THE *E. COLI* RpoS REGULON: IDENTIFICATION AND REGULATION

IDENTIFICATION OF NOVEL MEMBERS OF THE RpoS REGULON IN *ESCHERICHIA COLI*: THE ALTERNATIVE SIGMA FACTOR, RpoS IS REGULATED AT THE TRANSCRIPTIONAL LEVEL BY BarA, A MEMBER OF THE FAMILY OF TWO-COMPONENT RESPONSE REGULATORS

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Abstract

The stationary phase-specific expression of many genes in free living bacteria such as *Escherichia coli* is controlled at the level of transcription by the alternative sigma factor RpoS encoded by the *rpoS* gene. This central regulator of *E. coli*'s stationary phase regulon (and several stress response regulons) is known to be required for the induction of over 30 proteins in stationary phase cultures and proteins induced in response to environmental stresses such as carbon starvation or osmotic upshift. To date, several RpoS-dependent genes have been identified in the literature. However, since no single inducer exists to which all members of the regulon respond, identifying RpoS-dependent genes based on phenotypic screening (e.g. carbon starvation inducible genes) methods may not provide us with a complete enumeration of the regulon. The present study is a continuation of previous work done with a previously-generated bank of 5,000 promoter-*lacZ* operon fusion mutants which were screened for RpoS-dependence by introducing an *rpoS* null allele into these strains and scoring for reduced β-galactosidase activity. The identities of several of these RpoS-dependent promoter-*lacZ* fusions were determined by DNA sequencing and subsequent sequence analysis using the BLAST algorithm. The RpoS- and growth-phase-dependence of several of the genes identified in this study was verified by Northern blot analysis. The genes identified here fall in into three groups: (*i*) genes previously shown to be RpoS-dependent; (*ii*) genes of known function that were

not previously known to be RpoS-dependent; and (*iii*) ORFs not previously known to be RpoS-dependent.

Expression and activity of RpoS itself is subject to regulation that occurs at the levels of transcription, translation, and protein stability. How these different levels of control interact to affect the activation of RpoS and the RpoS regulon is only partially elucidated. This study identifies BarA as the first two-component transcriptional regulator required for the activity of *rpoS* and provides evidence that signal(s) may be present in exponentially growing cultures that lead to early exponential phase stimulation of *rpoS* and subsequently, the RpoS regulon. An *E. coli* strain with a mutation in *barA* exhibits a hydrogen peroxide sensitive phenotype resulting from reduced levels of HPI and HPII catalase (which is under the control of RpoS). The reduction in HPII activity is a result of a reduction in the levels of *katE* message (encoding HPII) in a *barA* strain. Western blots probed with anti-RpoS antisera and Northern blots probed with an *rpoS*-specific probe demonstrate that this deficiency for the HPII catalase is caused by a decrease in the levels of the regulator, RpoS, present in the *barA* strain. Northern analysis and promoter-*lacZ* fusion expression data provide evidence for a model of early exponential phase expression of the RpoS regulon. Signal(s) responsible for this induction may be present in early exponential phase cultures and may ultimately lead to RpoS-dependent gene expression in stationary phase.

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List of Abbreviations

^{o}C	degrees Celsius
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleotide-5'- triphosphate
λ	lambda
mg	milligram
ml	millilitre
mM	millimolar
mRNA	messenger RNA
nm	nanometre
OD_{600}	optical density at 600 nm
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
% w/v	percent weight per volume
P.F.U.	Plaque Forming Unit
RNA	ribonucleic acid
rpm	revolutions per minute
µg	microgram
µl	microlitre
UV	ultra violet

CHAPTER 1: INTRODUCTION

1.1 Overview

Free-living, Gram negative bacteria such as *Escherichia coli* and *Salmonella typhimurium* cultured in rich media, will grow exponentially until such time that factors such as cell density, nutrient limitation, or build-up of toxic secondary metabolites limits further growth. The transition into this period of slow/no growth is referred to as the stationary phase (Siegele and Kolter, 1992). Up until the beginning of this decade, there had been a historical bias towards studying genes expressed in exponentially growing cultures. However, the study of genes expressed in stationary phase and their regulation has come more to the forefront in recent years. Two-dimensional gel electrophoresis studies (McCann et al., 1991) have shown that as many as 30 proteins are induced as an exponentially growing culture enters stationary phase including BolA (Lange and Hengge-Aronis, 1991a) which is involved in changes cellular morphology to a more condensed, spherical shape; the catalase HPII (Loewen et al., 1985) which decomposes hydrogen peroxide into oxygen and water; and Dps (Almiron et al., 1992), a DNA binding protein that may protect the cell from DNA damage (for a review of other stationary phase-induced genes see (Loewen and Hengge-Aronis, 1994)). Induction of these genes may help to impart cellular resistance to environmental stresses and may increase the viability and longevity of the culture. The central regulator of this stationary

phase regulon is a sigma factor known as σ^S, σ^{38}, or RpoS, encoded by the *rpoS* gene (Lange and Hengge-Aronis, 1991b; Mulvey and Loewen, 1989). This gene was originally isolated and characterized as several different mutations that rendered cells sensitive to various environmental stresses such as: (*i*) *nur* (Tuveson, 1981) which was isolated as a mutation that confers near-UV sensitivity to strains; (*ii*) *katF* (Loewen and Triggs, 1984) which is required for catalase activity and rendered cells sensitive to oxidative stress; (*iii*) *appR* (Touati E, 1986), required for acid phosphatase activity; and *csi*-2 (Lange and Hengge-Aronis, 1991b) which renders cells sensitive to carbon starvation. RpoS has also been shown to be a required virulence factor in *Salmonella* (Fang et al., 1992). RpoS shows high homology to the vegetative sigma factor RpoD which led to its postulated role as an alternative sigma factor (Mulvey and Loewen, 1989). However, unlike the promoter sequence determinants of RpoD and other, better characterized sigma factors like RpoN, the RpoS consensus promoter sequence remains poorly understood. This regulator (hereto referred to as RpoS) is required for the induction of many genes expressed in stationary phase (for a review see (Loewen and Hengge-Aronis, 1994)), however, it is believed that the RpoS regulon has yet to be entirely delineated. Also, it is still not entirely known what role this regulon plays in stationary phase cultures since it does not seem to have a single unifying characteristic. It is a widely held view that RpoS may be a global stress response regulator in *E. coli* (for a review see (Hengge-Aronis, 1996)) as opposed to a stationary phase-specific regulator. Regardless of this, study of the RpoS regulon is important because discovery of and research into novel RpoS regulated genes, their regulation, and the regulation of RpoS itself could be a potential

source of new industrial products or possible courses for treating bacterial infections in the future.

1.2 Identification of Novel Members of the RpoS Regulon

1.2.1 Carbon starvation inducible genes

Since different environmental stresses/signals are putative inducers of members of the RpoS regulon, there have been several different screening techniques employed to identify and characterize new members of this stationary phase regulon based on a response to one of these signals. Weichart *et al.* (1993) used an identification screen based on carbon starvation induction. A bank of λp*lac*Mu promoter-*lacZ* transcriptional and translational fusion mutants were grown on minimal media plates containing the indicator dye X-gal and expression was compared at low (0.02 or 0.04%) and high (0.4%) concentrations of glucose. All fusions that showed higher levels of induction on the 'starvation' plates were selected and characterized (Weichart et al., 1993). Several of the RpoS-dependent genes identified in this study included *csi*-5 which mapped to *osmY* (encodes a putative periplasmic protein of unknown function), *csi*-17 which mapped to *glpD* (encodes aerobic glycerol-3-phosphate dehydrogenase), and *csi*-18 which mapped to *glgA* (encodes aerobic glycogen synthase) (Weichart et al., 1993). This method, while being very thorough, was not exhaustive in identifying all of the members of the RpoS regulon.

1.2.2 Identification of RpoS-dependent Salmonella typhimurium *genes*

Similar work to characterize members of the RpoS regulon in *S. typhimurium* was carried out using a MudJ transposon library with the expression of the *rpoS* gene under

the control of an arabinose inducible promoter (Fang et al., 1996). By replica-plating strains carrying the MudJ transcriptional-*lacZ* fusion and *rpoS* under an inducible promoter onto media +/- 0.1% arabinose and scoring for increased β-galactosidase activity, RpoS dependent fusions were identified and characterized by DNA sequencing (Fang et al., 1996). The only previously identified RpoS-dependent gene isolated in this work was the *otsA* gene, all of the others mapped to ORFs of unknown function, some of which show homology to proteins of known function (Fang et al., 1996). The paucity of previously identified RpoS-dependent genes isolated by this screening method may indicate that the RpoS regulon is probably larger in size that originally thought (McCann et al., 1991).

1.2.3 Identification of RpoS-dependent genes based on promoter searches

Another novel screen for identifying RpoS-dependent genes is based on cloning putative promoter regions into an expression vector and testing for activity of the reporter *lacZ* gene in *rpoS*$^+$ and *rpoS* strains (Talukder et al., 1996). This study examined a previously-constructed promoter library for increased β-galactosidase activity upon entry into stationary phase (Talukder et al., 1996), these clones were then tested for RpoS-dependence upon introduction into an *rpoS* background. The only gene identified in this study that was positively regulated by RpoS was of unknown function, the other five genes were all negatively regulated by RpoS (Talukder et al., 1996). A potential criticism of a study of this nature is the fact that since the true nature of the promoter element recognized by holoenzyme (E) complexed with σ^S is only poorly understood, the likelihood of a standard promoter library containing true RpoS-recognized promoter is

probably low.

1.2.4 RpoS-dependent genes induced by internal acidification

Transcriptional fusions to *lacZ* have not been the sole reporter gene fusion used in the identification of RpoS-dependent genes, Van Dyk *et al.* (1998) utilized a promoter-less expression vector system that expressed the *Photorhabdus luminescens luxCDABE* operon and screened for increases in bioluminescence. This type of expression system is amenable to studying gene expression because it can be done in real time without having to lyse the cells (Van Dyk et al., 1998) as is required for determination of β-galactosidase activity in whole cells. This screen originally attempted to identify genes that are induced when acetolactate synthase (ALS, the first common enzyme in isoleucine and valine biosynthesis) are inhibited by the herbicide, sulfometuron methyl (SM). Restriction enzyme products of the *E. coli* chromosome were cloned into a *lux* expression system and transformed cultures were incubated in the presence of sublethal doses of SM (Van Dyk et al., 1998). Six of the genes identified in this study that were induced by SM were also found to be RpoS-dependent. There were two previously identified genes *osmY* and *poxB* and four novel RpoS-dependent genes *f253b*, *ldcC*, *yciG*, and *yohF* (Van Dyk et al., 1998). The constructs carrying these promoters were also induced in the presence of the weak acid salicylate and it was thus postulated that incubating cultures in the presence of sublethal doses of SM may decrease the internal pH of the cell (Van Dyk et al., 1998). This provides evidence that weak acids and/or internal acidification may be a signal for induction of RpoS-dependent proteins.

1.2.5 'DNA chip' technology

The advent of DNA sequencing and sequence analysis technology, has led to a concentrated effort in determining the sequences of entire organismal genomes. The entire sequence of the *E. coli* genome has been determined (Blattner et al., 1997) and has been used to develop new methods of determining differences in gene expression. In what is known as Panorama™ (Genosys Biotechnologies Inc. TX.), every *E. coli* ORF is represented on a membrane ('DNA chip'). Using this technology, cultures can be grown under varying conditions (e.g. an exponentially growing culture and a stationary phase culture) and total RNA can be extracted. These RNA pools can be used in RT-PCRs to create radiolabelled cDNA probes that can then be hybridized to the 'DNA chip'. If the 'chip' is then subjected to phosphoimaging analysis, determinations of which genes are expressed under which conditions will be readily determined. Use of this type of technology will likely make the task of defining entire regulons or stimulons a much easier one.

1.3 Co-regulators of RpoS-dependent Genes

There are several examples of RpoS-dependent genes that are subjected to differential regulation depending on the phase of growth or exposure to environmental stress that requires other regulatory factors. This idea is discussed below.

1.3.1 Co-regulation by the Leucine-Responsive Protein (Lrp)

The leucine-responsive protein (Lrp) encoded by the *lrp* gene is a DNA-binding protein that has been shown to act as a repressor or activator of gene expression (Calvo and Matthews, 1994). The expression of several RpoS-dependent genes has been shown

to be dependent on Lrp. Landini *et al* (1996) demonstrated Lrp-dependent repression of the RpoS-dependent *aidB* gene (*aidB* may be a homolog of a human enzyme, isovaryl-coenzyme A dehydrogenase, involved in leucine decomposition (Landini et al., 1994)). The activity of a transcriptional, promoter-*lacZ* fusion to the *aidB* gene was greater in an *lrp* mutant strain compared to an isogenic wild type strain (Landini et al., 1996), indicating that Lrp is a specific repressor of *aidB* transcription. This result was verified using an *in vitro* transcription assay which demonstrated that *aidB* transcription by holoenzyme complexed with σ^S is severely inhibited in the presence of the Lrp protein (Landini et al., 1996). Lrp-dependent regulation of several genes can either be enhanced or inhibited in the presence of the amino acid leucine (Calvo and Matthews, 1994). A transcriptional promoter-*lacZ* fusion to *aidB* increases in activity when cultures are grown in the presence of increasing concentrations of leucine (Landini et al., 1996) indicating that accumulation of leucine may relieve Lrp-mediated repression of *aidB*. Finally, DNaseI protection and gel retardation assays in which Lrp was incubated with DNA containing the putative *aidB* promoter region confirmed Lrp binding to this region (Landini et al., 1996) which likely leads to inhibition of expression.

The osmotically induced, RpoS-dependent *osmY* (Lange et al., 1993; Yim and Villarejo, 1992) and *osmC* (Bouvier et al., 1998) genes are also co-regulated by Lrp. *osmC* transcription is driven from two overlapping promoters, $osmC_{p1}$ and $osmC_{p2}$, (Bouvier et al., 1998) of which only $osmC_{p2}$ is RpoS-dependent. Gel retardation assays using $osmC_{p2}$ DNA as the binding substrate demonstrated that cell extracts from *lrp* wild type and mutant strains display different binding profiles (Bouvier et al., 1998). When

these gel shift assays are performed in the presence of leucine, Lrp binding efficiency decreases (Bouvier et al., 1998). Point mutations that allow for the activity of each promoter to be assayed independently, used in conjunction with promoter-*lacZ* fusions, demonstrated that strains carrying an *lrp* mutation, exhibited increased $osmC_{p2}$ induction during exponential growth and that stationary phase induction is greatly diminished (Bouvier et al., 1998). This result indicated that Lrp may be required to repress expression of $osmC_{p2}$ during exponential growth (Bouvier et al., 1998) and is required for expression in stationary phase. To test if the observed increase in $osmC_{p2}$::*lacZ* activity is a result of an Lrp-dependent increase in RpoS levels, RpoS accumulation was assayed by immunoblot analysis in wild type and mutant *lrp* strains (Bouvier et al., 1998). RpoS levels were increased in all phases of growth, however, the activity of an *rpoS::lacZ* transcriptional fusion was unaffected, indicating that the increase in RpoS levels is due to a post-transcriptional affect such as increased protein stability (Bouvier et al., 1998). Bouvier *et al* (1998) speculate that the Lrp affect on the $osmC_{p2}$ promoter is likely due to a direct affect on the promoter even though RpoS levels are increased since the overall expression is lower in stationary phase in the *lrp* mutant background.

Induction of the RpoS-dependent *osmY* gene as cells enter stationary phase is postulated to be dependent on positive activation by RpoS and alleviation of repression by Lrp (Lange et al., 1993). In rich media, introduction of an *lrp::Tn*10 resulted in an increase in expression of an *osmY::lacZ* transcriptional fusion during the transition from mid-exponential to stationary phase (Lange et al., 1993). In minimal media, an *lrp* mutation causes a five-fold increase in *osmY* expression during exponential growth

(Lange et al., 1993) indicating that Lrp prevents *osmY* from being expressed until the culture enters stationary phase. Together, these results demonstrate that expression of the RpoS regulon is complex and involves different factors which can repress or activate expression in concert with RpoS.

