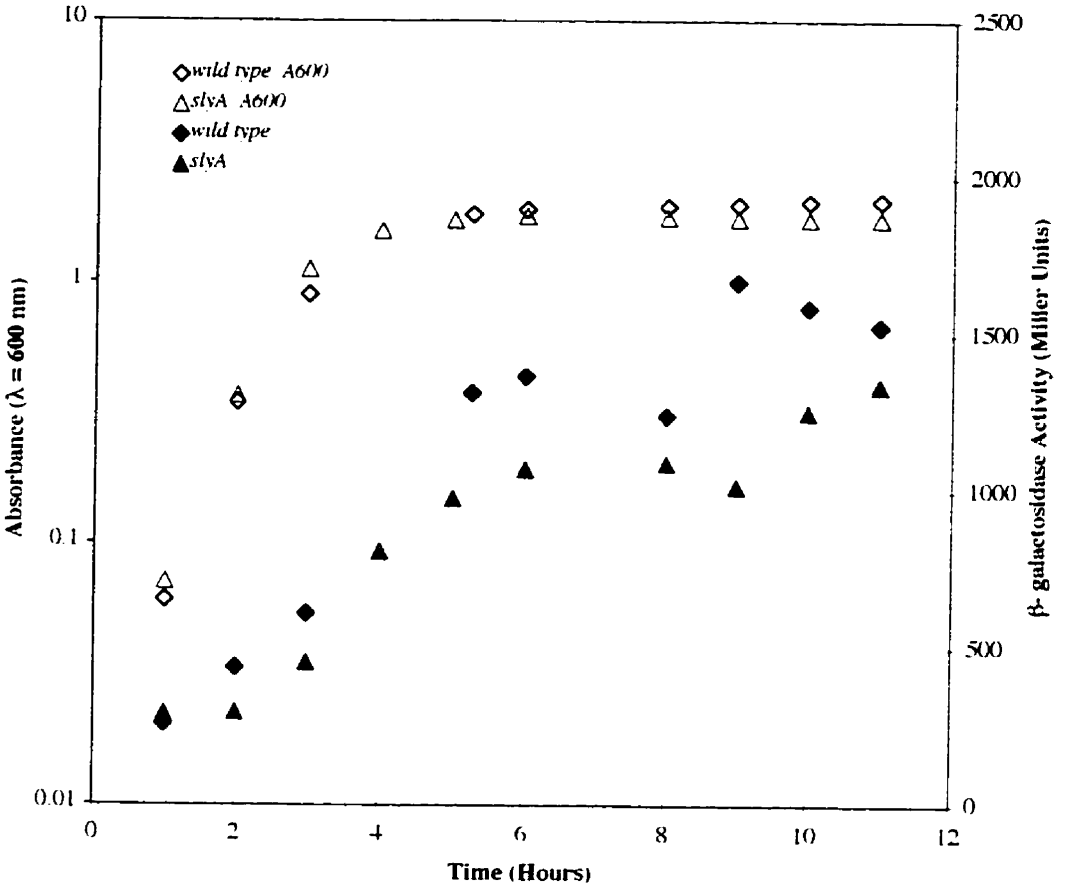
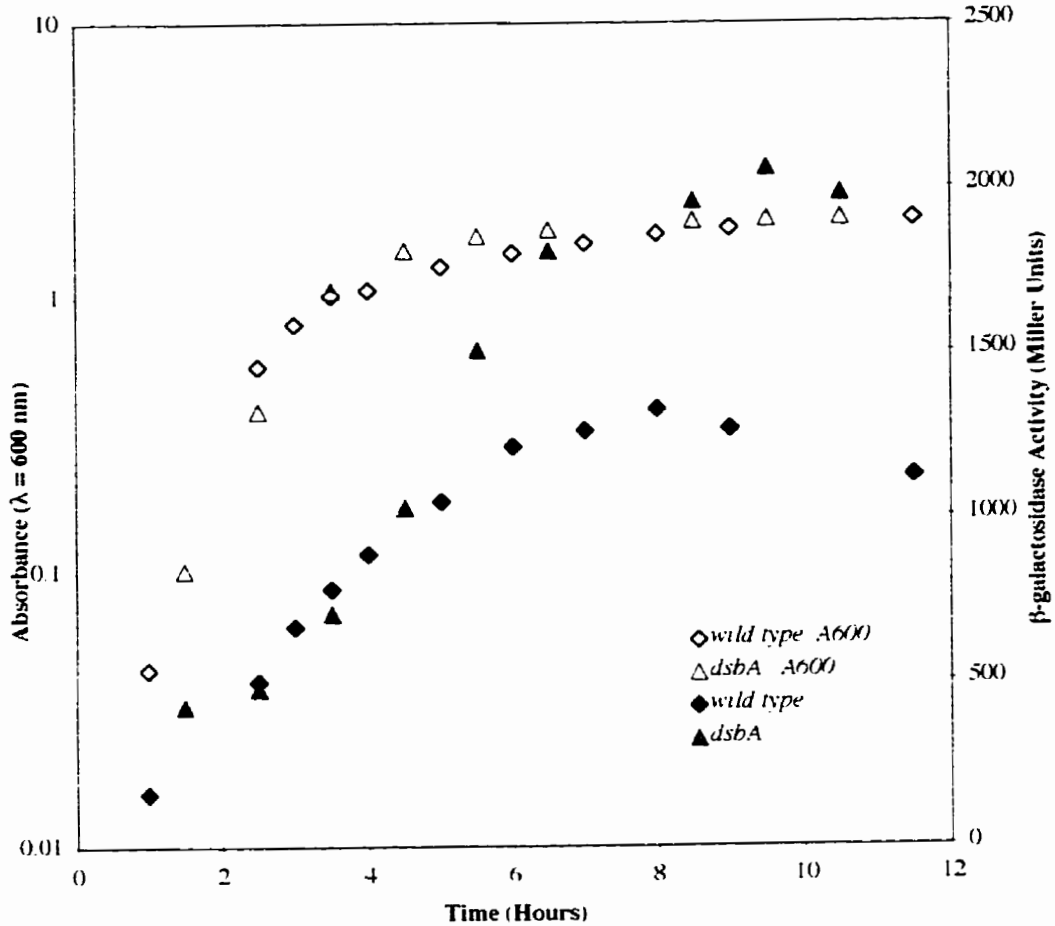

wild type A600
slyA A600
wild type
slyA
Absorbance (λ = 600 nm)
β- galactosidase Activity (Miller Units)
Time (Hours)
10
1
0.1
0.01
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12

**Figure 17.** Expression of a *dsbA::lacZ* transcriptional fusion in wild type (blue) and *dsbA* null (red) *S. typhimurium* SL1344 during growth in an aerated LB culture. The open symbols represent the growth curve, solid symbols represent β-galactosidase activity.