The *csiD* gene is interesting to note since its expression is solely dependent on RpoS and is only expressed when cells are carbon-starved (Marschall et al., 1998). When an *lrp::Tn*10 mutation is introduced into a strain harboring a *csiD::lacZ* transcriptional fusion there was about a 20 % decrease in activity compared to a wild type strain (Marschall et al., 1998). Further, the addition of leucine to the growth media resulted in a slight induction of the *csiD::lacZ* fusion in stationary phase cultures (Marschall et al., 1998) indicating that Lrp is a positive activator of *csiD* expression and activation is enhanced in the presence of leucine. DNaseI protection assays provided evidence that Lrp is able to bind several different sites upstream of the *csiD* transcriptional start site (Marschall et al., 1998).

1.3.2 Co-regulation by the cyclic AMP-CRP complex

Bacteria such as *E. coli* posses several different metabolic pathways to produce energy in the form of ATP from a variety of carbon sources. The cAMP-CRP complex is a key regulatory complex which is involved in a phenomenon known as catabolite repression. Catabolite repression involves the inhibition of many different enzymes that control the expression of the genes that control these various metabolic pathways when a culture is grown in media containing a carbon source such as glucose (reviewed in (Brock and Madigan, 1996)). When a bacterial culture experiences starvation, (i.e. the primary

carbon source (glucose) is exhausted) intracellular cAMP levels produced by adenylate cyclase (coded for by the *cya* gene) increase. When cAMP is complexed with the cAMP regulatory protein (CRP, coded by the *crp* gene) it will act as a transcriptional activator or repressor of a variety of genes. In the case of catabolite repression, this complex can stimulate or derepress the transcription of genes that will allow the cell to utilize other carbon sources. Since several RpoS-dependent genes are carbon starvation inducible, it seems logical that some of these RpoS-dependent genes may be regulated by the cAMP-CRP complex. Lange and Hengge-Aronis (1991) demonstrated that the RpoS-dependent, carbon starvation inducible gene *bolA* is negatively regulated by cAMP-CRP (Lange and Hengge-Aronis, 1991a). Utilizing a *bolA::lacZ* transcriptional gene fusion, Lange and Hengge-Aronis (1991) were able to demonstrate that the addition of exogenous cAMP to a growing *E. coli* culture results in a decrease in *bolA* transcription. Further, a *bolA::lacZ* fusion exhibited higher activity in strains that also carry a mutation in the *cya* gene (Lange and Hengge-Aronis, 1991a) encoding the enzyme adenylate cyclase which produces cAMP. The original screen for carbon starvation inducible genes performed by Weichart *et al* (1993) was done in a *cya* mutant background in an attempt to select for starvation inducible genes other than those that require cAMP-CRP for activation or repression. These workers then incubated their identified *csi* genes in media supplemented with cAMP to assess the affects. Promoter-*lacZ* transcriptional fusions to *csi*-12 and *csi*-16 showed increased activity after the addition of cAMP, however, the affect was not immediate. In fact, the increase in activity did not occur until after the cultures had entered stationary phase indicating that the cAMP-CRP complex may be

responsible for stationary phase-dependent, co-regulation of these RpoS-dependent genes (Weichart et al., 1993). Further study of *csi*-16 (*csiE*) (Marschall and Hengge-Aronis, 1995) revealed that expression of a *csiE::lacZ* fusion is only partially RpoS-dependent. In minimal and LB media, mutations in *cya*, *crp*, or *rpoS* result in significant reduction in *csiE::lacZ* expression during entry into stationary phase (Marschall and Hengge-Aronis, 1995). An *rpoSΔcya* double mutant exhibited almost no expression of *csiE* reiterating the idea that both RpoS and cAMP-CRP positively regulate *csiE* (Marschall and Hengge-Aronis, 1995). Interestingly, addition of exogenous cAMP to the growth media of the *rpoSΔcya* mutant resulted in an almost immediate induction of a *csiE::lacZ* fusion in exponential and early stationary phase cultures (although not to the levels of an *rpoS* wild type strain) (Marschall and Hengge-Aronis, 1995) supporting the authors' claim that *csiE* is only partially RpoS-dependent.

Another RpoS-dependent gene isolated in the original screen by Weichart *et al* (1993), *csiD*, has been recently mapped to an *E. coli* ORF *o360* (Marschall et al., 1998). As previously mentioned, *csiD* is solely dependent on RpoS for expression and is only induced under conditions of carbon starvation (Marschall et al., 1998). *In vivo* studies using *lac* reporter fusions to the *csiD* gene indicate that *csiD* is highly expressed when cells are carbon starved (Marschall et al., 1998). Further, introduction of an *rpoS* null mutation results in almost complete abolishment of expression (Marschall et al., 1998). Introduction of *cya* or *crp* mutations led to further reductions in activity (Marschall et al., 1998). Gel retardation assays demonstrated that in the presence of cAMP, CRP bound DNA containing the *csiD* promoter region (Marschall et al., 1998). DNaseI protection

assays confirmed CRP binding to the DNA upstream of the transcriptional start site and localized binding to be centered 68.5 nucleotides upstream of the transcriptional start site (Marschall et al., 1998).

Promoter-*lacZ* fusions to two other genes, *osmY* (*csi*-5) and *csi*-32 showed a reduction in activity when cultures were incubated in the presence of exogenous cAMP (Weichart et al., 1993) demonstrating that several RpoS-dependent genes are activated or repressed by this complex. Subsequent experiments involving the introduction of a *cya* mutation into a strain harboring an *osmY::lacZ* transcriptional fusion resulted in an increase in expression in exponential phase in minimal media that is abolished upon the addition of exogenous cAMP to the media (Lange et al., 1993). Since rich media such as LB may contain traces of cAMP that may complement a *cya* mutant, these workers introduced a *cya crp* double mutation into a strain carrying an *osmY::lacZ* fusion (Lange et al., 1993). This mutation resulted in early induction of *osmY*, however, the absolute levels of activity remained unchanged compared to an isogenic wild type strain indicating that the cAMP-CRP complex may play a role in determining timing of induction of *osmY* during entry into stationary phase (Lange et al., 1993). Since previous studies revealed a potential role for the cAMP-CRP complex in *rpoS* regulation, expression of an *osmY::lacZ* fusion was examined in an *rpoS cya* double mutant (Lange et al., 1993). Expression was still induced in early exponential phase (although not to the same magnitude as in the wild type strain) and expression could be reduced upon the subsequent addition of exogenous cAMP, indicating that cAMP-CRP may be having an RpoS-independent affect on *osmY* (Lange et al., 1993). These experiments illustrate the

complex levels of regulation to which the RpoS regulon is subjected.

Another RpoS-dependent gene, *aldB*, encoding an aldehyde dehydrogenase, has been demonstrated to be cAMP-CRP activated (Xu and Johnson, 1995a). Expression of an *aldB::lacZ* transcriptional fusion is reduced about twenty-fold in a *crp* (encoding the CRP protein) mutant relative to a wild type strain (Xu and Johnson, 1995a). Further, DNaseI footprinting assays demonstrated that CRP binds to DNA that contains the *aldB* promoter region (Xu and Johnson, 1995a) confirming its role as a potential transcriptional activator.

Several RpoS-dependent *S. typhimurium* genes are postulated to be cAMP-CRP dependent. MudJ transcriptional fusions highly dependent on *rpoS* for expression were tested in a *crp* mutant strain (Fang et al., 1996). All of the fusions identified in this study except for the *otsA::lacZ* fusion were derepressed in a *crp* mutant in exponential phase cultures (Fang et al., 1996). The exponential phase expression of these fusions in the *crp* mutant strains were comparable to the stationary phase activity in the wild type strain (Fang et al., 1996). These studies demonstrate that CRP represses the exponential phase expression of several RpoS-dependent *S. typhimurium* genes (Fang et al., 1996).

1.3.3 Co-regulation by the histone-like nucleoid protein (H-NS)

The histone-like nucleoid protein H-NS (encoded by the *hns* gene) is thought to bind DNA and can act to repress expression of many different genes including *rpoS* (Barth et al., 1995; Yamashino et al., 1995) (to be discussed). The affect of an *hns* mutation on the expression of the two previously-discussed *osmC* promoters has been studied using the afore mentioned promoter-*lacZ* fusions that can measure expression

driven from each of the promoters independently (Bouvier et al., 1998). The *hns* mutant displayed increased activity of both an $osmC_{p1}$::*lacZ* and $osmC_{p2}$::*lacZ* fusions relative to the wild type strain (Bouvier et al., 1998). Thus, H-NS is a putative negative regulator of both *osmC* promoter elements, however, this repression must occur via two distinct mechanisms since both promoters are subject to differential regulation. Since an *hns* mutant has increased levels of RpoS protein (Barth et al., 1995; Yamashino et al., 1995), the positive affect on $osmC_{p2}$ may be due to this increase (Bouvier et al., 1998). However, since $osmC_{p1}$ is largely RpoS-independent, the H-NS-mediated repression must occur in some other manner (Bouvier et al., 1998). The question of how exactly these two promoters are repressed H-NS remains to be elucidated.

1.3.4 Co-regulation by Crl

It has been recently shown that the product of the *crl* gene (coding for the Crl protein), which was originally thought to encode the curlin subunit of the cell surface structures known as curli which are formed in stationary phase cultures during periods of low temperature or osmolarity (Olsen et al., 1993), is required for the positive activation of RpoS in stationary phase (Pratt and Silhavy, 1998). In an initial screen based on alleviation of OmpR-mediated repression of an *ompF*::*lacZ* fusion (repression is RpoS-dependent) to identify factors involved in RpoS regulation (Pratt and Silhavy, 1996), mutations in the *crl* gene were identified (Pratt and Silhavy, 1998). These *crl* mutations were then tested on other RpoS-dependent promoter-*lacZ* fusions. Introduction of this mutant *crl* gene resulted in a moderate reduction of expression of several RpoS-dependent genes including *katE* and *bolA* (Pratt and Silhavy, 1998). Strains harboring

both *crl* and *rpoS* mutations showed no cumulative affect on the RpoS-dependent *katE::lacZ* fusion suggesting that Crl and RpoS function in the same genetic pathway (Pratt and Silhavy, 1998). In reference to the mode of action of Crl, the authors state the Crl protein shows no homology to any DNA-binding proteins nor does it appear to complex with RpoS as determined by gel shift assays (Pratt and Silhavy, 1998). The manner in which Crl stimulates expression of RpoS-dependent genes is thus unclear.

1.3.5 Co-regulation by Fis

Fis (encoded by the *fis* gene) is a small DNA-binding protein which was originally isolated in studies on site-specific DNA inversion reactions. Expression of *fis* increases in response to increases in nutrient concentrations (Ball et al., 1992) and Fis levels are low in mid exponential and stationary phase cultures. An initial screen for Fis repressed promoter-*lacZ* fusions led to the identification of some novel RpoS-dependent genes when these Fis repressed functions were subsequently tested for RpoS-dependence (Xu and Johnson, 1995c). The expression of RpoS-dependent *aldB::lacZ* (encoding the previously mentioned aldehyde dehydrogenase) *frg*-502*::lacZ*, and *frg*-734*::lacZ* fusions are reduced when *fis* is over-expressed from an IPTG-inducible vector (Xu and Johnson, 1995c). DNaseI footprinting assays demonstrated that Fis will bind the *aldB* promoter region (Xu and Johnson, 1995a) and this likely leads to repression during exponential growth.

The RpoS-dependent *proP* gene which codes for a transporter of the osmoprotectants proline and glycine betaine is positively-regulated by Fis (Xu and Johnson, 1995b). *proP* transcription is driven from two promoters, $proP_{p1}$ and $proP_{p2}$

which is RpoS-dependent (Xu and Johnson, 1995b). Over-expression of *fis* from an inducible plasmid resulted in a greater than eight-fold induction of a *proP::lacZ* gene fusion (Xu and Johnson, 1995b). Gel shift and DNaseI footprinting assays confirmed Fis binding to the *proP* promoter region (Xu and Johnson, 1995b) indicating that binding of Fis may lead to activation of *proP* expression.

All of these results, taken *in toto*, demonstrate a complex pattern of regulation of the RpoS regulon that is not merely as simple as-- RpoS-dependent genes are expressed when cells enter stationary phase-- but that expression of the RpoS regulon involves complex repression during exponential phase and subsequent activation upon entry into stationary phase or upon exposure to various forms of environmental stimuli.

1.4 RpoS Promoter Sequence Determinants

Even though many RpoS-dependent genes have been identified to date by several different groups of researchers, there is still no definitive data elucidating what constitutes the consensus RpoS-recognized promoter elements. One possibility that could explain this paucity of information is the fact that many of the RpoS-dependent genes identified in other studies are not that highly dependent on RpoS. Screens (such as the one presented in this work) that identify a group of highly RpoS-dependent genes may be a better set to work with to determine the consensus sequence. Work involving error prone PCR to study affinity binding of $E\sigma^S$ to different consensus sequences may be able to provide some information to help solve this problem. Subjecting sequences of DNA that exhibit preferential (*in vitro*) binding of $E\sigma^S$ over $E\sigma^D$ to several rounds of PCR and subsequently assaying for increased affinity for $E\sigma^S$ over $E\sigma^D$, could lead to the

identification of potential RpoS-recognized promoter sequences.

Many *E. coli* promoters identified to date can be separated into three groups: (*i*) those promoter recognized by $E\sigma^D$ only; (*ii*) those recognized preferentially by $E\sigma^S$; and (*iii*) promoters recognized by either $E\sigma^D$ or $E\sigma^S$ (Tanaka et al., 1995). Examples of these three types of promoters include the *alaS*, *fic*, and *lacUV5* promoters respectively.

1.4.1 The -10 region

One of the approaches taken to study this question involved using the above mentioned promoters to attempt to separate them into upstream elements (UE) including the -35 region and downstream elements (DE) consisting of the -10 region (Tanaka et al., 1995). The promoter sequences of *alaS*, *fic*, and *lacUV5* were amplified using PCR primers which would introduce specific restriction enzyme recognition sites. These promoter fragments were then digested and ligated to produce chimeric promoters (Tanaka et al., 1995). *In vitro* transcription assays using reconstituted $E\sigma^S$ and $E\sigma^D$ and these chimeric promoters containing various combinations of UE and DE from the three promoter classes determined that the DE from *fic* or *lacUV5* alone was enough to confer $E\sigma^S$ recognition (Tanaka et al., 1995). A potential problem akin to *in vitro* assays using reconstituted holoenzyme is that these systems may not contain other regulatory factors that may be involved in *in vivo* discrimination between RpoD- and RpoS-recognized promoters and that the discriminatory signals may not solely reside in the -10 region as eluded to by Tanaka *et al* (1995) and the affects of these other, ancillary binding factors may be mediated through interactions with RNA polymerase. However, the inability to generate mutants in the -10 region that were still able to drive RpoS-dependent

transcription (Utsumi et al., 1995) may indicate the significance of this region in conferring RpoS-dependence to a gene.