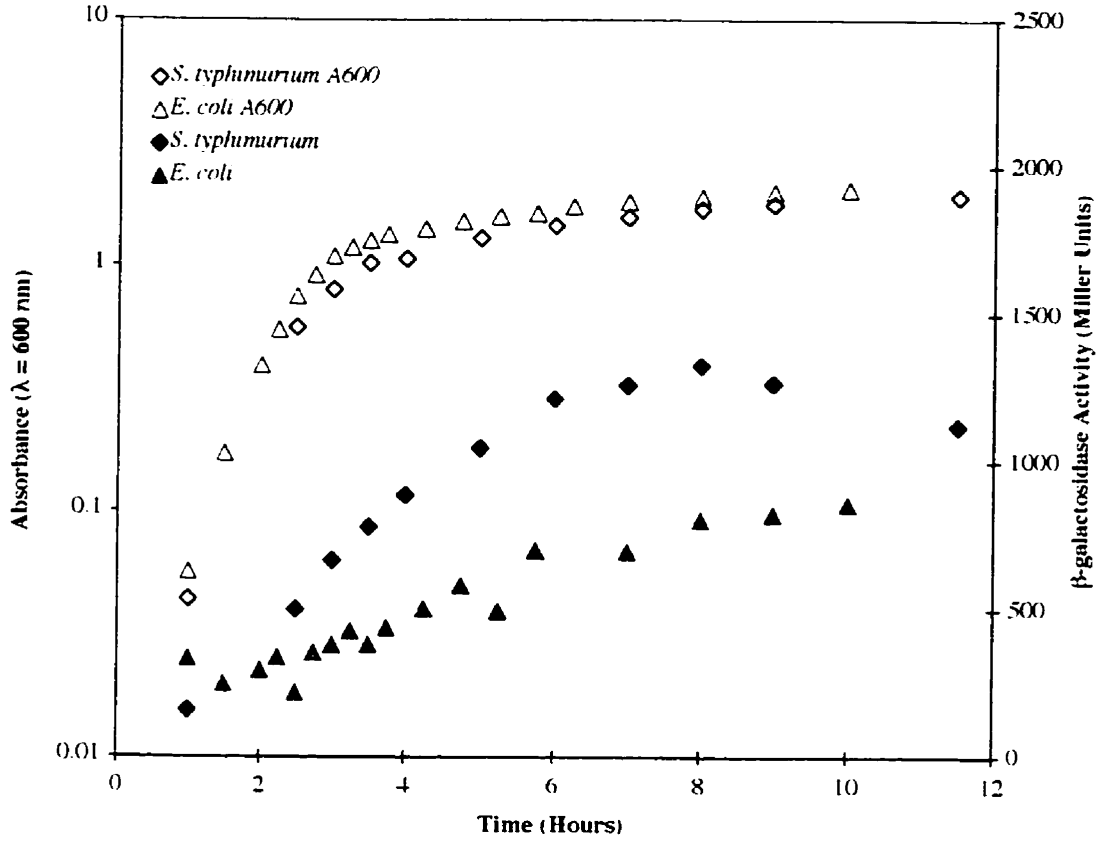

10
1
0.1
0.01
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
Absorbance (λ = 600 nm)
β-galactosidase Activity (Miller Units)
Time (Hours)
wild type A600
dsbA A600
wild type
dsbA

strain. Closer examination of the data reveals that *dsbA* expression during log phase is similar in both strains. Instead of leveling off around 1300 units, the *dsbA* null strain continues to express the *dsbA::lacZ* fusion until reaching approximately 2000 units. In addition, activity remains the same as an early stationary phase culture even after 24 hours (Table 4).

To see if *dsbA* expression from this cloned promoter region was regulated in the same manner in *E. coli*, both pMP190 and pMEG2 were electroporated into an *E. coli* MC4100 strain (NLM166). Interestingly, the same rate of increase of *dsbA* expression was not observed in *E. coli* (Figure 18). Expression from the *dsbA* promoter region increased linearly throughout growth. The rate was much lower, and the activity after a few hours in stationary phase was also approximately 500 units less than in *S. typhimurium*. After 24 hours, β-galactosidase activity decreased approximately 100 units, which is only 100 units less than in *S. typhimurium* at the same time point (Table 4).

During an infection and in nature, *S. typhimurium* can encounter a variety of stressful conditions, including nutrient limitation, temperature fluctuations, decreases in pH and oxygen concentration. These conditions have been previously shown to affect the regulation of a number of systems in *S. typhimurium* (Ernst *et al.*, 1990; Miller *et al.*, 1989; Lee *et al*, 1995). Two of these conditions, decreased oxygen concentration and pH, were chosen to see if they affected the expression of *dsbA*. To mimic lower oxygen concentrations, a static culture with *S. typhimurium* SL1344 containing pMEG2 was used. Under this condition bacterial growth differs from that in an aerated culture. The bacteria undergo log phase growth during the first three hours after subculturing, when

**Figure 18.** Expression of a *dsbA::lacZ* transcriptional fusion in *S. typhimurium* SL1344 (blue) and *E. coli* MC4100 (red) during growth in an aerated LB culture. The open symbols represent the growth curve, solid symbols represent β-galactosidase activity.


◇S. typhumurium A600
△E. coli A600
◆S. typhumurium
▲E. coli
Absorbance (λ = 600 nm)
β-galactosidase Activity (Miller Units)
Time (Hours)
10
1
0.1
0.01
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12

there is still a fair amount of oxygen in the medium (Figure 19). Thereafter, the growth rate decreases greatly as the oxygen in the media becomes depleted. Expression of *dsbA* under this condition is very low. β-galactosidase activity increases over the course of the assay, however, it remains below 500 units (Figure 19). Even after 24 hours, activity does not exceed 500 units (Table 4).

To assess the effect of decreased pH on *dsbA* expression, *S. typhimurium* SL1344 containing pMEG2 was grown overnight in minimal E glucose media pH 7.6 supplemented with 1X amino acids. For the β-galactosidase assay, a 1 in 1000 dilution of the overnight culture was used. The cells were grown to an $OD_{600}$ of approximately 0.2 in minimal E glucose media pH 7.6 supplemented with 1X amino acids, and then half of this culture was subcultured 1 in 1 in minimal E glucose media pH 4.5 supplemented with 1X amino acids. In minimal E glucose media pH 7.6, the stationary phase induction of *dsbA* expression is observed, however in minimal E glucose media pH 4.5, the stationary phase induction of *dsbA* expression does not occur (Figure 20). Also, the overall level of β-galactosidase activity seen is low (< 500 units) (Figure 20).

4. Examination of DsbA expression by Western immunoblotting

To complement the β-galactosidase assays which used a *dsbA*::*lacZ* transcriptional fusion to assess expression from the *dsbA* promoter, Western blots were done to examine DsbA levels during the different phases of growth. The amount of protein loaded on these gels was normalized using the $OD_{600}$ values of the cell cultures. From the Coomassie blue stained protein gel in Figure 21A, it is clear that approximately the same amount of protein was loaded in each well. The Western blot in Figure 21B

**Figure 19.** Expression of a *dsbA::lacZ* transcriptional fusion in *S. typhimurium* SL1344 in aerated (blue) and static (red) LB cultures. The open symbols represent the growth curve, solid symbols represent β-galactosidase activity.