Other studies to determine -10 consensus elements have involved using computer-based searches (Espinosa-Urgel et al., 1996). Sequences upstream of the translational start site for thirty-three RpoS-dependent genes identified to date were subjected to an alignment algorithm to determine regions of sequence similarity (Espinosa-Urgel et al., 1996). The putative -10 consensus determined by this method was CTATACT with frequency of appearance scores assigned to each position (Espinosa-Urgel et al., 1996). The C in the first position appeared in 70% of the promoters studied (Espinosa-Urgel et al., 1996). The authors speculate that a method consisting of utilizing this consensus sequence to search the *E. coli* database may provide a means to identify novel RpoS-dependent genes (Espinosa-Urgel et al., 1996). This method was used to identify a homologous sequence in the *frd* gene, coding for fumarate reductase (Espinosa-Urgel et al., 1996). When a *rpoS::Tn*10 is introduced into a wild type strain that is subsequently assayed for fumarate reductase activity, the activity decreased confirming that this gene is in fact RpoS-dependent (Espinosa-Urgel et al., 1996). A potential pitfall in this type of analysis is the fact that some promoters that may show poor homology to the RpoS consensus may actually be RpoS-dependent in the presence of ancillary activating factors. Searching for promoter elements alone would most likely exclude this class of genes.

1.4.2 The -35 region

Although there is considerable evidence demonstrating the necessity of the -10 region, the identity of and even the requirement for the -35 region remains unclear and there are some conflicting ideas present in the literature. Sequence alignment analysis which determined a putative -10 consensus failed to find a consensus -35 element common to the 33 RpoS-dependent genes analyzed (Espinosa-Urgel et al., 1996). Instead, it has been suggested that intrinsic DNA curvature in the upstream element is required for RpoS-dependent promoter recognition (Espinosa-Urgel and Tormo, 1993). Computer analysis of this region predicts intrinsic bending of the DNA upstream of the -10 region (Espinosa-Urgel and Tormo, 1993; Espinosa-Urgel et al., 1996) in the promoter region of RpoS-dependent genes. DNA migration assays also provided evidence that the -35 region of RpoS-dependent promoters has intrinsic curvature. DNA with intrinsic curvature that is separated on polyacrylamide gels run at different temperatures will have different migration patterns, specifically, curved DNA moves more slowly at 4 ^{o}C and thus appear larger than expected. The same DNA separated at 60 ^{o}C will migrate closer to its expected size. These migration assays demonstrate that DNA containing putative RpoS-dependent promoter sequences do migrate as larger bands at 4 ^{o}C (Espinosa-Urgel and Tormo, 1993) indicating that intrinsic curvature may be required for RpoS-dependent promoter recognition. These studies confirmed computer-generated models which predicted curvature in the promoters of these genes.

Conflicting evidence exists in the literature that describes the necessity of certain nucleotides in the -35 region (Utsumi et al., 1995; Wise et al., 1996). Mutational analysis

of the RpoS-dependent *fic* promoter (Utsumi et al., 1995) illustrates this point. A *fic::lacZ* protein fusion vector was mutagenized by treatment with hydroxylamine, transformed into an *rpoS* mutant strain and scored for restoration of β-galactosidase activity (Utsumi et al., 1995). No mutants in the -10 region were recovered, again underscoring the importance of this region (Utsumi et al., 1995), however, mutants harboring point mutations in the -35 region were isolated (Utsumi et al., 1995). Two mutants, one containing a single C–>T substitution at position -34 and a second mutant containing two C–>T substitutions as positions -34 and -38 showed restored β-galactosidase activity in an *rpoS* mutant background (Utsumi et al., 1995). Transcription assays verified that $E\sigma^D$ could recognize the promoter as efficiently as $E\sigma^S$ *in vitro* (Utsumi et al., 1995). Other site-directed mutagenesis studies have also demonstrated the importance of nucleotides in the -35 region (Wise et al., 1996). Computer analysis revealed that there appears to be a conserved CCG sequence in the -35 region of several RpoS-dependent promoters (Wise et al., 1996). Using site directed mutagenesis, a TT pair was substituted in favor of a CC pair in an RpoD-recognized *proU* promoter. The resulting $proU_{CC}$ promoter was now expressed in stationary phase and was RpoS-dependent (Wise et al., 1996). Attempts to create an RpoD-recognized promoter by creating an $osmY_{TT}$ promoter did not result in abolition of RpoS-recognition although the activity of this promoter was much lower in stationary phase cultures (Wise et al., 1996). Further, an $osmY_{AA}$ promoter exhibited no activity indicating that the phenomenon of $E\sigma^S$ promoter recognition may encompass more than simple exclusion of $E\sigma^D$ from RpoS-dependent promoters (Wise et al., 1996). Perhaps recognition of RpoS-dependent

promoters requires a combination of a -10 site, a -35 site, and intrinsic DNA curvature. More work using newly identified and very highly RpoS-dependent genes may hold the answer to the question of what constitutes an RpoS-recognized promoter.

1.5 Regulation of RpoS Occurs at the Levels of Transcription, Translation, and Protein Stability

Regulation of *rpoS* is quite complex and occurs at many different levels (see Fig. 1 for an overview). Although many different factors have been implicated in *rpoS* regulation, the manner in which they interact to determine the expression pattern of this stationary phase regulator is only poorly understood. Initial studies to characterize expression of *rpoS* have been undertaken utilizing transcriptional and translational promoter-*lacZ* fusions to the *rpoS* gene (Lange and Hengge-Aronis, 1991b; Lange and Hengge-Aronis, 1994; Mulvey et al., 1990; Schellhorn and Stones, 1992) demonstrating that *rpoS* is maximally induced at the transcriptional level as cells enter stationary phase. Translational fusions have exhibited similar patterns of induction (Lange and Hengge-Aronis, 1994). The stability of the σ^S protein which is subject to rapid degradation in exponential phase (Yamashino et al., 1995) also increases in stationary phase cultures (Lange and Hengge-Aronis, 1994; Yamashino et al., 1995) from a half-life of about 1.4 minutes in exponential phase to about 16.5 minutes in stationary phase (Lange and Hengge-Aronis, 1994). The various factors and small molecules involved in this complex regulation are discussed below.

Figure 1. Regulation of RpoS occurs at the levels of transcription, translation and protein stability. Modified from Lange *et al.*, (1994). Regulatory factors denoted in blue represent those identified since the publication of the original figure. Regulatory factor denoted in red was identified in this study.

Legend:

acetyl-P: acetyl-phosphate

cAMP: cyclic AMP-cAMP Regulatory Protein complex

ClpXP: protease

DnaK: heat shock chaperonin

DsrA: small, untranslated RNA

HF-I: RNA-binding protein required for phage Qβ replication

H-NS: histone-like nucleoid DNA-binding protein

HSL: homoserine lactone

OxyS: small, untranslated RNA

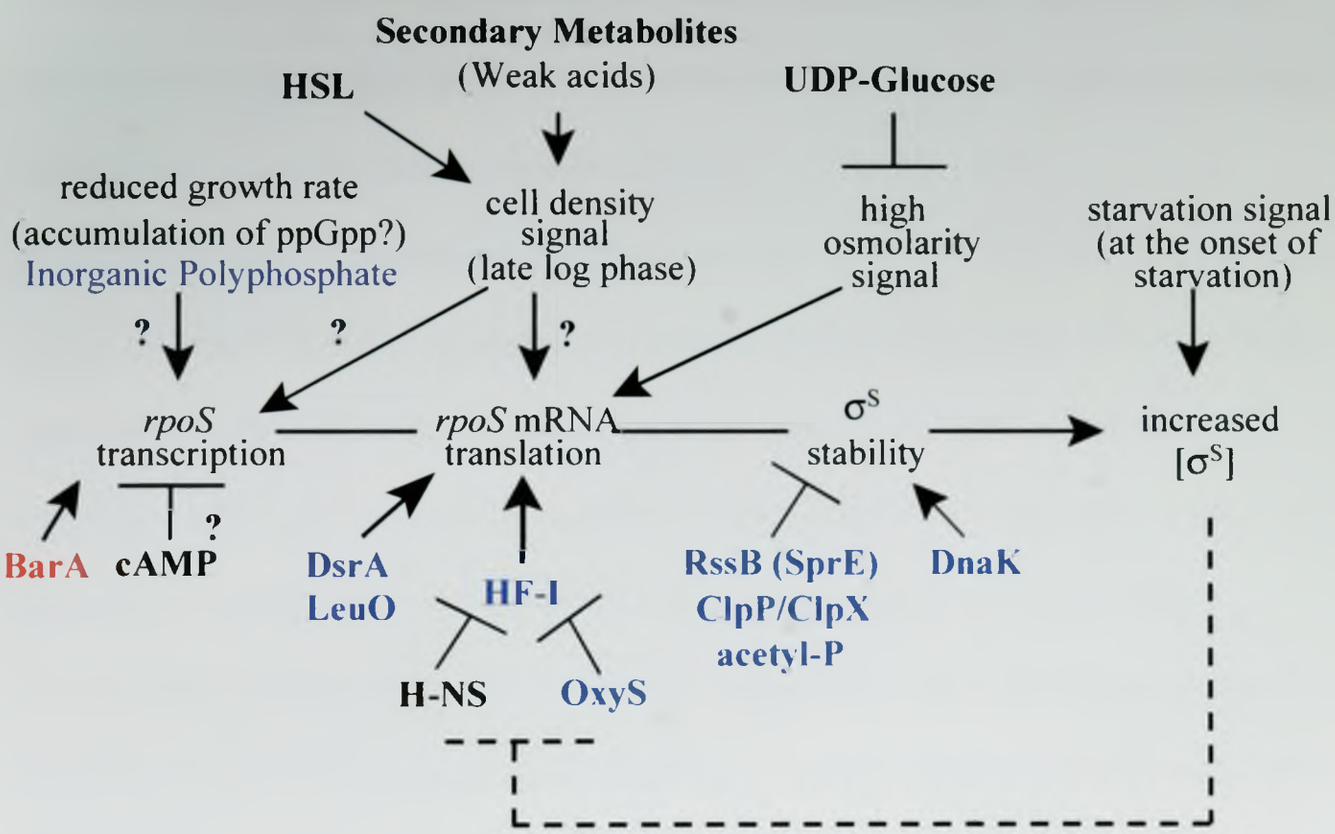
ppGpp: guanosine 3',5'-bispyrophosphate ("alarmone")

RssB (SprE): response regulator protein

UDP-glucose: precursor of the osmoprotectant trehalose

→ denotes positive activator

⊣ denotes repression


Secondary Metabolites
(Weak acids)
HSL
UDP-Glucose
reduced growth rate
(accumulation of ppGpp?)
Inorganic Polyphosphate
cell density
signal
(late log phase)
high
osmolarity
signal
starvation signal
(at the onset of
starvation)
?
?
?
rpoS
transcription
rpoS mRNA
translation
σS
stability
increased
[σS]
?
BarA
cAMP
DsrA
LeuO
HF-I
H-NS
OxyS
RssB (SprE)
ClpP/ClpX
acetyl-P
DnaK

1.5.1 Transcriptional control of rpoS

Several small signaling molecules are postulated to play a role in transcriptional regulation of *rpoS*. Potential inducers include guanosine 3',5'-bispyrophosphate (also known as alarmone and ppGpp) (Gentry et al., 1993; Lange et al., 1995), weak acids (Schellhorn and Stones, 1992), homoserine lactone (Huisman and Kolter, 1994), and inorganic phosphate (Shiba et al., 1997). UDP-glucose is suspected to play an inhibitory role in *rpoS* regulation (Bohringer et al., 1995).

1.5.1.1 Guanosine 3',5'-bispyrophosphate (ppGpp)

Alarmone (ppGpp) is produced as a result of ribosome stalling and its synthesis is dependent on the *relA* and *spoT* genes (Stephens et al., 1975). ppGpp^{O} (*ΔrelAspoT*) mutants exhibit a phenotype that is similar to that of an *rpoS* mutant (Gentry et al., 1993; Lange et al., 1995). The well characterized RpoS-dependent production of acid phosphatase (encoded by *appA*) is moderately impaired in a ppGpp^{O} mutant compared to its wild type parental strain during the transition to stationary phase (Gentry et al., 1993). Further, accumulation of RpoS protein as cells enter stationary phase is abolished in a ppGpp^{O} mutant (Gentry et al., 1993). *spoT* mutants which produce high basal levels of ppGpp exhibit increased levels of RpoS (Gentry et al., 1993). Comparable results were obtained when *relA* was placed under the control of a inducible promoter in an expression vector system (Gentry et al., 1993). Introduction of a ppGpp^{O} mutation into a strain carrying an *rpoS::lacZ* transcriptional fusion resulted in a decrease in expression (Lange et al., 1995). Taken *in toto*, these results indicate that ppGpp may act as signal that the cell is entering stationary phase and subsequently stimulating *rpoS* transcription.

1.5.1.2 Weak acids

Weak acids such as acetate are a by-product of basic cellular metabolism and have been shown to be inducers of an *rpoS::lacZ* transcriptional fusion (Schellhorn and Stones, 1992) indicating that weak acid may be a signal of late exponential phase growth. These workers suggest that internal pH may actually be the inducing signal. If this is the case then it could be hypothesized that any weak acid with a pKa similar to that of acetate, such as benzoate, will induce *rpoS* expression. Benzoate and propionate, which are not products of *E. coli* fermentation but have pKas similar to acetate, were able to induce the expression of an *rpoS::lacZ* fusion (Schellhorn and Stones, 1992). *E. coli* fermentation products and other weak acids with pKas different from acetate (Schellhorn and Stones, 1992) were unable to induce *rpoS* expression, as expected. Re-suspending an exponential phase cells in the supernatant from a stationary phase culture resulted in an almost immediate activation of an *rpoS::lacZ* fusion (Schellhorn and Stones, 1992) indicating that something produced and excreted by the cells (perhaps weak acids) may be a signal of the onset of stationary phase.

1.5.1.3 Homoserine lactone

The *rspA* gene was identified in a mutant screen for its ability to repress the expression of an RpoS-dependent *bolA::lacZ* transcriptional fusion (Huisman and Kolter, 1994). It was postulated that RspA, a putative lactonizing enzyme, may reduce the levels of some starvation signaling molecule responsible for *rpoS* induction (Huisman and Kolter, 1994), indicating that homoserine lactone (HSL) may be an inducer of RpoS activity. The affect of over-expression of *rspA* on *bolA* was due to its affect on *rpoS*

transcription as demonstrated by a reduction in the activity of an *rpoS::lacZ* transcriptional fusion (Huisman and Kolter, 1994). Mutants unable to synthesize homoserine exhibit reduced catalase activity (the major HPII catalase of *E. coli* is RpoS-dependent) that could be reversed upon the addition of homoserine or HSL to the growth media (Huisman and Kolter, 1994). Further, mutants unable to synthesize HSL produce low levels of RpoS as determined by immunoblot analysis (Huisman and Kolter, 1994). This reduction in RpoS levels can be reversed upon the addition of exogenous HSL indicating that HSL may be a starvation signal molecule whose accumulation leads to an induction of RpoS (Huisman and Kolter, 1994).

1.5.1.4 Inorganic polyphosphate

Recently, a role in *rpoS* regulation has been proposed for inorganic polyphosphate [poly(P)] (Shiba et al., 1997). Placing a yeast exopolyphosphatase (PPX1) under the control of an inducible promoter that allows for its over-expression results in a reduction of poly(P) levels almost below detection (Shiba et al., 1997). Over-expression of this phosphatase resulted in hypersensitivity to exposure to hydrogen peroxide that appeared to be HPII-dependent (Shiba et al., 1997). This result was verified by examining the expression of a *katE::lacZ* transcriptional fusion (*katE* codes for HPII) which demonstrated that over-production of poly(P)ase resulted in decreased expression of *katE* (Shiba et al., 1997). Since *katE* expression is RpoS-dependent, levels of RpoS were analyzed under conditions of low poly(P) levels. Western blot analysis revealed that over-expression of poly(P)ase resulted in reduced levels of RpoS (Shiba et al., 1997). To assess the level at which poly(P) is stimulating RpoS activity, poly(P)ase was over-

expressed in a strain harboring an *rpoS::lacZ* transcriptional fusion resulting in decreased expression. These findings indicate that poly(P) is required for *rpoS* activation (Shiba et al., 1997) and that accumulation of poly(P) levels may act as an inducing signal of *rpoS* expression.

1.5.1.5 UDP-glucose

UDP-glucose is an example of a signal molecule responsible for the inhibition of *rpoS* expression (Bohringer et al., 1995). In a screen to isolate factors other than RpoS that control the expression of an *osmY::lacZ* transcriptional fusion, Bohringer *et al* (1995) isolated a mutation in the *pgi* gene which encodes phosphoglucose isomerase (PGI). Since mutations in *pgm* and *galU* are pleiotropic to *pgi* (i.e. they are all impaired in synthesis of UDP-glucose) and also had an affect on the expression of *osmY* and other RpoS-dependent genes, UDP-glucose may be involved in regulation of *rpoS* (Bohringer et al., 1995). A *pgi::Tn*10 mutation resulted in an increase in expression of an *osmY::lacZ* fusion during exponential growth as well as resulting in an increase in the levels of RpoS in the cell (Bohringer et al., 1995). These results indicated that UDP-glucose may negatively regulate RpoS. Since UDP-glucose is a precursor for the osmoprotectant trehalose, these authors suggested that UDP-glucose levels are high in exponential phase cultures and thus represses RpoS activity and when cells face osmotic challenge, UDP-glucose levels drop in favor of trehalose synthesis therefore alleviating *rpoS* repression allowing for the expression of RpoS-dependent genes (Bohringer et al., 1995).

1.5.2 Translational control of RpoS

Recently, much work has been done to identify novel regulatory factors that are responsible for RpoS regulation, to elucidate their various modes of action and to determine how they interact to form the complex regulatory network that ultimately leads to the expression of the RpoS-dependent, stationary phase regulon.

1.5.2.1 The roles of H-NS, HF-I, & OxyS in the translational control of RpoS

As mentioned above, the histone-like nucleoid protein, H-NS, is a DNA binding protein that is responsible for the silencing of many genes including *rpoS* (Barth et al., 1995; Yamashino et al., 1995). An *hns* mutant exhibits increased levels of RpoS protein during exponential growth (Barth et al., 1995; Yamashino et al., 1995). However, the *hns*-mediated increase in RpoS levels was not a result of increased transcription since an *rpoS::lacZ* transcriptional fusion did not show an increase in activity in this mutant background (Barth et al., 1995). This result seemed curious since H-NS is a DNA-binding protein. It was therefore suggested that H-NS-mediated regulation of RpoS must be occurring at a post-transcriptional level. *rpoS::lacZ* translational fusions showed increased activity in an *hns* background indicating that H-NS is acting at the translational level (Barth et al., 1995; Yamashino et al., 1995). Recent studies have shown that H-NS may be involved in the same regulatory pathway as an RNA binding protein HF-I (Muffler et al., 1997). HF-I is encoded by the *hfq* gene and was originally isolated and characterized in its role in replication of the *E. coli* RNA phage Qβ (Carmichael et al., 1975). Mutations in *hfq* have no affect on an *rpoS::lacZ* transcriptional fusion (Muffler et al., 1997), however, *rpoS::lacZ* translational fusions in both *E. coli* and *S.*

typhimurium exhibit significantly lower activity in an *hfq* mutant background (Brown and Elliott, 1996; Muffler et al., 1997). Evidence presented by Muffler *et al* (1997) suggests that H-NS may regulate RpoS translation by regulating the transcription of *hfq*. HF-I in turn is proposed to alleviate secondary structure in the *rpoS* mRNA that sequesters the ribosome binding site and thus allows for efficient translation to occur (Muffler et al., 1997). If HF-I is acting at a post-transcriptional level then replacement of the native *rpoS* promoter should not affect HF-I-mediated regulation. In fact, placing an *rpoS::lacZ* fusion under the control of the *tac* or *lacUV5* promoter allowed for normal transcription and HF-I regulation to occur (Cunning et al., 1998). Deletion analysis studies of the region upstream of the transcriptional start site indicate that nucleotides that are required for HF-I-mediated regulation of RpoS are greater than 100 nucleotides upstream of the putative antisense structure indicating that HF-I may do more that simply interact with the RNA and melt the secondary structure (Cunning et al., 1998). The small RNA OxyS may also have a role in this pathway (Zhang et al., 1998). OxyS was originally isolated as a small, untranslated RNA, whose expression is dependent on OxyR (Altuvia et al., 1997), that could repress induction of RpoS. Strains carrying a constitutively- expressed plasmid-borne copy of *oxyS* exhibited decreased stationary phase expression of an *rpoS::lacZ* translational fusion (Zhang et al., 1998). These results were confirmed by immunoblot analysis which demonstrated that RpoS protein levels were also reduced when *oxyS* is over-expressed (Zhang et al., 1998). Since several other factors are involved in RpoS regulation, mutations in the genes encoding these factors were tested in conjunction with over-expression of *oxyS* to establish if any are required for OxyS-

mediated RpoS regulation. OxyS is able to repress expression of *rpoS* in all but an *hfq* mutant background in rich media, indicating that OxyS requires a functional HF-I (Zhang et al., 1998). Does OxyS affect RpoS regulation by affecting the levels of HF-I? Immunoblot analysis indicated that HF-I levels are unaffected by over-expression of *oxyS* (Zhang et al., 1998). A 27 base pair linker region between stem loops b and c of OxyS has been demonstrated to be required for regulation of RpoS (Altuvia et al., 1998). The authors propose that HF-I which binds A-rich regions may bind the A-rich regions in this OxyS linker (Zhang et al., 1998). This hypothesis was tested by mutagenizing the linker region. All of the OxyS linker mutants exhibited a reduced ability to repress RpoS (Zhang et al., 1998) indicating that HF-I may bind OxyS. This idea was confirmed by co-immunoprecipitation and gel retardation analyses which demonstrated HF-I's ability to bind OxyS (Zhang et al., 1998). It would be tempting to speculate that OxyS competes with *rpoS* mRNA for HF-I binding leading to *rpoS* repression however, equal amounts of *rpoS* mRNA could be precipitated even under conditions of *oxyS* over-expression precluding the possibility that inhibition by competition is occurring (Zhang et al., 1998). The authors speculate that under conditions of oxidative stress, OxyS is induced and prevents HF-I from activating RpoS. This may function to prevent redundant systems from being activated since OxyR, which is a transcriptional activator of genes (including *oxyS*) required to survive oxidative stress (Tartaglia et al., 1989), has already been induced (Zhang et al., 1998). This would prevent redundant expression of genes that are regulated by OxyR and RpoS (e.g. *katG* (Mukhopadhyay and Schellhorn, 1994)). Together, these three factors: H-NS, HF-I, and OxyS form a complex system of *rpoS*

translational regulation. The manner in which they interact with one another to regulate RpoS is not known.

1.5.2.2 The roles of DsrA & LeuO

As mentioned above, RpoS is induced in response to exposure to a variety of environmental stresses (for a review see (Hengge-Aronis, 1996)) including exposure to sub-optimal temperatures (Sledjeski et al., 1996). This 'cold shock' induction of RpoS is dependent on a small, untranslated RNA, DsrA (Sledjeski et al., 1996). In a *dsrA* mutant background, an *rpoS::lacZ* translational fusion exhibits only very low levels of induction when cultures are grown at 20 ^{o}C (Sledjeski et al., 1996) indicating that DsrA is required for RpoS expression at low temperatures. Over-expression of *dsrA* from a multicopy, inducible expression vector works to counteract H-NS-mediated gene silencing (Sledjeski et al., 1996). Until recently, the mode of action of DsrA was unknown. DsrA is thought to regulate RpoS via an anti-antisense mechanism mediated by direct RNA:RNA interactions (Lease et al., 1998; Majdalani et al., 1998). The *dsrA* gene was placed under the control of an arabinose-inducible promoter and this vector was mutagenized to assess the role of each of the three stem loops (SL1, SL2, & SL3) in the DsrA RNA (Majdalani et al., 1998). These mutant vectors were then transformed into strains carrying *rpoS::lacZ* translational fusions to examine their various affects, if any (Majdalani et al., 1998). This analysis determined that SL1 is absolutely required for RpoS activation at lower temperatures and that replacement of SL3 (which appears to function as a transcriptional terminator) with another termination sequences had no affect on DsrA's ability to induce RpoS (Majdalani et al., 1998). A computer search of the *E. coli* database

revealed that *dsrA* shows short stretches of homology to many *E. coli* genes including homology in SL1 to *rpoS*, dubbed *rpoS'* (Lease et al., 1998). This provided the first clue that DsrA may function by RNA:RNA interactions. When base pair changes were introduced into the *rpoS'* region of DsrA, reduced activity of an *rpoS::lacZ* translational fusion resulted at lower temperatures (Lease et al., 1998) confirming the results of Majdalani *et al* (1998) that SL1 is required for RpoS regulation at sub-optimal temperatures. Although this evidence is still largely indirect, the authors hypothesize that DsrA base pairs with the *rpoS* transcript to prevent intrastrand base pairing that frees the ribosomal binding site thus increasing the efficiency of *rpoS* translation (Lease et al., 1998; Majdalani et al., 1998).

The LysR-like regulator LeuO is thought to be involved in a regulatory pathway in conjunction with DsrA and H-NS in regulating RpoS (Klauck et al., 1997). A Tn*10* insertion in the *leuO* gene that placed *leuO* under the control of the P_{out} promoter of Tn*10* (this promoter drives the transcription of an antisense RNA that prevents excision ((Klauck et al., 1997) and references therein) that results in increased *leuO* expression. This insertional mutant was isolated in a screen for mutants in which expression of an *osmY::lacZ* fusion is reduced (Klauck et al., 1997). When the *leuO* insertional mutation was tested for affect on RpoS, a reduction in the activity of *rpoS* translational fusions was revealed (Klauck et al., 1997). This affect was much more pronounced when cells were grown at 25 ^{o}C and the authors postulate that LeuO is a repressor of *rpoS* translation at lower temperatures (Klauck et al., 1997). Over-expression of *leuO* from an inducible vector confirmed these results (Klauck et al., 1997). Since LeuO belongs to the LysR

family of transcriptional regulators and is more affective at low temperatures, the authors proposed and tested for an indirect role for LeuO in the regulation of *rpoS* translation involving DsrA (Klauck et al., 1997). The affects of a *dsrA* mutation coupled with *leuO* over-expression on an *rpoS::lacZ* translational fusion did not exhibit additivity, indicating that DsrA and LeuO may be involved in the same genetic pathway (Klauck et al., 1997). Further evidence in support of this came from studies on the affect of *leuO* over-production on DsrA. Introduction of a *leuO* mutation strongly reduced the activity of a *dsrA::lacZ* transcriptional fusion indicating that LeuO can repress *dsrA* at low temperatures (Klauck et al., 1997). A role for H-NS has also been proposed for this pathway although it is quite complex. It appears that H-NS represses expression of *leuO* and only in an *hns* mutant background does LeuO exhibit temperature-dependent repression of *dsrA* (Klauck et al., 1997), although the role of each member of this intricate and complex pathway remains to be fully explained.

1.5.3 Post-translational control of RpoS

1.5.3.1 DnaK

It has been well documented that the RpoS protein is subject to regulation at the level of protein stability. RpoS has an exponential phase half-life of 1.4 minutes that increases to about 16.5 minutes at the onset of stationary phase (Lange and Hengge-Aronis, 1994). Further, exponential phase stability of RpoS also increases in response to environmental stresses such as osmotic challenge (Lange and Hengge-Aronis, 1994). The heat shock chaperone protein DnaK has been implicated as a regulator of RpoS stability (Muffler et al., 1997). Translational fusions to *rpoS* that retain a putative turn-over (T/O)

element subjecting the RpoS-LacZ hybrid protein to proteolysis in a manner similar to that of the wild type RpoS protein, indicated that DnaK is required for accumulation of RpoS when cells are exposed to elevated temperatures (Muffler et al., 1997). Expression of translational *rpoS::lacZ* fusions that are missing this turn-over element were not affected by mutations in DnaK (Muffler et al., 1997) indicating that DnaK is required for RpoS activity at high temperature. Since RpoS does not appear to have a role in protecting the cell from heat shock, the authors suggest that RpoS may be induced to confer cross protection to other environmental stress that may accompany exposure to elevated temperatures (Muffler et al., 1997).

1.5.3.2 RssB/SprE/MviA, ClpXP, & acetyl-phosphate

The *E. coli* proteins RssB (Muffler et al., 1996) also known as SprE (Pratt and Silhavy, 1996) and the *S. typhimurium* homolog MviA (Bearson et al., 1996) are putative members of the response regulator family of two-component regulators that regulate RpoS stability. This protein has a novel C-terminus output domain with unknown function. The cognate sensor kinase for this response regualtor has not been identified (Bearson et al., 1996; Muffler et al., 1996; Pratt and Silhavy, 1996). RssB has been shown to be required for rapid degradation of RpoS during exponential growth (Bearson et al., 1996; Muffler et al., 1996; Pratt and Silhavy, 1996). An RssB mutant exhibited an almost ten-fold increase in exponential level of RpoS as determined by immunoblot analysis (Muffler et al., 1996) indicating that RssB plays a negative role in RpoS stability in exponential growth. These results were confirmed utilizing the above mentions *rpoS::lacZ* translational fusion containing the turn over element. RpoS-LacZ activity was

higher in an *rssB* mutant background (Muffler et al., 1996). Further, overproduction of RssB led to a decrease in RpoS levels during all phases of growth (Pratt and Silhavy, 1996). Strains of *S. typhimurium* with mutations in the *mviA* gene show increased resistance to acid shock in all phases of growth, which was due to elevated levels of RpoS in these cells (Bearson et al., 1996) confirming the idea that RssB regulates RpoS stability in exponential phase.

The ability of RssB to regulate RpoS stability has been linked to the ClpXP protease (Zhou and Gottesman, 1998). *clpXP* mutants are phenotypically similar to *rssB* mutants in that they exhibit elevated exponential phase levels of RpoS (Zhou and Gottesman, 1998). Studies comparing ClpXP-mediated turn-over of RpoS and the λO protein (which is also subject to ClpXP degradation) indicate that RssB may somehow be modifying RpoS, making it a target for degradation, since λO protein turn over was unaffected in *rssB* wild type and mutant backgrounds (Zhou and Gottesman, 1998). These results indicate that RssB is acting on RpoS as opposed to somehow stimulating the activity of ClpXP. To date, no cognate sensor kinase has been identified that can potentially activate RssB. A role has been proposed for acetyl phosphate [acetyl(P)] as a potential phosphate donor for RssB (Bouche et al., 1998). Impairing the cell's ability to synthesize acetyl(P) results in an increase in the exponential phase stability of RpoS as indicated by increased stability of the RpoS-LacZ hybrid protein with the T/O element intact although, degradation is not completely abolished (Bouche et al., 1998). Phosphotransfer to aspartic acid residue D58 of RssB by radiolabelled acetyl(P) was also demonstrated (Bouche et al., 1998) indicating that this molecule may be acting as the

phosphate donor that activates RssB. The authors postulate that as cells become carbon starved, acetyl(P) levels drop which may lead to a reduction in RssB-mediated degradation of RpoS in turn allowing RpoS levels to increase (Bouche et al., 1998) thus leading to the induction of the RpoS regulon. Whether or not phosphorylated RssB transfers the phosphate group to RpoS, thus 'marking' it for degradation remains to be determined.

Taken *in toto*, these results demonstrate that regulation of RpoS and ultimately, members of the RpoS regulon, is a complex process and is subject to multiple levels of control. The necessity for these multiple levels of control is still only poorly understood.