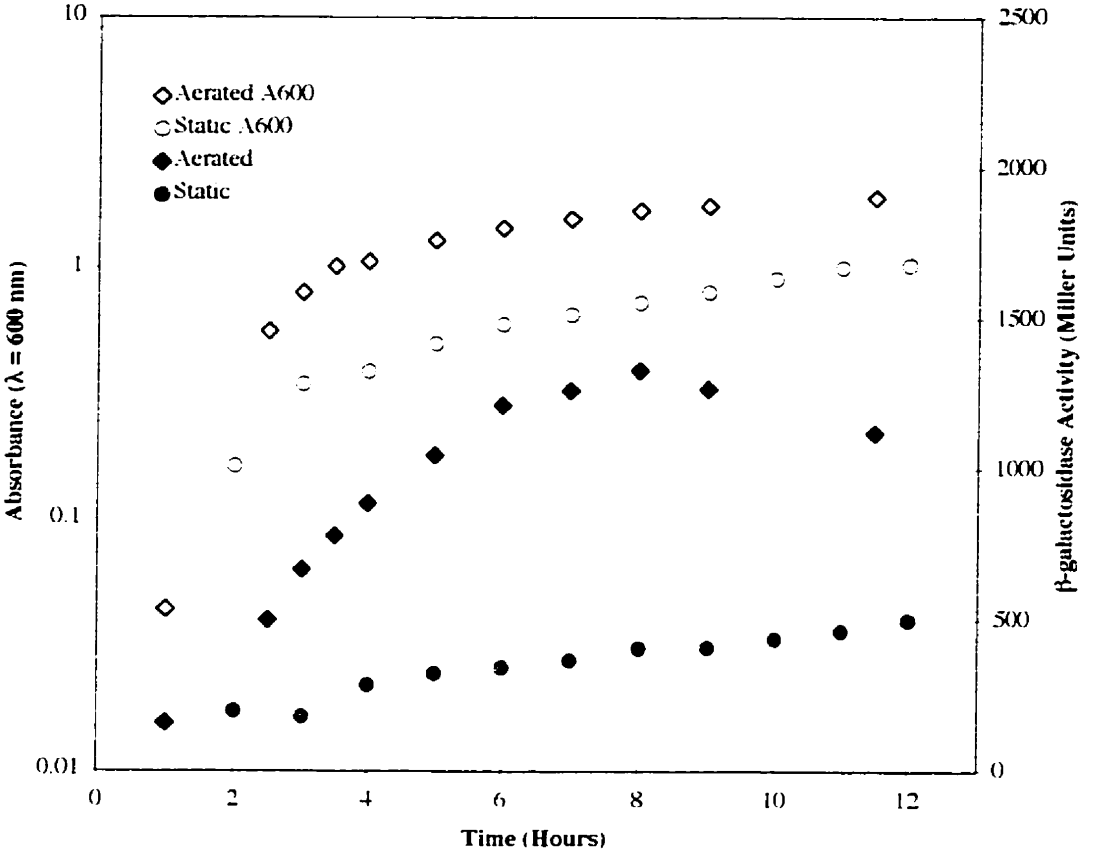

Aerated A600
Static A600
Aerated
Static
Absorbance (λ = 600 nm)
β-galactosidase Activity (Miller Units)
Time (Hours)
10
1
0.1
0.01
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12

**Figure 20.** Expression of a *dsbA::lacZ* transcriptional fusion in *S. typhimurium* SL1344 in minimal E glucose media buffered at pH 7.6 (blue) or 4.5 (red). The open symbols represent the growth curve, solid symbols represent β-galactosidase activity. The arrow indicates when the cells were subcultured 1:1 into minimal E glucose media pH 4.5. A 1/1000 dilution of an overnight culture was used to start the cultures for this assay.

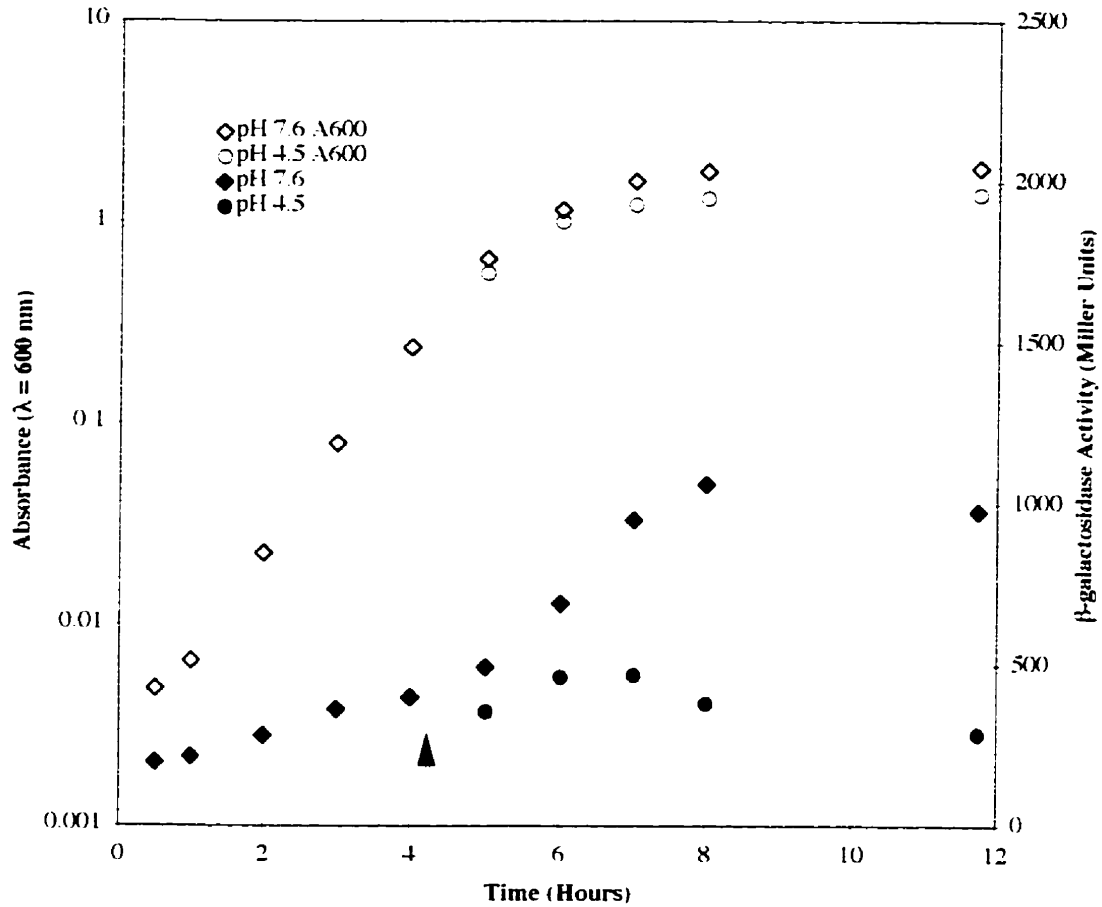
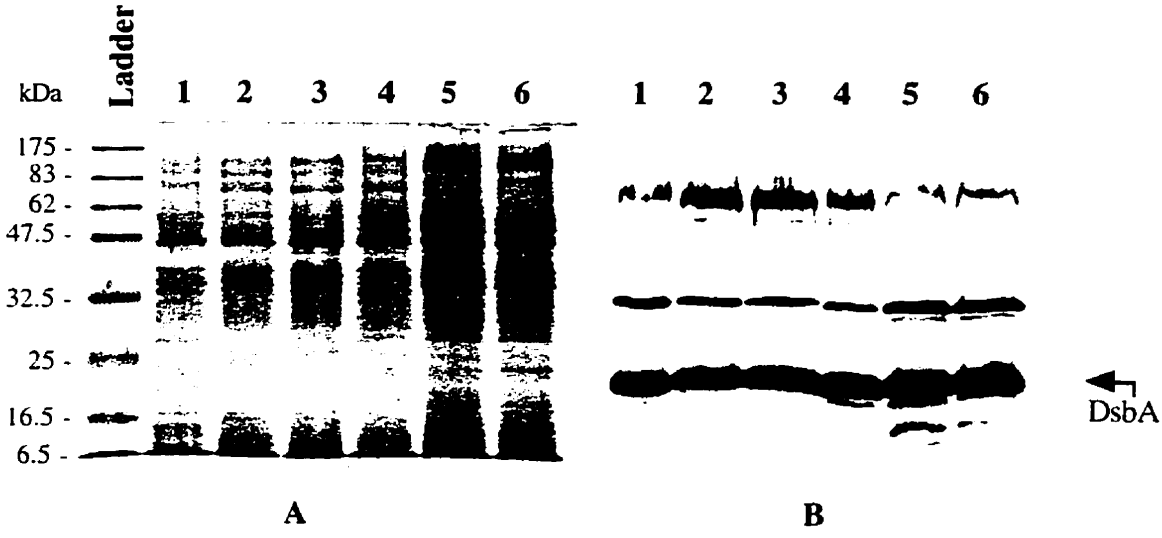