1.6 BarA is a Member of the Family of Two-component Response Regulators

In the course of the work that follows, we identified BarA as a possible transcriptional regulator of *rpoS*. The *barA* gene was first identified as a multicopy suppressor of an *envZ* deletion (Nagasawa et al., 1992) by its ability to restore activity of promoter-*lacZ* fusions to the outer membrane porin encoding genes *ompC* and *ompF*. EnvZ and OmpR are an example of a sensor kinase and a response regulator that affect gene expression (they affect the expression of the previously mentioned porin encoding genes *ompC* and *ompF*). BarA shows homology in both amino and carboxyl termini to the sensor kinase and response regulator domains of the family of two-component regulators (Nagasawa et al., 1992) and was named BarA for **b**acterial **a**daptive **r**esponse protein. However, since *in vitro* attempts to assay for BarA-dependent phosphorylation of OmpR were unsuccessful (Nagasawa et al., 1992), it is unclear how BarA complemented the *envZ* deletion. Two-component regulatory systems have been well

studied and are responsible for induction of proteins that control several cellular responses including chemotaxis, nitrate respiration, and osmotic sensing (for review see (Parkinson, 1993; Stock et al., 1989; Stock et al., 1990)). They consist of two members: the sensor kinase is usually membrane bound and can have autophosphorylation, kinase, and phosphatase capabilities. It is able sense 'environmental stimuli' which causes its subsequent activation. After autophosphorylation, the sensor can then activate the second member, the response regulator which, depending on its phosphorylation status, can affect gene expression (for reviews see (Parkinson, 1993; Stock et al., 1989; Stock et al., 1990)). Sensors contain a conserved C-terminus histidine residue which is the site of autophosphorylation and response regulators contain a conserved N-terminus aspartic acid residue which is the phospho-accepting site. Sensors often possess phosphatase capability and can affectively turn the response regulator 'on' or 'off'. BarA belongs to a unique class of two-component regulators which possess both an N-terminus sensor domain and a C-terminus regulator domain containing the conserved histidine and aspartic acid residues respectively (Nagasawa et al., 1992) and also posses two putative membrane spanning domains. Other examples of members of this class are ArcB, BvgC, VirA and FrzE, all of which possess both sensor and response regulator domains (Nagasawa et al., 1992).

barA was identified in another study as a required virulence factor in uropathogenic *E. coli* strains and was re-named *airS* for attachment and iron regulation sensor (Zhang and Normark, 1996). This gene was identified in an RT-PCR based screen of mRNAs in virulent *E. coli* cells (Zhang and Normark, 1996). The cDNA library that

was generated was sequenced and *barA* was isolated as a gene expressed under these conditions (Zhang and Normark, 1996). The authors propose a role for BarA in regulation of iron sensing and/or uptake based on the inability of *barA* mutant to grow on iron-limited media (Zhang and Normark, 1996). Previous work in our laboratory by Suman Mukhopadhyay (Ph.D. Thesis) demonstrated that a *barA* mutant exhibits a hydrogen peroxide sensitive phenotype and that the levels of catalase activity (which is RpoS-dependent) in this mutant are reduced compared to an isogenic wild type strain. A newly proposed role for BarA as a transcriptional regulator of *rpoS* will be discussed.

1.7 Project Objectives and Outline

The following presentation is work that was a continuation of two separate projects in the laboratory. Chapters two, three, and appendix one are continuations of work done to identify novel members of the RpoS regulon by Linda Wei (M.Sc. Thesis). This screen involved introduction (by conjugation) of a null allele of a trans-acting regulator (in this case an *rpoS*::*Tn*10) into a bank of strains which were previously mutagenized with a randomly-inserting, promoterless *lacZ* expression vector (λp*lac*Mu53). The resulting transconjugants were screened for reduced β-galactosidase activity and tested for complementation with a plasmid-borne copy of *rpoS* (pMM*katF3*).

The present study concentrated on determining the identities of these RpoS-dependent genes. This involved a sequencing strategy (Roy et al., 1995) employing the properties of the lambdoid-like expression vector. Exposure of cultures carrying these promoter-*lacZ* fusions to UV radiation results in induction of the lambda excision system. When the prophage excises itself from the chromosome, it can carry and package nascent host DNA. Utilizing a sequencing primer that will hybridize to the Mu c arm of the expression vector, nascent DNA sequence can be determined by DNA sequencing and analysis. The sequences of several novel RpoS-dependent genes are described here. The RpoS- and growth-phase-dependence of these genes was verified by Northern analysis of bacterial RNA isolated from wild type and *rpoS* mutants during growth. Since several RpoS-dependent genes are known to be co-regulated by other regulatory factors such as Lrp, cAMP-CRP, and Fis, we also tested ten of our RpoS-dependent gene fusions for co-regulation by the cAMP-CRP complex.

The work presented in chapter four is a continuation of work done by Suman Mukhopadhyay (Ph.D. Thesis) on the identification of hydrogen peroxide sensitive mutants in *Escherichia coli*. In this screen he identified *barA*, a previously identified multicopy suppressor of an *envZ* deletion and a bacterial virulence factor (see introduction), as a gene which is required for resistance to hydrogen peroxide challenge. A *barA* mutant exhibited reduced levels of the *E. coli* hydroperoxidase enzymes, HPI and HPII (which is under the control of RpoS). This work attempts to determine the manner in which a *barA* mutation results in this decrease in catalase activity by examining *katE* (codes for HPII) mRNA levels in wild type and *barA* mutant strains and by examining the mRNA and protein levels of the main regulator of *katE*, RpoS, by Northern and Western analysis respectively.

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CHAPTER 2.

PREFACE

The following manuscript entitled: "Identification of Conserved, RpoS-Dependent Stationary-Phase Genes of *Escherichia coli*" was accepted for publication in the Journal of Bacteriology on 24 September, 1998 and published in the December issue (*180*(23): 6283-6291).

Jonathon P. Audia performed all of the Northern blot experiments, isolated DNA for sequencing of the HS1010, HS1095, and HS1098 fusion junctions, and was also involved in the editing of the manuscript and in preparing a response to one of the reviewer's comments.

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December 21, 1998

Ms. Linda Illig
Director Publications

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Dear Ms. Illig:

On behalf of two of my students, Mr. Jonathon Audia and Ms. Lily Chang, I would like to request permission to include an article we recently published in J. Bacteriol (JB 731-98) in two MS theses. The students are planning to submit their theses early in the near year. The full citation for the paper is:

Schellhorn HE, et al. Identification of conserved, RpoS-dependent stationary-phase genes of Escherichia coli. J Bacteriol. 1998 Dec;180(23):6283-91

Please contact me if you require any additional information.

Sincerely,

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Herb E. Schellhorn
Associate Professor of Biology

HES/mb

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Identification of Conserved, RpoS-Dependent Stationary-Phase Genes of *Escherichia coli*

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During entry into stationary phase, many free-living, gram-negative bacteria express genes that impart cellular resistance to environmental stresses, such as oxidative stress and osmotic stress. Many genes that are required for stationary-phase adaptation are controlled by RpoS, a conserved alternative sigma factor, whose expression is, in turn, controlled by many factors. To better understand the numbers and types of genes dependent upon RpoS, we employed a genetic screen to isolate more than 100 independent RpoS-dependent gene fusions from a bank of several thousand mutants harboring random, independent promoter-*lacZ* operon fusion mutations. Dependence on RpoS varied from 2-fold to over 100-fold. The expression of all fusion mutations was normal in an *rpoS*/*rpoS*$^+$ merodiploid (*rpoS* background transformed with an *rpoS*-containing plasmid). Surprisingly, the expression of many RpoS-dependent genes was growth phase dependent, albeit at lower levels, even in an *rpoS* background, suggesting that other growth-phase-dependent regulatory mechanisms, in addition to RpoS, may control postexponential gene expression. These results are consistent with the idea that many growth-phase-regulated functions in *Escherichia coli* do not require RpoS for expression. The identities of the 10 most highly RpoS-dependent fusions identified in this study were determined by DNA sequence analysis. Three of the mutations mapped to *otsA*, *katE*, *ecnB*, and *osmY*—genes that have been previously shown by others to be highly RpoS dependent. The six remaining highly-RpoS-dependent fusion mutations were located in other genes, namely, *gabP*, *yhiUV*, *o371*, *o381*, *f186*, and *o215*.

Like many other free-living bacteria, *Escherichia coli* lives in environments that may change rapidly with respect to both nutrients and physical conditions. To survive stresses associated with starvation, *E. coli* expresses many stationary-phase-specific genes whose expression depends largely on an alternative sigma factor, σ^S, encoded by *rpoS* (27, 30). Inactivation of this gene renders the cell sensitive to heat shock (25, 29), oxidative stress (25, 29), osmotic challenge (29), and near-UV light (40). Proteins that depend on RpoS include catalase HPII (33, 39, 42) and catalase HPI (32), exonuclease III (39), penicillin-binding proteins (15), and osmoprotective proteins (21, 22, 53). RpoS is required for virulence (17) and acid tolerance (6) in *Salmonella typhimurium*. Although the signal(s) giving rise to increased expression of RpoS itself is not completely understood, homoserine lactone (23), UDP-6-glucose (10), and weak acids, such as acetate (42), have been shown to be inducers of RpoS.

Several approaches have been used to enumerate and identify RpoS-regulated functions. Many of these genes, however, are probably still unidentified. Two-dimensional gel electrophoresis studies of proteins expressed in wild-type and *rpoS* strains have revealed that the RpoS regulon is quite large (30). Mutagenesis with random *lacZ* (16, 51) or *lux* insertions (46), coupled with screening for RpoS-related characteristic phenotypes, has also been successfully employed to identify new RpoS-regulated genes (51). However, unlike other regulons, the RpoS regulon does not have a single unifying characteristic or differentiating phenotype that all members share. These factors, in addition to its suspected large size, have delayed complete characterization of the regulon. To circumvent the problems associated with the characteristics described above, we have employed a mutant identification scheme in which an *rpoS* null allele is introduced into strains containing random promoter-*lacZ* fusions to directly identify RpoS dependency. Since this procedure does not rely on a phenotype specific for the regulon (e.g., carbon starvation), this method should be of general use in the identification of members of any regulon for which a null allele of a positive-acting regulator is available.

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MATERIALS AND METHODS

Bacterial strains, phage, and plasmid. The bacterial strains, phage, and plasmid used in this study are listed in Table 1.

Chemicals and media. All chemicals were supplied by either Fisher Scientific, Ltd. (Toronto, Ontario, Canada), Sigma Chemical Co. (St. Louis, Mo.), or Gibco BRL (Burlington, Ontario, Canada). Antibiotics and other nonautoclavable stock solutions were filter sterilized with Gelman Sciences (Ann Arbor, Mich.) Acrodise sterile filters (pore size, 0.45 μm). Liquid and solid media were prepared as described by Miller (31). Cultures were routinely grown in Luria-Bertani (LB) rich broth. The concentrations of antibiotics used were as follows: kanamycin, 50 μg/ml; streptomycin, 50 μg/ml; tetracycline, 15 μg/ml; and ampicillin, 100 μg/ml. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used at a concentration of 50 μg/ml.

Growth conditions. All growth and survival assays were performed with GC4468 derivatives. Cultures were grown overnight in LB medium containing the appropriate antibiotics. Cell growth was monitored spectrophotometrically (UV-VIS spectrophotometer, model UV-1201; Shimadzu Corporation, Kyoto, Japan) by optical density at 600 nm (OD_{600}). For expression studies, bacterial cultures were maintained in the early exponential phase (OD_{600} of <0.2) for at least 8 generations prior to the start of the experiment. Cultures were grown in flasks at 37°C at 200 rpm, sampled, and assayed for β-galactosidase activity at the times indicated.

For survival assays, bacterial cultures were incubated for 10 days in LB broth at 37°C in sealed microtiter plates (to minimize evaporation). Following this period, cultures were diluted in M9 salts buffer, plated on LB medium, and enumerated after 24 h of incubation.

Enzyme assays. β-Galactosidase activity was assayed as described by Miller (31). Units of activity were calculated as [1,000 × OD_{420}]/[time of incubation (min) × volume (ml) × OD_{600}] and were expressed as Miller units. Catalase activity was measured spectrophotometrically by monitoring hydrogen peroxide decomposition at OD_{240} as described previously (42).

Genetic methods. The phenotypic screen for RpoS dependence is based on the observation that introduction, by transduction, of *rpoS*::Tn*10* into a strain containing a *katE*::*lacZ* fusion abolishes β-galactosidase activity (41). Since conju-

TABLE 1. *E. coli* strains, plasmid, and bacteriophage used in this study

Strain, plasmid, or bacteriophage	Genotype	Source or reference
Strains		
MC4100	Δ(*argF-lacZ*)*205 araD139 flbB5301 relA1 rpsL150 thi ptsF25*	42
GC4468	Δ*lacU169 rpsL*	42
KL16	Hfr (PO45) *relA1 spoT1 thi-1*	K. B. Low
NC4468	As GC4468, but ϕ(*katE*::*lacZ*$^+$)131	41
NC122	As NC4468, but *rpoS13*::Tn*10*	41
HS180	Like KL16, but *rpoS13*::Tn*10*	$P1_{vir}$ (NC122) × KL16→Tet^r
GC122	As GC4468, but ϕ(*rpoS*13::Tn*10*)	41
GC202	As GC4468, but ϕ(*katG*::Tn*10*)	42
HS1001–HS1105	As GC4468, but carrying *RpoS* dependent promoter-*lacZ* fusions	This study
HS1001T–HS1105T	As HS1001-HS1105, but *rpoS*13::Tn*10*	This study
13C10	As GC4468, but carrying a growth-phase-dependent, RpoS-independent promoter-*lacZ* fusion	This study
Plasmid; pMM*katF3*	Carries *rpoS* (*katF*) gene	33
Bacteriophage; $P1_{vir}$	Generalized transducing phage	Laboratory collection

gation is much more efficient and amenable to large numbers of transfers than P1-mediated transduction, we reasoned that the use of an appropriate Hfr donor containing a null mutation in the *rpoS* gene close to the point of origin could facilitate simultaneous testing of several thousand colonies for dependence on RpoS. Since the point of origin of transfer in the Hfr strain KL16 is located at 64 min and DNA is transferred during mating in a counterclockwise direction, *rpoS*, located at 62 min, should be transferred shortly after initiation of conjugation. The *rpoS13*::Tn*10* mutation was introduced into Hfr KL16 from strain NC122 (*rpoS13*::Tn*10*; *katE*::*lacZ*) by P1-mediated transduction. Transductants were selected on media supplemented with tetracycline (to select against the recipient) and streptomycin (to select against the donor). Transductants were flooded with 30% hydrogen peroxide to confirm transfer of the *rpoS13*::Tn*10* mutation (*E. coli* colonies normally evolve gas bubbles when flooded with hydrogen peroxide because of the activity of catalase HPII, the catalase encoded by *katE*). This Hfr donor was confirmed to be HPII negative and was designated HS180. The Hfr transfer capability of HS180 was tested with the control strains NC4468 (*katE*::*lacZ*) and MC4100. All transconjugants exhibited reduced catalase levels, and as expected, transconjugants produced by mating HS180 with NC4468 also exhibited reduced β-galactosidase activity.

Plasmid transformations were performed with the TSS (transformation and storage solution) method of transforming recipient cells (13). To select for transformation of the pMM*katF3* plasmid, LB agar plates containing kanamycin, streptomycin, and ampicillin were used. Transformed *rpoS* transconjugants were selected on the same medium supplemented with tetracycline.

Identification of RpoS-dependent fusions. To isolate promoter-*lacZ* fusions that depend on RpoS, we used a previously constructed collection of 5,000 independent transcriptional *lacZ*$^+$ mutants as F$^-$ recipients (42). These LacZ$^+$ mutants, in an MC4100 background, harbor randomly inserted λp*lac*Mu53 phage that also confer kanamycin resistance (12). The donor strain (HS180) was grown to the exponential phase (OD_{600} = 0.3) in LB broth, and 200-μl samples of culture were placed into microtiter plate wells. Recipient strains were grown to saturation in microtiter plate wells containing 200 μl of LB broth. Aliquots (20 μl) of strain HS180 were mated with each F$^-$ recipient directly in microtiter wells for 30 min and replica plated onto selective plates containing streptomycin, kanamycin, tetracycline, and X-Gal. Transfer of the *rpoS* allele was confirmed by testing the resulting transconjugants for catalase activity (see above). Putative RpoS-dependent (*rsd*) fusions were identified by comparing the levels of β-galactosidase activity of the fusions in *rpoS*$^+$ and *rpoS* strains on LB plates containing X-Gal. Recipients were then purified, and one clone from each was tested for RpoS dependency. Complementation tests were done by transforming each transconjugant with pMM*katF3*, a plasmid containing the *rpoS* gene (33). To ensure that strains contained single-copy chromosomal *lacZ* insertions, the fusions were transduced into GC4468 and retested for RpoS dependence.

Induction of λ lysogens. Because the phage used to generate the mutant bank was a lambda derivative (12), bacterial DNA proximal to the introduced promoter-*lacZ* mutation can be isolated by UV induction of the lambdoid prophage from the bacterial mutants (38). A single clone was inoculated into LB medium containing streptomycin and kanamycin and grown overnight at 37°C. Cells were subcultured into 50 ml of fresh medium (1/10) the next morning, grown to an OD_{600} of 0.4, centrifuged, and resuspended in 10 ml of 10 mM $MgSO_4$. Induction of the lambdoid prophage was performed by irradiating the culture at 25 mW for 7 s (approximately 35 J/m^2). Five milliliters of 3xLL (38) medium was added, and the irradiated culture was shaken vigorously in a petri plate until lysis was observed (3 to 5 h). The lysate was transferred to a 30-ml glass Corex tube. Chloroform was added, the phage lysate was mixed vigorously, and cell debris was removed by centrifugation at 10,000 × *g* for 20 min. DNase (10-μg/ml final concentration) was added to remove traces of chromosomal DNA.

Sequencing of bacterial DNA proximal to the λ fusion junction. Preparation and sequencing of DNA from UV-induced lysates were performed as previously described (38) with a 25-mer primer (5′-CCCGAATAATCCAATGTCCTCCC GG-3′) located 30 nucleotides from the Mu c end boundary. DNA sequencing was performed by the MOBIX central facility at McMaster University. The amount of DNA used in each sequencing reaction was approximately 0.5 to 1.0 μg. Sequences were compared to those in the GenBank database by using the BLASTN alignment algorithm (1). Bacterial homologs of identified *E. coli* RpoS-dependent genes were determined by using the gapped BLASTX alignment algorithm (2).

RNA extraction and Northern blot analysis. Cultures were grown as described above, and aliquots were removed from exponential- and stationary-phase cultures. RNA was extracted with an RNeasy Midi Kit (Qiagen, Inc., Valencia, Calif.). Northern analysis was performed with equal amounts of RNA from the different samples by standard methods (43). Total RNA was blotted onto BIOTRANS nylon membranes (ICN, Montreal, PQ, Canada) as described in reference 43 and fixed by baking at 80°C for 2 h. Prehybridization and hybridization were performed at 42°C with gentle agitation. When necessary, blots were stripped by boiling in 0.1 to 0.5% sodium dodecyl sulfate according to the manufacturer's (ICN) instructions for reprobing.

Oligonucleotide primers were synthesized and used in PCRs to generate specific probes to five identified *rsd* genes and to a control non-*rsd* gene control (*rnA*) *katE*, forward (with respect to the open reading frame [ORF]), 5′-CAAAGCG GATTTCCTCTCAGATC-3′, and reverse, 5′-TGTCAAATGGCGTCTGACTT AG-3′; *osmY*, forward, 5′-CTGCTGGCTGTAATGTTGACCTC-3′, and reverse, 5′-CATCTACCGCTTTGGCGATACTT-3′; *gabD*, forward, 5′-GAAAGGCGA AATCAGCTACGC-3′, and reverse, 5′-CTTCGATGCCATACTTCGAACCT-3′; *gabP*, forward, 5′-CCATCTGGTTATTTTCCCTCG-3′, and reverse, 5′-GG TAATAAAGCCGATGACTAGCCAG-3′; and *rnA*, forward, 5′-GTGCCCAG ATGGGATTAGCTAGTAG-3′, and reverse, 5′-GTCGAGTTGCAGACTCCA ATCC-3′. Each PCR tube contained 1× PCR buffer (500 mM KCl, 200 mM Tris [pH 8.4]), 50 pmol of each of the forward and reverse primers, 0.4 mM each of the four deoxynucleoside triphosphates, 4 mM $MgCl_2$, ~50 ng of *E. coli* DNA, and ~10 U of *Taq* polymerase in a final volume of 50 μl. Reactions were run for 25 cycles under the following conditions: (i) 96°C for 30 s, (ii) 61°C for 60 s, and (iii) 72°C for 90 s. PCR products were purified with the QIAquick PCR purification kit (Qiagen, Inc.) and radiolabelled with [α-^{32}P]dCTP (NEN Life Science Products, Inc., Boston, Mass.) by random priming. The identity of all PCR products was confirmed by DNA sequencing.

RESULTS

Isolation of σ^s-dependent fusion mutants. A diagrammatic representation of the screening procedure for the isolation of σ^s-dependent promoter-*lacZ* fusions is shown in Fig. 1. Putative σ^s-dependent fusions were identified by comparing the level of β-galactosidase activity of wild-type (with respect to *rpoS*) recipients to that of *rpoS*::Tn*10* transconjugants on LB plates containing X-Gal. From this screen of 5,000 mutants, 105 *rpoS*::Tn*10* transconjugants were identified that exhibited reduced β-galactosidase activity compared with that of the wild-type recipients. Putative RpoS-dependent (*rsd*) transcriptional fusions were transduced into GC4468 and retested for σ^s

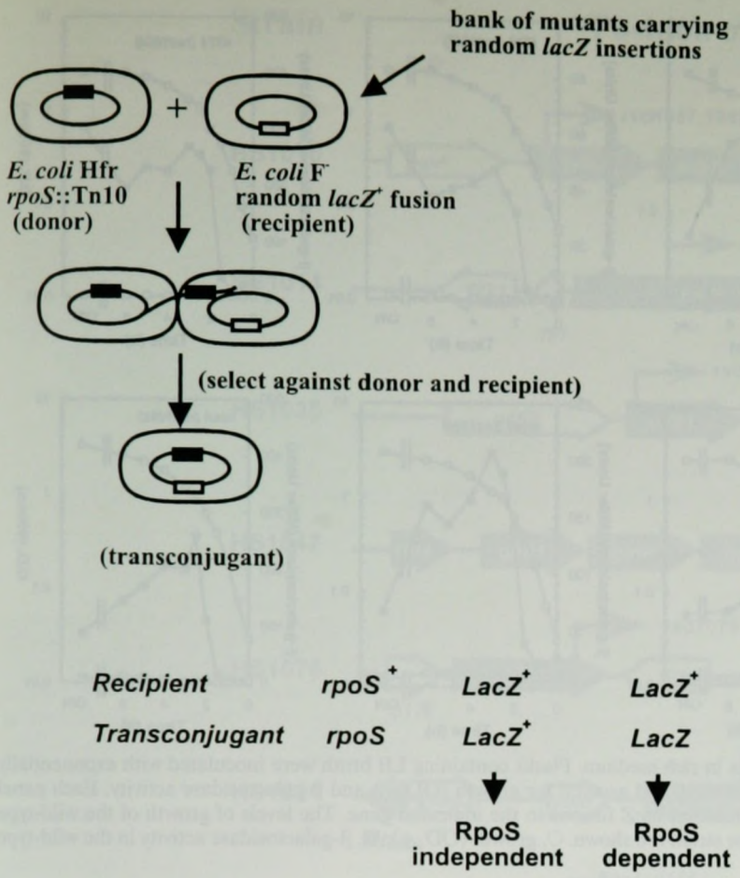

FIG. 1. Schematic representation of the strategy used to identify transconjugants harboring RpoS (σ^S)-dependent promoter-*lacZ* fusions.

dependency. A σ^S-dependent *katE*::*lacZ* fusion strain, NC4468, served as a positive control, and a strain carrying a σ^S-independent fusion, 13C10, was used as a negative control in subsequent mating procedures. The *lacZ* expression of all 105 transductants was RpoS dependent (Fig. 2).

To confirm that the lower β-galactosidase activity of the transconjugants was due to introduction of the *rpoS* null mutation and was not the result of a secondary mutation, transconjugants were transformed with pMM*katF3* containing a wild-type *rpoS* gene. Recipients were transformed in parallel, serving as controls for any variation in β-galactosidase levels due to the presence of the vector. In many cases, the transformed wild-type and *rpoS* strains exhibited higher levels of β-galactosidase activity than the nontransformed derivatives. This may be due to the increased levels of *rpoS* expression on multicopy plasmids, an observation reported by other investigators (39). As expected, all 105 mutants were efficiently complemented when transformed with plasmid-borne *rpoS* (data not shown).

Growth-phase expression of *rsd-lacZ* fusions. Many known RpoS-regulated genes are expressed at relatively low levels in the exponential phase but are induced as cells enter the stationary phase in rich medium (for review, see reference 19). We tested growth-phase induction of the *rsd* promoter-*lacZ* mutations isolated in this study. As expected, all fusions were maximally expressed in the early stationary phase or in 24-h cultures (Fig. 3). In each case, induction began before the cultures reached an OD_{600} of 0.3, suggesting that the signal(s) required for induction of these genes, whatever its nature, is present in early-exponential-phase cultures. We further examined growth-phase dependence in the other 95 fusion mutants and found that in each case, induction was initiated in the early exponential phase. We have previously observed that transcriptional induction of a single-copy *rpoS*::*lacZ* fusion occurs in the early exponential phase (42).

Identification of *rsd-lacZ* fusion junctions. λ DNA was prepared from induced lysogens as described in Materials and Methods. The Mu c end vector sequence (5′-AATACA-3′) was confirmed for all sequences, and the determined DNA sequence proximal to the fusion junction was compared to published *E. coli* sequences. We have identified 50 of the 105 RpoS-dependent fusions isolated in this study by sequencing DNA prepared from UV-induced phage lysates as previously described (38). It is well established that the promoters of many RpoS-dependent genes can also be recognized by RpoD (45), the main vegetative sigma factor of *E. coli*. Since we were primarily interested in identifying genes that specifically require RpoS for expression (as opposed to those genes having promoters that can be recognized by both RpoS and RpoD [45]), we initially characterized the 10 fusions which exhibited the highest degree of RpoS dependence. Three of the 10 most-highly-RpoS-dependent mutations mapped to genes previously shown to be RpoS regulated, including *katE* (*rsd1014*), the structural gene for HPII catalase; *otsA* (*rsd1098*), which en-

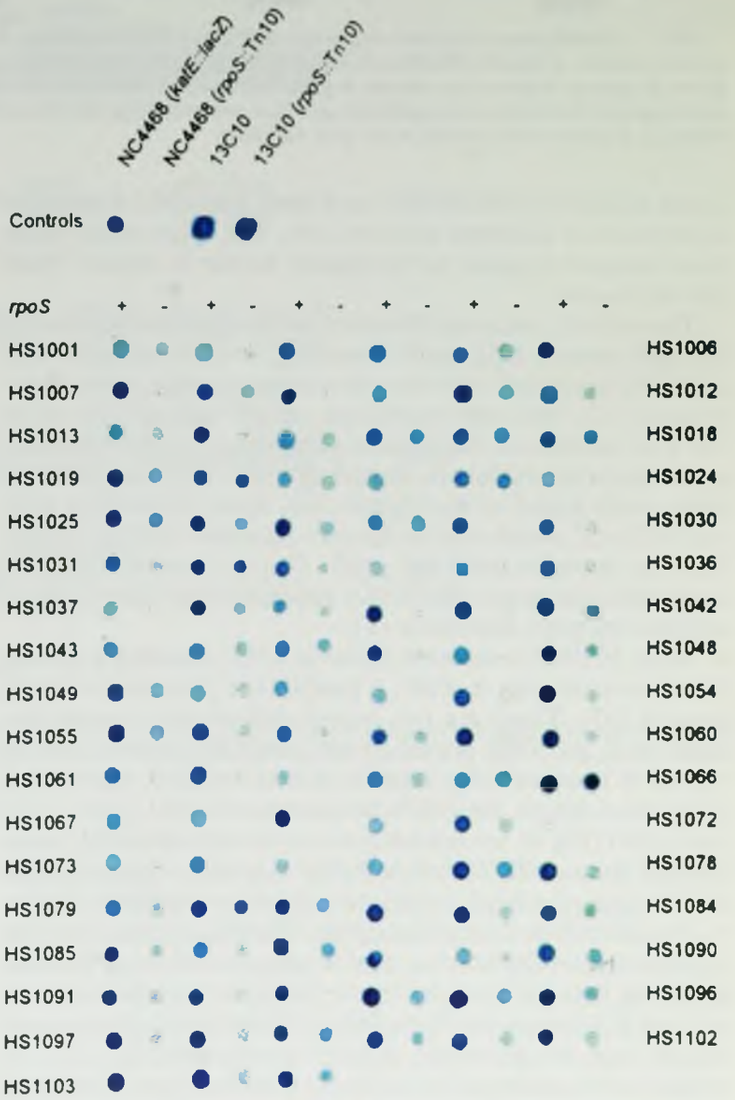

FIG. 2. The 105 recipient (*rpoS*⁺) and transconjugant (*rpoS*) pairs in a GC4468 background. Strains were plated on M9 minimal media supplemented with 0.4% glucose. The σ^S-dependent and -independent control strains NC4468 (*katE*::*lacZ*) and 13C10, respectively, were placed in the top row, with *rpoS* derivatives placed adjacent to them. *rpoS*⁺ and *rpoS* derivative pairs are adjacent to one another in rows, starting from the top left. The *rpoS* status of each column is shown on the top (+, *rpoS*⁺; −, *rpoS*).