◇pH 7.6 A600
○pH 4.5 A600
◆pH 7.6
●pH 4.5
Absorbance (λ = 600 nm)
β-galactosidase Activity (Miller Units)
Time (Hours)
10
1
0.1
0.01
0.001
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12

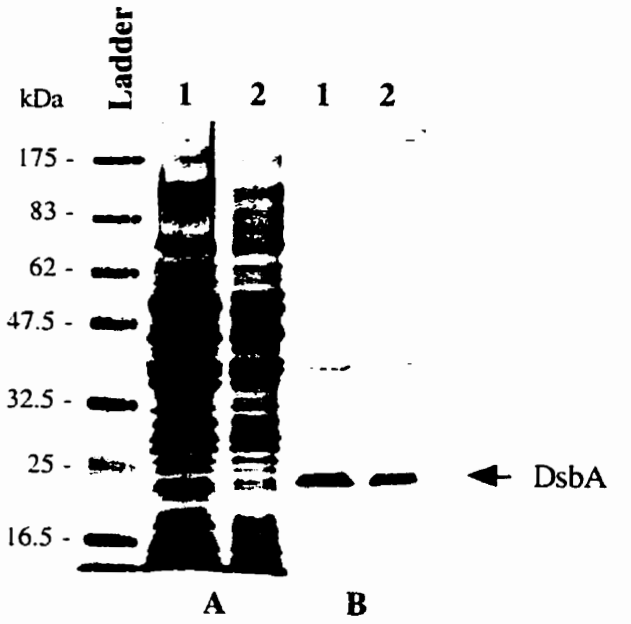
**Figure 21.** A 12% SDS-polyacrylamide gel (A) with whole cell lysates of cells harvested during different phases of growth. Lane 1: $OD_{600}$= 0.09, 2: $OD_{600}$= 0.2, 3: $OD_{600}$= 0.49, 4: $OD_{600}$= 1.07, 5: 1.96, 6: overnight culture ($OD_{600}$= 2.1). The amount of protein loaded was standardized using the $OD_{600}$ readings. The corresponding Western blot using an anti-DsbA antibody is illustrated in panel B. The arrow indicates DsbA.


kDa
Ladder
1
2
3
4
5
6
175 -
83 -
62 -
47.5 -
32.5 -
25 -
16.5 -
6.5 -
1
2
3
4
5
6
DsbA
A
B

illustrates that the steady-state level of DsbA does not change during growth, contrary to the information provided by the expression assays which used a transcriptional fusion.

A Western blot was also done to compare levels of DsbA in cells grown in a static culture versus in cells grown with aeration. Whole cell lysates of overnight cultures were used. Again, the amount of protein loaded on these gels was normalized using the $OD_{600}$ values of the cell cultures. From Figure 22, it is clear that when approximately equal amounts of protein is loaded, DsbA is present in almost equal amounts in aerated and static cultures. These results also differ from those obtained from the expression assays.

**Figure 22.** A 12% SDS-polyacrylamide gel (A) with whole cell lysates overnight aerated (Lane 1; $OD_{600}$= 1.7) and static (Lane 2; $OD_{600}$= 0.49) LB cultures of *S. typhimurium* SL1344. The amount of protein loaded was standardized using the $OD_{600}$ readings. The corresponding Western blot using an anti-DsbA antibody is illustrated in panel B. The arrow indicates DsbA.


kDa
Ladder
1
2
1
2
175 -
83 -
62 -
47.5 -
32.5 -
25 -
16.5 -
DsbA
A
B

# Chapter 4: Discussion

Disulfide bond formation plays an important role in protein folding and stability of disulfide bond containing proteins. The enzyme responsible for catalyzing this process in both *E. coli* and *S. typhimurium*, in addition to other Gram-negative bacteria is DsbA (Bardwell *et al.*, 1991; Kamitani *et al.*, 1992; Turcot, 1997; Peek and Taylor, 1992; Tomb, 1992). While its function as a disulfide oxidoreductase has been characterized (Bardwell *et al.*, 1991; Kamitani *et al.*, 1992; Wunderlich and Glockshuber, 1993, Zapun *et al.*, 1993), the regulation of expression of *dsbA* remains to be fully elucidated, especially in *S. typhimurium*. Thus, the objective of this research was to gain some understanding of the regulation of *S. typhimurium dsbA* expression.

The information available on the regulation of *dsbA* expression in *E. coli* was used to give direction to this research project. In *E. coli*, two promoters for *dsbA* have been identified (Belin and Boquet, 1994) and they are separated by 986 base pairs (Figure 5). Furthermore, the distal *dsbA* promoter, which precedes the *yihE* gene located immediately upstream of *dsbA* on the *E. coli* chromosome, has been shown to be regulated by the Cpx two-component signal transduction system (Pogliano *et al.*, 1997). In response to 1) overexpression of NlpE, an outer membrane lipoprotein (Danese *et al.*, 1995), 2) a lack of the major phospholipid, phosphatidylethanolamine, in the membrane (Mileykovskaya and Dowhan, 1997), and 3) a decrease in pH from 7.4 to 6.0 (Nakayama and Watanabe, 1995), the Cpx system is activated in *E. coli*. The Cpx signal transduction pathway has not been identified in *S. typhimurium*.

Keeping the information with respect to *E. coli dsbA* regulation in mind, work on examining the regulation of *S. typhimurium dsbA* began. To determine whether or not regions which correspond to a *yihE*-like gene with a second promoter and CpxR binding site are present upstream of *S. typhimurium dsbA*, one could look at the DNA sequence in this region. However, only the first 254 base pairs of the DNA sequence upstream of the *S. typhimurium dsbA* translational start site are known, thus it is not possible to locate a second promoter by this means.

A comparison of potential promoter regions is limited to the known upstream *dsbA* region of *S. typhimurium* and the same region in *E. coli* (Figure 5). If one assumes that the -10 and -35 boxes of the *S. typhimurium dsbA* promoter are located in the same position as in *E. coli*, and a comparison is made between these two regions, one discovers that they are only 66.7% identical. These differences in the promoter sequence may be of some significance. Studies using the *S. typhimurium dsbA*::*lacZ* transcriptional fusion vector pMEG2 in *E. coli* support the hypothesis that the *dsbA* proximal promoter differs between the two organisms. In *S. typhimurium*, expression of *dsbA* was induced maximally at the onset of stationary phase, this phenomenon was not observed in *E. coli* (Figure 18). The region immediately upstream of the putative -35 box of the *S. typhimurium dsbA* promoter is 86.6% identical to the same region in *E. coli*, which corresponds to the 3' end of *yihE* in *E. coli* (Figure 5). This suggests that a *yihE*-like gene may be present in *S. typhimurium*.

In order to learn more about the upstream *dsbA* region in *S. typhimurium*, an approximately 7 kb fragment containing *S. typhimurium dsbA* and approximately 4.8 kb of upstream sequence were cloned into the vector pBAD24. Several attempts were made

to sequence this clone, unfortunately all failed. Trouble-shooting to overcome this problem involved designing new primers with higher melting temperatures, and extracting the plasmid using various protocols, all to no avail. PCR amplification of the promoter region and restriction mapping have confirmed that the desired construct pMEG1 was created. The reason as to why pMEG1 cannot be sequenced has not been identified, however, it is possible that the secondary structure of the plasmid in this region is complex enough inhibit sequencing. As described in Appendix I, steps are being taken to subclone a segment of pMEG1 into another vector, and perhaps this construct will yield sequence data. A second option exists, this is to sequence the upstream *dsbA* region from the 51 kb cosmid pLAFR2-31 using the primer NM40.

Another means used to try to determine whether or not two promoters existed for *S. typhimurium dsbA* was Northern analysis. If the genomic arrangement of *dsbA* is the same in *E. coli* and *S. typhimurium*, in *S.typhimurium* two mRNA transcripts should be made as long as the correct growth conditions are used. These two transcripts, differing in size by almost 1 kb, should be detectable on a Northern blot. This rationale was applied to *S. typhimurium*. If two *dsbA* promoters exist, then two transcripts should be detectable. Thus, RNA was isolated from *S. typhimurium* and Northern hybridization was performed.

The techniques involved in these procedures brought with them their own obstacles. Due to a lack of familiarity with RNA work and the necessary protocols, several RNA isolation techniques and many attempts using these protocols were necessary to be able to routinely isolate high quality RNA. With help from Dr. A. Kropinski (personal communication), the mystery as to why three bands in addition to the

16S rRNA band were visible after electrophoresis of *S. typhimurium* RNA was solved. In *S. typhimurium*, fragmentation of the 23S rRNA occurs, which results in a different rRNA banding pattern on an agarose-formaldehyde gel than is seen with *E. coli* rRNA (Hsu *et al.*, 1992). Reasons why *S. typhimurium* degrades its 23S rRNA have been proposed by Hsu *et al.* (1994). They suggest that tight control of rRNA synthesis and degradation allows the organism to quickly adapt to rapidly changing growth conditions, especially in an animal host.

If *S. typhimurium dsbA* has two promoters located in approximately the same positions as in *E. coli*, and given that *dsbA* is about 680 base pairs, two transcripts of at least 0.7 kb and 1.7 kb would be expected. The exact transcript sizes could vary slightly from these hypothetical values, given the fact that the transcription termination site has not been elucidated, nor is the measurement of transcript sizes from a Northern blot 100% accurate. Interestingly, two transcripts of approximately 0.85 kb and 1 kb were detected by Northern analysis under all conditions examined (Figure 8). No transcript of approximately 1.7 kb was detected. The discovery that *S. typhimurium* has two transcripts differing in size by only approximately 200 base pairs is novel. This indicates that two *dsbA* promoters may also exist in *S. typhimurium*, however they would be more closely spaced in this organism than in *E. coli*.

There is also a second possible explanation for the existance of the two closely spaced transcripts. Two transcriptional stops sites could be present, and one, located 0.85 kb from the transcriptional start site, is inefficient allowing transcription to continue until it reaches a stronger termination site further downstream. Several examples of transcriptional stop sites with differing efficiencies have been described (Richardson and

Greenblatt, 1996). This, however, does not necessarily account for the difference in abundance of the 0.85 kb transcript versus the 1 kb transcript under static LB conditions or in the *htrA* null strain. In order to conclusively determine whether or not two promoters exist, further analysis such as RNase protection assays, could be carried out.

In Figure 8, it appears as though the abundance of transcript is greater under some conditions versus others. To a small extent, this is due to the amount of RNA loaded, which varies from lane to lane. Even when the same amount of RNA was loaded in each well (based on $OD_{260}$ readings), it often did not appear that the same amount of RNA was present in each lane after electrophoresis (personal observation). Therefore, the Northern blots in this study were not necessarily used to quantitate abundance of transcript between conditions examined. Future work in this area could include an appropriate internal control, such as a probe for rRNA, which could then be used to quantitate transcription products.

In the cases of RNA isolated from a minimal E glucose media pH 7.6 culture and from the *htrA* null strain however, the difference in overall abundance of transcript is not solely due to the variations in the amount loaded. Coincidently, the same amount of RNA was loaded for these two samples. These results suggest that some component(s) of the minimal E glucose media pH 7.6 may be enhancing overall transcription of *dsbA*, since both transcripts are more abundant in this media. The decrease in transcript abundance in the *htrA* null strain is discussed below.

Comparisons can be made between the abundance of the 0.85 kb transcript and the 1 kb transcript for each condition. Under conditions such as static culture and lack of HtrA, it seems that there is more 0.85 kb transcript in relation to the 1 kb transcript. This

finding suggests that if two *S. typhimurium dsbA* promoters exist, then under these conditions transcription from the more distal promoter is regulated differently than from the proximal promoter , thereby accounting for the difference in transcript abundance. Under low oxygen conditions, a factor required for transcription from the more distal promoter may be limiting due to other regulatory mechanisms. This then results in decreased synthesis of the 1 kb transcript. Also, more 0.85 kb transcript is present in the static culture cells than in the aerated culture cells. Although this seems inconsistent with the *dsbA*::*lacZ* transcriptional fusion results discussed later, the cells in the aerated LB culture were not in the growth phase at which they maximally express *dsbA*, when the RNA was isolated. This could account for the difference in the abundance of the 0.85 kb transcript under these two conditions. Future work should include analysis of *dsbA* transcription by Northern hybridization using aerated stationary phase cultures.

HtrA is an endopeptidase located in the periplasm. It is necessary in *E. coli* for survival at elevated temperatures, presumably because this condition may cause an increase in the formation of misfolded proteins (Lipinska *et al.*, 1989, 1990; Strauch *et al.*, 1989). In *E. coli*, lack of HtrA results in an upregulation of the Cpx system which then causes increased induction of the levels of DsbA (Danese and Silhavy, 1997). Johnson *et al.* (1991) discovered that *S. typhimurium htrA* mutants are not temperature sensitive, and also showed that they are highly attenuated for virulence. This finding suggests that HtrA may have different activities in *E. coli* than in *S. typhimurium*, and may also be regulated differently. The results of the Northern blot in Figure 8 support this suggestion. In the *S. typhimurium htrA* null strain, there was no significant increase in the levels of the *dsbA* transcripts, as would be expected if the lack of HtrA caused an

accumulation of misfolded proteins thereby causing a stress response. Furthermore, the observation that the 0.85 kb transcript was more abundant than the 1 kb transcript again supports the the idea that there are two promoters for *S. typhimurium dsbA* which are regulated differently.

An attempt was made to probe *E. coli* RNA with a *S. typhimurium dsbA* DNA probe to see if *dsbA* specific transcription products could be detected. It had been established in a previous study that an *E. coli dsbA* DNA probe bound to *S. typhimurium dsbA* DNA in Southern blots (Turcot, 1997). Since it is known that *E. coli dsbA* has two promoters (Belin and Boquet, 1994), one would expect to see two mRNA transcripts differing in size by about 1 kb, however no *E. coli dsbA* transcript was detected on the Northern blots. One reason for the inability to detect *E. coli dsbA* mRNA could be that the stringency of the hybridization was too great. A hybridization temperature of approximately $55^0$C was used because *S. typhimurium* RNA was being probed at the same time, and the probe is 100% homologous to *S. typhimurium dsbA*. This temperature may have been too high given that *E. coli* and *S. typhimurium dsbA* are only 79 % identical. Had a lower temperature been used, the *E. coli dsbA* transcripts may have been detected. Additionally, an *E. coli dsbA* DNA probe could have been used.