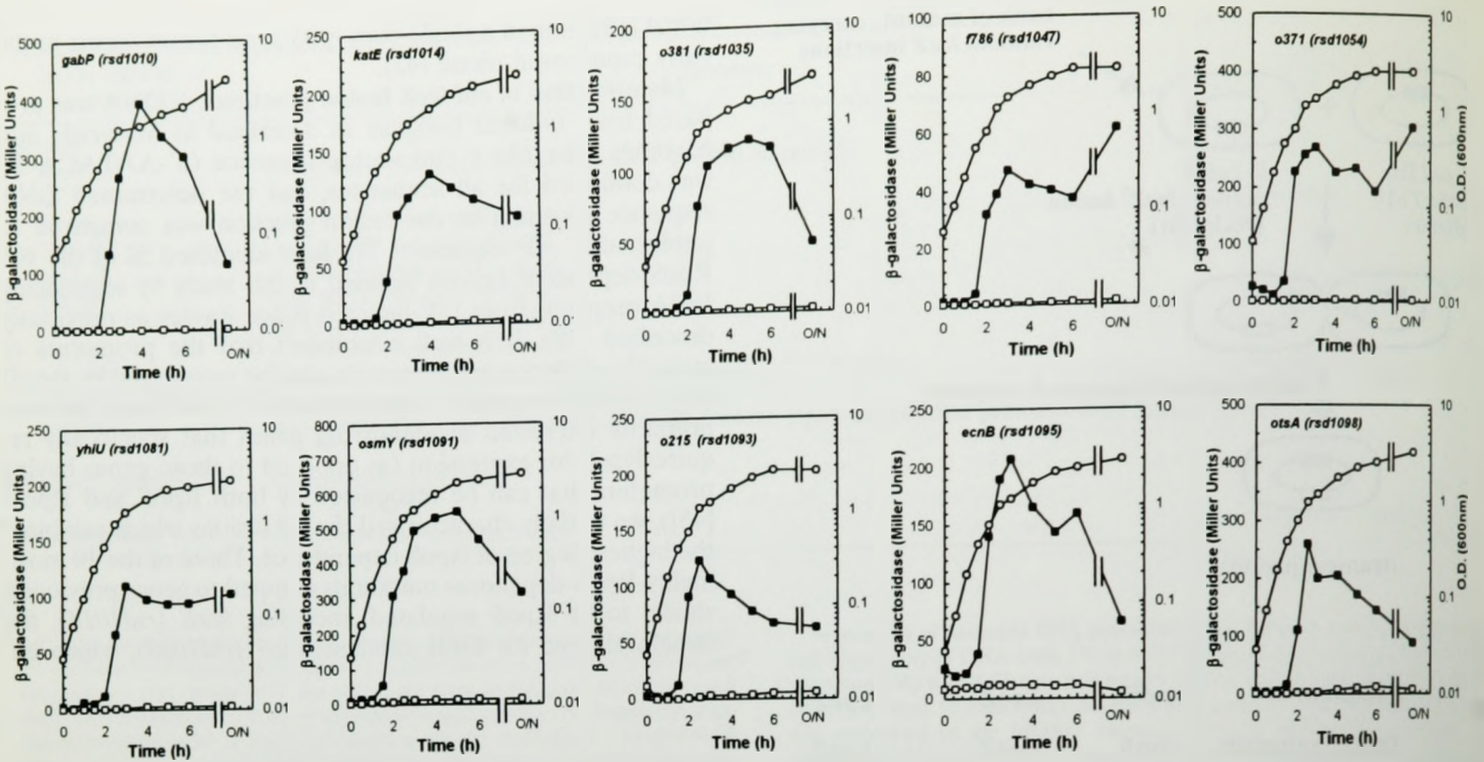

FIG. 3. Growth-phase-dependent expression of 10 highly-RpoS-dependent fusions in rich medium. Flasks containing LB broth were inoculated with exponentially growing cultures as described in Materials and Methods, sampled periodically as indicated, and assayed for growth (OD_{600}) and β-galactosidase activity. Each panel shows the growth of the culture and the β-galactosidase activity in strains carrying promoter-*lacZ* fusions to the indicated gene. The levels of growth of the wild-type strain and *rpoS* derivatives were equivalent, and thus only growth data for the wild-type strain are shown. ○, growth (OD_{600}); ■, β-galactosidase activity in the wild-type strain; □, β-galactosidase activity in the *rpoS* derivative.

codes trehalose synthase (21); and *osmY* (*rsd1091*), a probable lipoprotein of unknown function (54). The other seven mutations mapped to genes not previously known to require RpoS for expression.

The *rsd1010* mutation is located in the terminal member of the *gab* operon (35), *gabP*, encoding a γ-aminobutyric acid (GABA) permease that can also transport other amine compounds (11). Two other mutations, *rsd1057* and *rsd1058*, are in the first member of the operon, *gabD* (Fig. 4), which encodes a succinate semialdehyde dehydrogenase. The two *gabD* fusions were found to be slightly less RpoS dependent than *rsd1010-lacZ*, which may be due to a repetitive (REP) element that lies between *gabT* and *gabP*. This is consistent with the suggestion that these short DNA sequences may have a role in attenuating gene expression (5).

Strain HS1035 contains a fusion in *o381*, encoding a protein that is homologous to PotF, a periplasmic putrescine-binding protein (37). There are two known polyamine transport systems in *E. coli*. The *potABCD* and *potFGHI* operons are involved in transport of putrescine and spermidine, respectively (37). Interestingly, the ORFs downstream of *o381* (*o337*, *o313*, and *o264*) (Fig. 4) are homologous to the corresponding members of the *potABCD* and *potFGHI* operons, suggesting that *o381* is part of a third, conserved polyamine transport operon.

The *rsd1047-lacZ* mutation (strain HS1047) is located in *f786*, a gene of unknown function that is conserved in other bacteria matching hypothetical membrane proteins from *Synechocystis* sp. and *S. typhimurium*. This gene is immediately downstream of *dps* and the glutamine uptake operon, *glnHPQ* (Fig. 4), which are also induced in stationary-phase cultures. Combined with the *dps* gene (3) and the *glnHPQ* operon (52), these genes, *dps-glnHPQ-f786*, may constitute a large stationary-phase-specific operon.

The *rsd1076* mutation mapped to *o371*, a reading frame of unknown function, that is homologous to glucose dehydrogenase B from *Acinetobacter* sp. (14).

The *rsd1095* mutation mapped to the newly described *ecnB* locus, a gene coding for a bacteriolytic protein that may play a role in the selective elimination of moribund cells in stationary-phase populations (7) (Fig. 4). The expression of this gene is now known to be RpoS dependent (7). The *ecnB* gene was originally described as part of a longer gene, *sugEL*, a suppressor of GroEL chaperone function in *E. coli* (44). The *sugEL* gene was reported to have two promoters, one of which is induced in stationary-phase cells (44). An adjacent divergently transcribed reading frame encodes the Bcl lipoprotein, whose expression is also RpoS regulated (8).

One mutation (*rsd1081*) mapped to *yhiU* (Fig. 4), the first member of an operon encoding a probable two-member drug efflux pump that is homologous to AcrAB and EnvCD. A second fusion (*rsd1077*) mapped to *yhiV*, previously shown to be RpoS dependent (4). These membrane-bound complexes coordinate the energy-dependent transport of a wide variety of noxious compounds (for review, see reference 36).

Several of the other identified fusions were located in known RpoS-dependent genes, including *rsd1004*, which mapped to *ldcC*, encoding a lysine decarboxylase of *E. coli* (45, 49), and *rsd1082*, which mapped to *aidB* (49). The remaining fusions mapped to genes whose regulation was not previously known to be controlled by RpoS and will be reported elsewhere.

Expression of highly-RpoS-dependent genes in rich and minimal media. The expression of highly-RpoS-dependent fusions was examined in rich (Table 2) and in minimal (Table 3) media. The growth of the *rpoS* derivatives tested was similar to that of the *rpoS*$^+$ strains in both rich and minimal media. As expected, the expression of all promoter fusions was dependent on RpoS in the stationary phase in rich media. In the exponential phase, the expression of most fusions was several-fold higher in a wild-type strain than in an *rpoS* strain, suggesting that RpoS may be important for the expression of these genes in exponentially growing cells. This was true of cells growing in minimal medium (Table 3) as well as those grown in

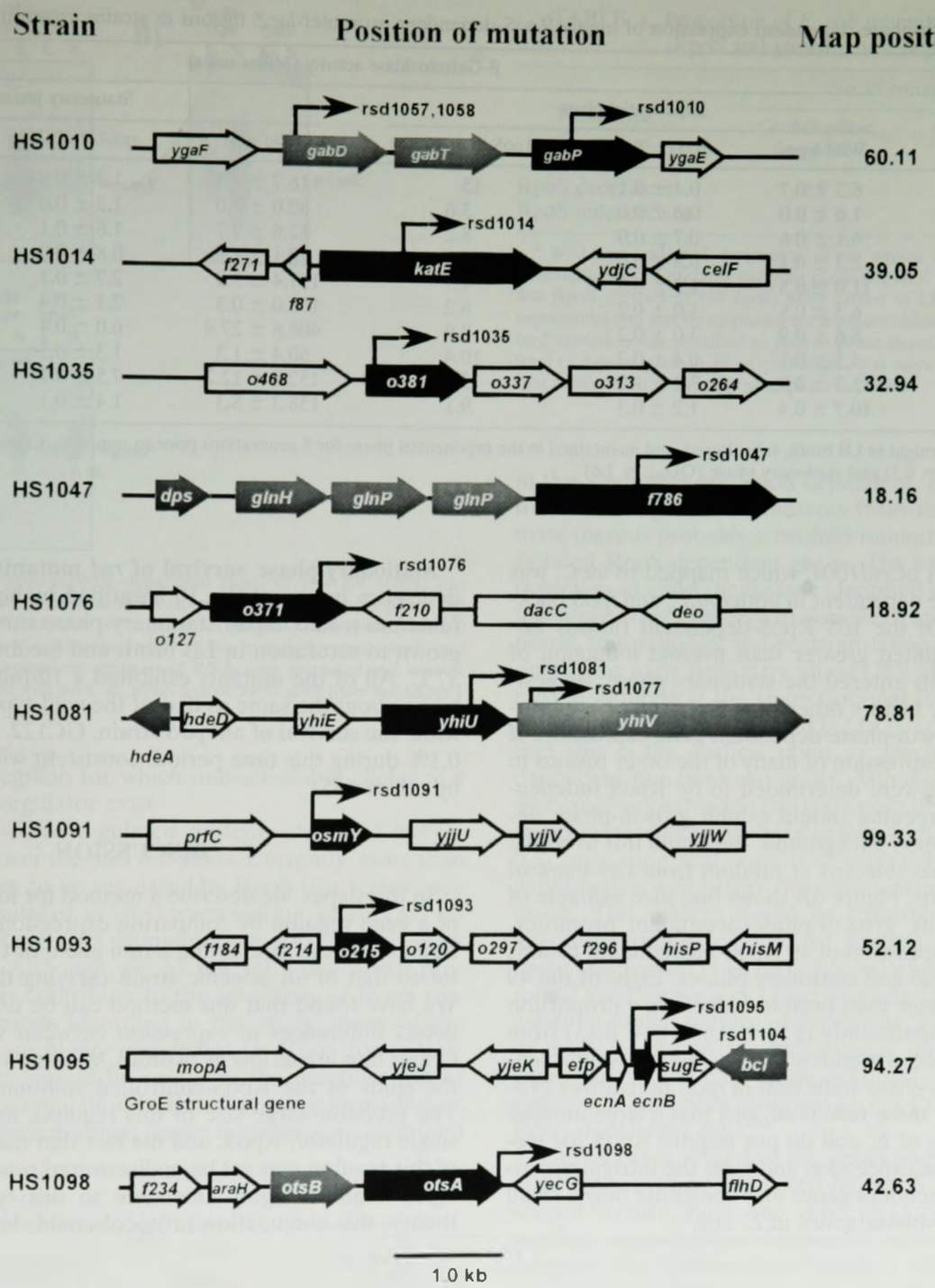

FIG. 4. Location of points of insertion of transcriptional fusions in RpoS-regulated genes identified in this study. Arrows indicate the direction of transcription of genes. ■, highly RpoS-dependent genes carrying the promoter-*lacZ* fusions identified in this study; ■, other RpoS-dependent genes (see text); □, genes not known to require RpoS for expression.

rich medium (Table 2). The expression of *rsd1095* (*ecnB*) was moderately growth-phase dependent, even in an *rpoS* background, as previously shown (18).

Levels of mRNA of identified genes expressed in the exponential and stationary phases of growth. To confirm that the identified promoter-*lacZ* fusions accurately represented RpoS-dependent gene control in a wild-type strain, RNA was prepared from wild-type (GC4468) and *rpoS* (GC122) strains sampled in the exponential (OD_{600} = 0.2) and stationary (OD_{600} = 1.6) phases and hybridized to PCR-amplified probes specific for *gabP*, *osmY*, and *katE* (Fig. 5). *katE* and *osmY*, two well-characterized RpoS-dependent genes, were highly expressed in the stationary phase in the wild-type strain, but they were expressed at low levels in the exponential phase and were largely absent in the *rpoS* strain (Fig. 5). Similarly, the expression of *gabP* was also growth phase dependent only in the wild-type strain and was expressed at low levels in an *rpoS* strain (Fig. 5). Since *gabP* is part of an operon that includes another identified *rsd* gene, *gabD* (Fig. 4), we also performed Northern analysis with a probe specific to *gabD*. As expected, the expression pattern of this gene was similar to that of *gabP* (data not shown). The expression of the other identified highly-RpoS-dependent genes was also confirmed, by Northern analyses, to be growth phase and RpoS dependent (data not shown).

RpoS-independent, growth-phase-dependent gene expression. Surprisingly, many of the fusions were found to be growth-phase dependent even in an *rpoS* background. For ex-

TABLE 2. Growth-phase-dependent expression of highly-RpoS-dependent promoter-*lacZ* fusions in strains grown in rich medium[a]

Mutation	Identified gene	β-Galactosidase activity (Miller units)					
		Exponential phase			Stationary phase		
		Wild type	*rpoS13*::Tn*10*	RpoS dependence	Wild type	*rpoS13*::Tn*10*	RpoS dependence
rsd1010	*gabP*	6.3 ± 0.7	0.4 ± 0.1	15	116.7 ± 8.8	1.3 ± 0.0	92.0
rsd1014	*katE*	1.6 ± 0.0	0.4 ± 0.0	3.6	60.0 ± 2.0	1.2 ± 0.0	52.0
rsd1035	*o381*	6.1 ± 0.6	0.7 ± 0.0	8.2	52.6 ± 7.7	1.6 ± 0.1	33.7
rsd1047	*f786*	2.3 ± 0.1	0.3 ± 0.0	8.6	40.1 ± 1.6	0.8 ± 0.0	52.4
rsd1076	*o371*	11.0 ± 0.8	1.5 ± 0.3	7.5	137.4 ± 3.9	2.7 ± 0.1	50.9
rsd1081	*yhiU*	6.3 ± 0.5	1.0 ± 0.2	6.2	108.0 ± 0.3	2.1 ± 0.4	50.7
rsd1091	*osmY*	8.6 ± 0.9	3.0 ± 0.2	2.9	408.8 ± 27.8	6.0 ± 0.4	67.6
rsd1093	*o215*	4.5 ± 0.1	0.4 ± 0.2	10.4	60.4 ± 1.3	1.3 ± 0.3	45.0
rsd1095	*ecnB*	11.3 ± 0.9	3.1 ± 1.0	3.8	152.8 ± 12.2	7.5 ± 0.1	20.4
rsd1098	*otsA*	10.7 ± 0.4	1.2 ± 0.3	9.1	158.3 ± 8.3	1.4 ± 0.1	115.3

[a] Strains were grown overnight in LB broth, subcultured, and maintained in the exponential phase for 8 generations prior to sampling. Cultures were sampled at the exponential phase (OD_{600} = 0.3) and stationary phase (OD_{600} = 1.6).

ample, the expression of *rsd1004*, which mapped to *ldcC*, was strongly growth-phase dependent in both *rpoS*$^+$ and *rpoS* backgrounds (Fig. 6A). Of the 105 RpoS-dependent fusions isolated, 15 strains exhibited greater than fivefold induction of β-galactosidase as cells entered the stationary phase, suggesting that regulation by factors other than RpoS may be important in control of growth-phase-dependent gene expression. If this is true, then the expression of many of the other fusions in the mutant bank that were determined to be RpoS independent in the initial screening should exhibit growth-phase dependence even in an *rpoS* background. We found this to be the case for many mutants selected at random from our bank of transcriptional mutants. Figure 6B shows one such example of an RpoS-independent, growth-phase-dependent promoter. We then examined expression of 49 RpoS-independent fusions in both the exponential and stationary phases. Eight of the 49 fusions exhibited greater than fivefold induction, a proportion that does not differ significantly ($\chi_{1,1}$ = 0.609, ρ = 0.43) from the proportion of RpoS-dependent fusions that showed a similar degree of growth-phase induction in *rpoS* derivatives (Table 4). Taken in toto, these results suggest that a large number of nonessential genes of *E. coli* do not require RpoS for elevated stationary-phase expression and raise the intriguing possibility that RpoS-dependent genes may constitute only a small fraction of stationary-phase genes in *E. coli*.