From the Northern analyses of *S. typhimurium* RNA, it is clear that two distinct *dsbA* mRNA transcripts of approximately 0.85 and 1 kb exist. The abundance of each transcript can vary with respect to each other, especially when the cells are grown under static conditions or in an *htrA* null strain. Future studies should be directed at determining the origin of each transcript.

A second aspect of this study involved creating a transcriptional fusion with the

putative proximal *S. typhimurium dsbA* promoter region. The region immediately upstream of the *dsbA* transcription start site was cloned in front of a promoterless *lacZ* gene, allowing expression from the promoter to be measured by β-galactosidase activity. Since an increase in β-galactosidase activity was seen from the *dsbA*::*lacZ* transcriptional fusion construct pMEG2, while virtually no activity was seen from the vector pMP190 lacking an insert (Figure 13), it can be concluded that the region immediately upstream of the *dsbA* translation start site (Figure 10) contains a promoter. Before the activity of this promoter was assessed under different growth conditions, two controls were performed. Chloramphenicol is required to maintain the plasmids pMP190 or pMEG2, and this antibiotic can cause an increase in plasmid copy-number. This would result in elevated levels of β-galactosidase activity which are not representative of *dsbA* expression. β-galactosidase assays were performed with and without chloramphenicol. From the results (Figure 11), it is clear that chloramphenicol is not affecting β-galactosidase activity, since activity was similar in the presence and absence of the antibiotic. Second, to confirm that the substrate ONPG used in the β-galactosidase assays was not limiting, a 1 in 10 dilution of the culture was assayed concurrently with a standard assay (Miller, 1972). The results of this control clearly illustrate that the substrate was not limiting (Figure 12). Had the ONPG been limiting under standard assay conditions, the β-galactosidase activity from the 1/10 diluted sample should have been identical to or greater than the activity from a standard assay.

The first information as to how *S. typhimurium dsbA* expression was regulated came from the β-galactosidase assays performed using LB cultures grown at 37$^{0}$C with aeration (Figure 13). A re-plotting of the data as the change in β-galactosidase activity

over time illustrates that the greatest change in the rate of β-galactosidase activity occurs at the onset of stationary phase (Figure 14). The standard deviation of the points on the change in rate of β-galactosidase activity curve in Figure 14 was quite large. This can be easily accounted for considering the fact that the data from the five cultures used to plot the graph were not precisely standardized for growth phase, since no two cultures grow exactly the same. Upon analysis of Figures 13 and 14, it is evident that activity is high half an hour after subculturing and then decreases. This evidence suggests that activity in an overnight culture is high compared to activity in an actively dividing culture. The decrease in activity which occurs could be attributed to two factors. Firstly, *S. typhimurium* does not encode the *lac* operon, therefore it does not produce β-galactosidase. Since β-galactosidase is not a native protein in *S. typhimurium*, it may not be very stable in this organism and therefore could have a high turnover rate. Secondly, expression of *dsbA* during this time may be virtually turned off. The combination of these two factors would result in the observed decrease in activity. Between two and three hours after subculturing, when cells are in late log phase, there is a great change in the rate of β-galactosidase activity (Figure 14). This is followed by another large change in rate in early stationary phase, between the third and fourth hour after subculturing. This phenomenon clearly illustrates that expression of *dsbA* is greatly induced during this time period. After four hours, only small increases in total β-galactosidase activity occur, and by the seventh hour, little change in activity occurs as the change in activity from one hour to the next fluctuates around zero. This implies that once *dsbA* expression has been induced, it remains fairly constant. Although Table 4 indicates that *dsbA* expression is lower in a 24 hour culture than in an eight hour culture, when one looks back at Figure

13, 865 units of activity is equivalent to that seen in an early stationary phase culture. This supports the claim made earlier that activity in an overnight culture is high. From the data presented in Figures 13 and 14, it can be concluded that expression of *dsbA* is growth phase dependent with maximal expression occurring during stationary phase. Furthermore, once *dsbA* expression is induced, it remains fairly constant for at least 24 hours. This discovery brings two questions to mind. Is this stationary phase induction of *dsbA* expression dependent on the stationary phase sigma factor RpoS? Why is expression of *dsbA* increased during stationary phase?

To answer the first question, the *dsbA::lacZ* transcriptional fusion construct was put into a *rpoS* null *S. typhimurium* strain. RpoS, the stationary phase sigma factor, regulates the expression of over 20 genes, many of which are involved in the development of the stress-resistant stationary phase state (Goodrich-Blair *et al.*, 1996). Some of the adaptive changes which occur during stationary phase are increased protection from osmotic shock, and increased resistance to oxidative damage and heat shock (Goodrich-Blair *et al.*, 1996). Perhaps expression of *dsbA* is increased during stationary phase because DsbA is required for protein-folding of certain components involved in the physiological changes which occur during this phase of growth.

The *rpoS* null was created in a *S. typhimurium* 14028s strain. Before activity in the *rpoS* mutant could be assessed, activity of the wild type was examined and compared to *S. typhimurium* SL1344. If Figures 13 and 15 are compared, the β-galactosidase activity curves are almost identical for the two strains. The difference between the curves seen after eight hours can be attributed to experimental variation between assays. It can be said that there is no significant difference in expression of the *dsbA::lacZ*

transcriptional fusion between *S. typhimurium* SL1344 and 14028s. Since *rpoS* is induced during late-exponential phase (Lange and Hengge-Aronis, 1991; Mulvey *et al.*, 1990; Schellhorn and Stones, 1992) , if it affects *dsbA* expression, one would expect to see this affect at the onset of stationary phase. From the results presented in Figure 15, it appears that RpoS does not regulate *dsbA* expression. Had RpoS been directly involved in regulating *dsbA* expression, then almost no induction of β-galactosidase activity at the onset of stationary phase should have been observed in the *rpoS* null strain. The question as to why expression of *dsbA* is increased during stationary phase still remains unanswered.

Recently, a transcriptional regulator, SlyA, was identified in *S. typhimurium* which is induced in stationary phase, is not dependent on RpoS, and may control the expression of genes required to adapt to the environment in the infected host, such as those required for resistance to oxidative stress (Buchmeier *et al.*, 1997). If certain proteins involved in the oxidative stress response pathway or other pathways regulated by SlyA require disulfide bonds, then the possibility exists that SlyA acts on this *dsbA* promoter. When activity of the *dsbA*::*lacZ* transcriptional fusion was assayed in a *slyA* null mutant (also created in *S. typhimurium* 14028s), induction of *dsbA* expression at the onset of stationary phase in the *slyA* null strain was still observed, and overall expression was only 200-300 units lower in the mutant than in wild type *S. typhimurium* 14028s (Figure 16). This value falls in the range of assay to assay variability. Even though this is only a preliminary result, there does not appear to be any significant difference in expression of *dsbA* between the *slyA* mutant and wild type. If SlyA was the key transcriptional regulator of *dsbA* expression, the high β-galactosidase activity seen during

stationary phase should not have been observed in the *slyA* null strain. SlyA and RpoS are not the only regulators of gene expression during stationary phase. Cyclic AMP levels have been shown to increase during stationary phase. These increases in cAMP levels have also been shown to increase expression of certain genes at the onset of stationary phase. One example of a gene regulated in response to cAMP levels is *cstA* which is involved in the utilization of peptides as carbon and energy sources (Schultz and Matin, 1991; Goodrich-Blair *et al.*, 1996). Future studies could look the affect of cAMP levels on the expression of *dsbA*.

To gain insight into other factors which may affect *dsbA* expression, β-galactosidase activity from pMEG2 was measured in 1) a *S. typhimurium dsbA* null strain, 2) an *E. coli* MC4100 strain, 3) a static LB culture, and 4) an acid stressed culture. In a *dsbA* null strain, if there is feedback regulation, one would expect to see an increase in β-galactosidase activity, corresponding to an increase in *dsbA* expression. For example, the lack of disulfide bond forming ability provided by DsbA in the periplasm could activate a signalling system which in turn stimulates the *dsbA* promoter to increase transcription. On the other hand, if excess DsbA has a repressive effect on *dsbA* transcription in the wild type strain, then in the absence of DsbA, β-galactosidase activity from pMEG2 could increase due to the lack of this repression. The results show that during exponential growth, the activity from pMEG2 in the wild type and null strains is almost identical (Figure 17). Upon entering into stationary phase, induction of *dsbA* is also seen in the null strain, however it is 600-700 units greater than in the wild type. Even after 24 hours, it remains greater than in the wild type strain (Table 4). Obviously, some type of regulatory mechanism must be stimulating this increase in *dsbA* expression.

However, from these results it cannot be determined whether it is the lack of DsbA activity or the lack of repression by DsbA activity which causes this increase. In addition, since the increase in the level of *dsbA* expression in a *dsbA* null strain only becomes obvious in stationary phase, it supports the idea that there appears to be a distinct need for DsbA during stationary phase.

Exciting observations were made when *dsbA* expression was measured from pMEG2 in an *E. coli* strain. Unlike in *S. typhimurium*, in *E. coli*, growth phase dependent expression of *dsbA* was not observed (Figure 18). Rather, β-galactosidase activity increased more gradually over the course of the experiment. Furthermore, the same level of β-galactosidase activity was not attained in the *E. coli* strain. This discovery seems to indicate that a different regulatory mechanism for *dsbA* transcription exists in *E. coli*, and it is less efficient at recognizing the *S. typhimurium dsbA* promoter. This is not surprising when one considers how different the two proximal promoter regions are (Figure 5). The findings from this experiment suggest that the regulation of expression of *dsbA* in *S. typhimurium* may be different than in *E. coli*. However, given that the β-galactosidase activity after 24 hours was similar when experimental variation is taken into consideration, the requirement for *dsbA* during stationary phase is conserved between the organisms.

When *S. typhimurium* causes an infection in a host, it travels through the gastro-intestinal tract where it can encounter drastic changes in pH, from lower than pH 2 in the stomach to pH 7 or above in the distal portion of the small intestine (Moffett *et al.*, 1993). Once in the small intestine, it penetrates the epithelial layer and can subsequently be ingested by macrophages (Finlay and Falkow, 1989). Inside a macrophage, the

organism must be able to resist both acid and oxidative stress if it wants to survive. Also, outside of the host this organism may confront acid stress in the form of industrial waste, acid mine drainage or in decaying organic matter (Bearson *et al.*, 1997). Acid stress and oxygen limitation, which occurs in both the host and in a heap of industrial sludge, can stimulate events that result in the synthesis of proteins required by the organism to survive under these less than ideal growth conditions (Ernst *et al.*, 1990; Miller *et al.*, 1989; Lee *et al*, 1995). To determine whether or not these two environmental factors could affect *dsbA* expression, β-galactosidase assays were performed with cultures grown under these conditions.

To approximate oxygen limiting conditions studies were performed with pMEG2 in *S. typhimurium* grown under static conditions. The growth of organisms in a static culture differs from in an aerated culture, as is evident when the two growth curves in Figure 19 are compared. During the first 3 hours after subculturing, the bacteria are in "pseudo-log" phase. Thereafter, their growth rate declines, and approximates stationary phase. The decrease in growth rate can be attributed to the fact that the oxygen in the media has started to become limiting. Under this condition, only a gradual increase in *dsbA* expression from the transcriptional fusion was observed (Figure 19). At 24 hours, activity had not increased above 500 units (Table 4). Thus, it appears that under oxygen limiting conditions there is no significant induction of *dsbA* expression. One reason for this might be that the cells in a static culture do not enter stationary phase for the same reason as aerated cultures. In static culture, cells enter stationary phase due to oxygen limitation, while in an aerated culture they enter stationary phase due to nutrient limitation. These two deprivation states may activate separate signalling pathways

leading to the synthesis of different proteins necessary for survival. It may be that when cells experience nutrient limitation, DsbA may be directly or indirectly required to assist in protein-folding of components in this stress response, whereas when oxygen is limiting DsbA is not required in large amounts. As will be discussed below, another more likely explanation for the decreased expression from this *dsbA* promoter under oxygen limiting conditions exists.

*S. typhimurium* has different acid tolerance responses (ATRs). During log phase growth, two types of ATRs can be mounted, transient or sustained (Lee *et al.*, 1995). In either case, the synthesis of a number of acid shock proteins is triggered when the bacteria are exposed to a pH around 4.5, and these proteins protect the cell when it is exposed to more acidic conditions (eg. pH 3.3) (Foster, 1993). If DsbA is directly or indirectly involved in the ATR, then one would expect that the transcription of *dsbA* would increase shortly after the cells were subcultured into minimal E glucose media pH 4.5. In terms of *dsbA* expression from pMEG2, as measured by β-galactosidase activity, one would expect to see a sharp increase in activity shortly after subculturing from minimal E glucose media pH 7.6 into minimal E glucose media pH 4.5, rather than at the onset of stationary phase. From Figure 20, it is clear that expression of *dsbA* from this promoter region was not stimulated by the decrease in pH. β-galactosidase activity increased only slightly after the cells were transferred to minimal E glucose media pH 4.5, and then decreased. Therefore, the regulation of expression of *dsbA* from this promoter is not affected upon activation of the log-phase acid tolerance response. Additionally, the stationary phase induction seen in minimal E glucose media pH 7.6 was not observed in minimal E glucose media pH 4.5. Thus, the regulatory mechanism acting

on this *dsbA* promoter in neutral pH, does not affect it under more acidic conditions. If a second promoter for *S. typhimurium dsbA* exists, as suggested by the Northern blots, it may be that the activity of this promoter is affected by acid stress.

Another approach which was used to complement the *dsbA::lacZ* transcriptional fusion assays was Western immunoblotting. Two different Western blots were done. The first compared DsbA levels during various stages of growth while the second compared DsbA levels in overnight static and aerated cultures (Figures 21 and 22, respectively). When approximately the same amount of protein is loaded for each sample, there appears to be no dramatic difference in the amount of DsbA present in the cells during the different stages of growth, or in a static versus aerated culture. These findings are not in agreement with the information obtained from the β-galactosidase assays performed using the *dsbA::lacZ* transcriptional fusion. The assays indicated that there was little *dsbA* expression in early log phase, and abundant expression in stationary phase. Similarly, in a static culture there was less *dsbA* expression, than in an aerated culture. A reason for this discrepancy could be that *S. typhimurium dsbA* does have two promoters, as indicated by the Northern blots. If two promoters exist, it is very likely that different regulatory mechanisms act on each promoter. Based on the information provided by the β-galactosidase assays, the promoter region which was cloned into pMEG2 seems to be growth phase regulated where maximal expression occurs in stationary phase, and less active under conditions of decreased oxygen concentration. If a second distal promoter located upstream of the one cloned into pMEG2 exists, it could be constitutively active or activated by factors which do not alter expression from the proximal promoter. By having two promoters for *dsbA*, the bacterium has finer control

over the level of DsbA present in the periplasm. According to the Western blots, it means *S. typhimurium* keeps DsbA levels constant.

In summary, this study has provided new and valuable insight into the regulation of the *S. typhimurium dsbA* expression. The Northern analyses have elucidated that there are two mRNA transcripts for *S. typhimurium dsbA*, and they differ in size by approximately 200 base pairs. These transcripts are present under the conditions assayed, including aerated and static LB cultures, minimal media, low pH and a *htrA* null *S. typhimurium* strain. This finding suggests that two promoters exist for *dsbA* in *S. typhimurium*. Studies using a plasmid-borne *dsbA*::*lacZ* transcriptional fusion have demonstrated that expression of *dsbA* from this putative proximal promoter is growth phase dependent, with maximum induction of expression occurring in stationary phase. This stationary phase expression of *dsbA*, however, is neither RpoS-dependent, nor is it regulated by the transcriptional regulator SlyA. In a *S. typhimurium dsbA* null strain the same growth phase dependent regulation is observed, while in an *E. coli* strain this induction of *dsbA* expression in stationary phase is less pronounced. Under acid stress and oxygen limiting conditions, no growth phase dependent regulation of *dsbA* expression is apparent. Western immunoblots of DsbA from different growth phases and from overnight aerated and static cultures show that steady-state DsbA levels remain relatively constant within the cell. This finding contradicts the results obtained with the *dsbA*::*lacZ* transcriptional fusion, however it does support the results from the Northern analyses which suggest the presence of two promoters. Some findings, such as the results obtained using the *dsbA*::*lacZ* transcriptional fusion under low pH and decreased oxygen

conditions seem to conflict considering there would be a physiological need for DsbA function under these conditions. However, the results from the Northern analyses suggest that there are two promoters. More work needs to be done to determine if this putative second promoter exists and how it is regulated. It may well be that under conditions when *dsbA* expression from the proximal promoter is low, expression from the second promoter may be high, thereby generating the increased levels of DsbA which one might expect under such stressful conditions.

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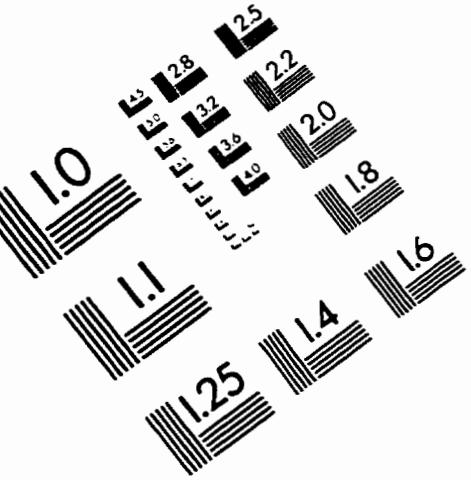
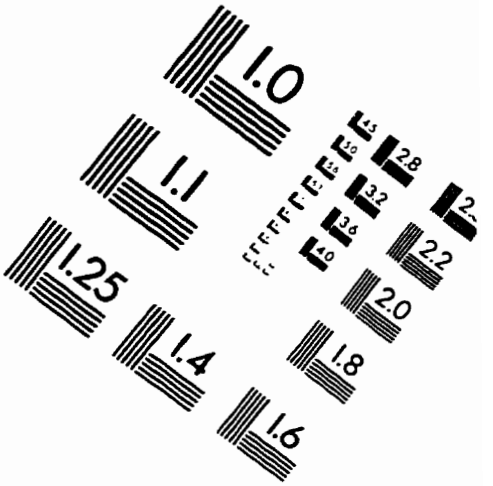
# Appendix I

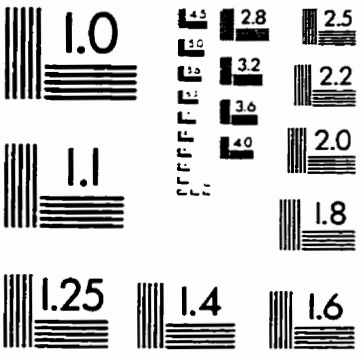
Since sequencing of pMEG1 has been unsuccessful, work on subcloning a segment of this plasmid has commenced. The new construct, which will be designated pMEG3, will not only be used for sequencing, but in the future will be used for creating a second transcriptional fusion as it contains sufficient DNA upstream of *dsbA* to contain a second promoter region.

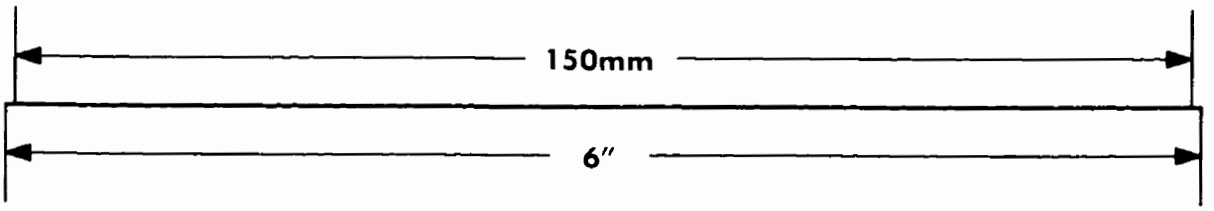
To construct pMEG3, the 1.3 kb *EcoR*V fragment of pMEG1 (Figure 7) will be cloned into the unique *Sma*I restriction site in pUC19. Since inserts into the multiple cloning site of this vector will disrupt the *lacZ* gene, blue/white screening can be used to determine whether or not a colony contains an insert in pUC19. Any white colonies will be mini-prepped, and the plasmid will be digested with *EcoR*I and *Hind*III to determine whether or not the plasmid contains the desired insert. Once pMEG3 has been constructed, it will be sequenced. The insert will then be incorporated into the low copy number transcriptional fusion vector pFZY1(R. Kelln, personal communication) and used to assay *dsbA* expression.

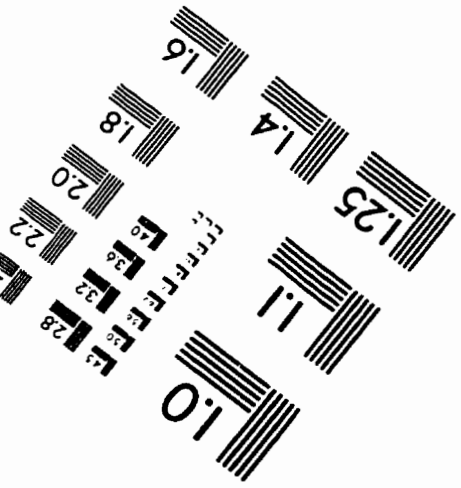
# IMAGE EVALUATION
# TEST TARGET (QA-3)

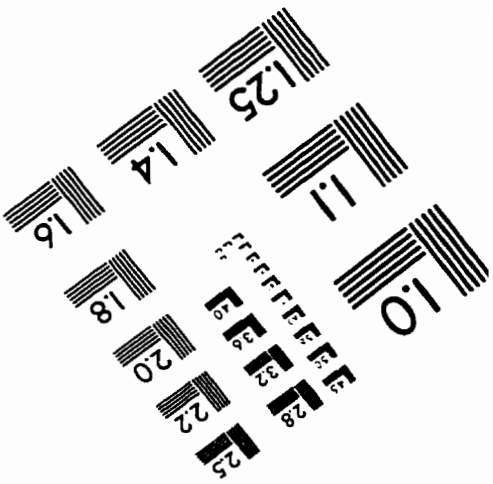
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