Stationary-phase survival of *rsd* mutants. To test whether deficiency in any of the 10 identified highly-RpoS-dependent functions would impair stationary-phase survival, cultures were grown to saturation in LB broth and incubated for 10 days at 37°C. All of the mutants exhibited a 10-fold reduction in viability, about the same as that of the wild-type strain (GC4468), while the survival of an *rpoS* strain, GC122, was approximately 0.1% during this time period, consistent with results obtained by others (55).

DISCUSSION

In this paper, we describe a method for identifying members of a gene regulon by comparing expression of *lacZ* fusions in transconjugants containing a null allele of the putative regulator to that of an isogenic strain carrying the wild-type allele. We have found that this method can be used to reproducibly detect differences in expression between wild-type and *rpoS* strains that are as low as twofold. We employed this method in the study of the RpoS-controlled stationary-phase response. The probable large size of this regulon, its dependence on a single regulator, RpoS, and the fact that many of the members of this regulon may yet be undiscovered render the study of the RpoS regulon highly amenable to this type of analysis. In theory, this conjugation protocol could, however, be used to

TABLE 3. Growth-phase-dependent expression of highly-RpoS-dependent promoter-*lacZ* fusions in strains grown in glucose minimal medium[a]

Mutation	Identified gene	β-Galactosidase activity (Miller units)					
		Exponential phase			Stationary phase		
		Wild type	*rpoS13*::Tn*10*	RpoS dependence	Wild type	*rpoS13*::Tn*10*	RpoS dependence
rsd1010	*gabP*	8.5 ± 0.2	0.9 ± 0.1	9.0	43.0 ± 9.0	1.4 ± 0.0	30.5
rsd1014	*katE*	7.9 ± 1.4	0.5 ± 0.1	15.4	53.8 ± 6.8	1.3 ± 0.1	42.7
rsd1035	*o381*	14.4 ± 0.9	0.6 ± 0.0	23.2	66.4 ± 3.4	0.9 ± 0.1	74.2
rsd1047	*f786*	5.1 ± 1.1	0.5 ± 0.1	10.6	38.3 ± 3.2	0.6 ± 0.1	63.0
rsd1076	*o371*	9.8 ± 0.7	1.0 ± 0.1	9.6	72.2 ± 7.4	1.9 ± 0.1	40.1
rsd1081	*yhiU*	13.2 ± 1.2	1.6 ± 0.2	8.1	53.1 ± 0.3	3.0 ± 0.4	17.4
rsd1091	*osmY*	12.8 ± 0.1	2.1 ± 0.2	6.2	107.9 ± 7.8	2.3 ± 0.1	46.3
rsd1093	*o215*	13.6 ± 1.0	0.7 ± 0.1	19.2	79.7 ± 15.5	0.9 ± 0.1	86.4
rsd1095	*ecnB*	5.9 ± 0.3	2.0 ± 0.1	2.9	95.6 ± 7.5	4.4 ± 0.2	21.8
rsd1098	*otsA*	10.1 ± 1.6	2.0 ± 0.1	4.8	98.2 ± 3.6	2.9 ± 0.2	34.2

[a] Strains were grown overnight in minimal medium, subcultured, and maintained for 8 generations in the exponential phase prior to sampling. Cultures were sampled in the exponential phase (OD_{600} = 0.2) and stationary phase (OD_{600} = 0.9).

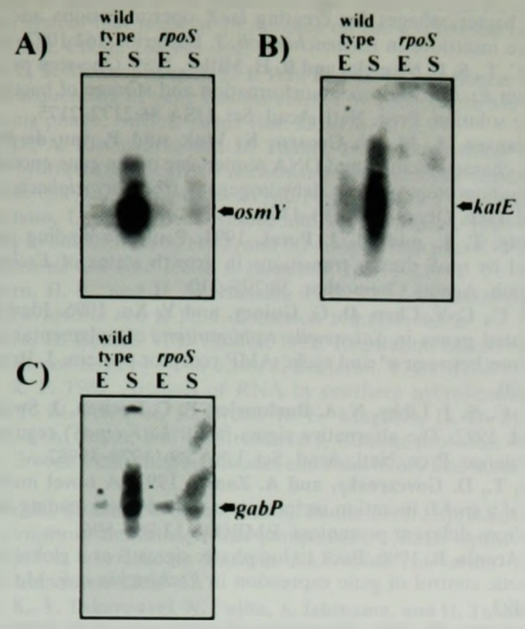

FIG. 5. RpoS- and growth-phase-dependent expression of *rsd* genes. The results of Northern analyses of total RNA isolated from exponential-phase (E) and stationary-phase (S) cultures of wild-type (GC4468) and *rpoS* (GC122) strains are shown. RNA was hybridized with probes specific for *osmY*, *katE*, and *gabP*. To confirm that equivalent amounts of RNA were extracted and loaded, control blots were probed with *rrnA*, an RpoS-independent gene (data not shown).

characterize any regulon for which null-selectable alleles in a single controlling regulator exist.

The number of RpoS-regulated proteins identified has increased markedly over the past few years. Currently, more than 40 genes are known to be regulated by RpoS (for review, see reference 20). Results of in vitro transcription assays (34) indicate that many RpoS (σ^S) promoters are also recognized by RpoD (σ^{70}) (45). Although we do not yet know which promoter determinants contribute to the specificity of RpoS recognition of the fusions identified in this study, many of the fusions identified are highly dependent on RpoS for expression. As such, they should be useful in the identification of factors important in regulation by this alternative sigma factor.

A total of 105 transcriptional fusion mutants were identified

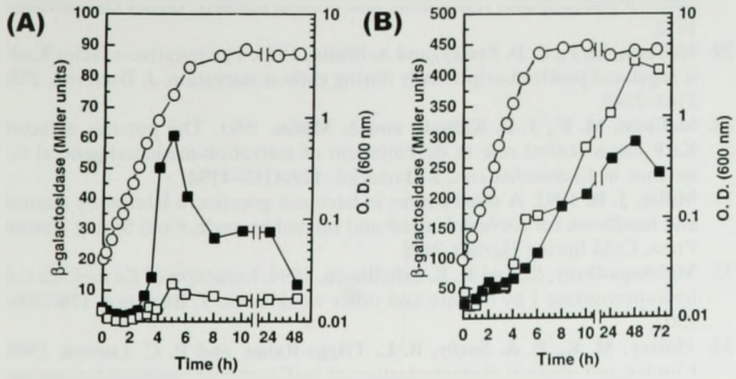

FIG. 6. Growth-phase-dependent expression of an RpoS-dependent fusion and an RpoS-independent fusion. Flasks containing LB broth were inoculated with exponentially growing cultures as described in Materials and Methods, sampled periodically as indicated, and assayed for growth (OD_{600}) and β-galactosidase activity. Each panel shows growth of the culture and β-galactosidase activity in strains carrying promoter-*lacZ* fusions to the indicated gene. The levels of growth of the wild-type strain and *rpoS* derivatives were equivalent, and thus only growth data for the wild-type strain are shown. (A) *rsd1004* (*ldcC*) (RpoS dependent). (B) 13C10 (RpoS independent). ○, growth (OD_{600}); ■, β-galactosidase activity in the *rpoS*$^+$ strain; □, β-galactosidase activity in the *rpoS* derivative.

TABLE 4. Proportion of *E. coli* mutants whose expression is RpoS and growth-phase dependent[a]

Fusion type	No. of mutants with expression:		
	Growth-phase dependent	Growth-phase independent	Total
RpoS dependent	15	90	105
RpoS independent	8	41	49

[a] Wild-type and *rpoS* derivatives of *rsd* strains (105 total) and a randomly selected group of mutants carrying promoter-*lacZ* fusions whose expression is not RpoS dependent (49 total) were grown in LB broth and assayed in the exponential and stationary phases for β-galactosidase activity. A given promoter-*lacZ* mutation was classified as growth-phase dependent if the stationary-phase level of β-galactosidase, in an *rpoS* strain, was more than five times greater than that observed in the exponential-phase cultures.

in this study that are RpoS dependent. Based on our previous work isolating catalase mutants from this bank (42), we estimate there is probably a twofold redundancy in the number of isolated RpoS-dependent genes. The early-exponential-phase induction of all of the fusions identified was somewhat surprising, since RpoS, subject to complex controls at the transcriptional, translational, and posttranslational levels, is fully active only in the early stationary phase (28). However, expression of *rpoS* is induced at the transcriptional level early in the exponential phase at an OD_{600} of 0.2 (42), consistent with the idea that this is the earliest point at which induction of RpoS-dependent functions can occur. Although maximal expression was usually observed in the stationary phase, the expression of all promoter fusions isolated in this study began at an OD_{600} of 0.3, suggesting that concerted expression of RpoS-regulated genes begins well before the commencement of the stationary phase. This pattern would be consistent with the idea that adaptive proteins required for survival during periods of nutrient deprivation must be produced while the cell is capable of robust gene expression. Other identified RpoS-regulated genes exhibit a similar pattern of expression. For example, induction of *dnaN* begins in the exponential phase but is maximally expressed in stationary-phase cultures (48). Similar patterns of expression have been observed for *bolA* (9, 23), another highly-RpoS-dependent gene.

The fact that several of the genes identified were not previously known to be regulated by RpoS may be explained by several factors. First, the gene product may be masked by another compensatory functional activity with the cell. For example, the physiological function of the *ecnB/sugE* gene product is probably masked in cells that produce GroEL, the major chaperonin in *E. coli*. A second possible explanation is that some proteins are expressed at levels too low to measure—LdcC, a second lysine decarboxylase in *E. coli*, is detectable only when expressed on a multicopy plasmid (24). Finally, the gene of interest may be one of the many ORFs (currently more than half of all ORFs) in *E. coli* that have not been assigned any function and thus not been previously studied (e.g., *o371*).

The fact that a large proportion of fusions in the mutant bank were found to be growth-phase regulated (both RpoS dependent and RpoS independent) cannot readily be explained by current models of growth-phase regulation. We estimate that almost 20% of the mutants in the bank (~1,000) carry growth-phase-inducible fusions, a relatively small fraction (105/1,000 [~10%]) of which are RpoS dependent. This suggests that a large proportion of the bacterium's genetic repertoire is involved in adaptation to nutrient deprivation or to some other growth-phase-related stimulus. The non-RpoS-

dependent component of this response has thus far received little attention, but its characterization is undoubtedly critical in understanding how bacteria adapt to suboptimal conditions. There are probably other transcriptional factors besides RpoS that lead to increased expression of certain genes during the stationary phase. Transcriptional control of "gearbox" promoters (9, 47) is tightly coupled to growth rate, and one of these promoters is known to be RpoS independent (9, 26). Additional sequence analysis and primer extension studies are required to determine if the promoters of the *rsd-lacZ* fusions are homologous to the proposed gearbox consensus promoter sequence (47). Factors affecting posttranslational stationary-phase expression have been described and include alterations in ribosome assembly (50) and differential protein degradation (28). These are, however, unlikely to be involved in the regulation of the fusions isolated in this study, since the mutagen employed (λp*lac*Mu) generates transcriptional promoter fusions (12).

The characterization of other fusion mutations isolated in this study should aid in the identification of genes that are expressed specifically in the stationary phase and may provide additional clues regarding the regulation and physiological function of the RpoS regulon.

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CHAPTER 3.

PREFACE

The following chapter entitled: "Other *Escherichia coli* RpoS-dependent genes identified in this study" is a continuation of work done by L. Wei (M.Sc. Thesis).

Jonathon P. Audia performed all of the Northern blot experiments and isolated DNA for sequencing of all of the RpoS-dependent promoter-*lacZ* fusions presented here.

Jonathon P. Audia, B.Sc. (Hons.)
December, 1998.

CHAPTER 3

3.1 Introduction

Free-living bacteria such as *Escherichia coli* exist in a constantly changing environment in which nutrients can be scarce and toxins may be present in growth-limiting concentrations. This phase of growth is referred to as stationary phase (see Chapter 1). The alternative sigma factor, RpoS, is a central regulator of genes expressed during this phase of growth (Lange and Hengge-Aronis, 1991; Mulvey and Loewen, 1989) that may provide resistance to environmental stresses and help extend the viability of the bacterium. The previous chapter detailed a screen whose goal was to identify novel members of the RpoS regulon using promoter-*lacZ* transcriptional gene fusions (see Chapter 2). The data presented here is an extension of that work which will report the identities of other RpoS-dependent genes, confirm their RpoS-dependence by Northern analysis and attempt to speculate on the possible functions of some of the ORFs of unknown function based on homology to proteins in other organisms.

3.2 Material and Methods

3.2.1 Bacterial strains and plasmids

All strains and plasmids used are listed in Table 1.

3.2.2 Chemicals, media, and growth conditions

All chemicals were purchased from either Sigma Chemical (St. Louis, MO), Gibco BRL (Burlington, ON) or Fisher Scientific Ltd. (Toronto, ON). Luria Bertani (LB) media was prepared as described (Miller, 1992). Antibiotics were added to growth media at the following concentrations: ampicillin, 100 μg/ml; streptomycin, 100 μg/ml;

Table 1. E. coli *strains and plasmids*.

Strains	Genotype	Source/Reference
A) Strains		
GC4468	Δ*lacU*169 *rpsL*	laboratory collection
NC122	as GC4468 but Φ(*katE::lacZ*$^+$)131 and *rpoS*::Tn*10*	laboratory collection
NC122(pMM*katF3*)	as NC122 but pMM*katF3*	laboratory collection
HS1001-HS1105	as GC4468 but carrying RpoS-dependent promoter-*lacZ* transcriptional fusions	L. Wei, M.Sc. Thesis
HS1001T-HS1105T	as HS1001-HS1105 but *rpoS*::Tn*10*	L. Wei, M.Sc. Thesis
B) Plasmids		
pMM*katF3*	carries a functional *rpoS* allele	(Mulvey et al., 1988)

kanamycin, 50 μg/ml; tetracycline, 15 μg/ml. The indicator dye 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was added at a concentration of 50 μg/ml.

For growth experiments, cultures were grown at 37 ^{O}C (with aeration at 200 rpm) overnight in LB media with appropriate antibiotics, subcultured the next morning and subsequent samples were removed for assay as described in the figure legends. All cultures were maintained in exponential phase (OD_{600}<0.2) for at least 8 generations prior to the start of any experiment. Culture growth was monitored by measuring sample density at 600 nm (OD_{600}) using a UV-VIS Spectrophotometer (model UV-1201, Shimadzu Co., Japan).

3.2.3 DNA extraction and genetic techniques

All RpoS-dependent promoter-*lacZ* fusions were identified and characterized as previously-described (L. Wei, M.Sc. Thesis). Excision of the lambdoid prophage was induced by exposing cultures harboring RpoS-dependent promoter-*lacZ* fusions to UV radiation and DNA was extracted from the lysate and sequenced as described (Roy et al., 1995) with the following modifications. A single clone was inoculated into LB media containing streptomycin and kanamycin and was grown overnight (with aeration at 200 rpm) at 37 ^{O}C. Cells were subcultured into 50 ml fresh media (1/10) the next morning, grown to an OD_{600} of 0.4, collected by centrifugation (3,200 rpm, 4 ^{O}C for 15 min) and resuspended in 10 ml 10 mM $MgSO_4$. The culture was spread evenly onto a 150 x 15 mm Petri plate and induction of the fusion-containing prophage was performed by irradiating the culture at 25 mW for 7 s (approximately 35 J/m^2). Five ml of warmed 3xLL media was added and the culture was incubated at 37 ^{O}C with gentle agitation until

lysis was observed (usually 3-5 h). Cell debris was removed by centrifugation (3,200 rpm, 4 ^{O}C for 15 min) and the supernatant was transferred to a 50 ml polypropylene FALCON tube (Becton Dickinson Labware, Lincoln Park, NJ). Several drops of chloroform was added and the phage lysate was mixed vigorously on a vortex. Cell debris removed by centrifugation (3,200 rpm, 4 ^{O}C for 15 min) and the phage-containing supernatant was removed and stored at 4 ^{O}C. Phage lysate titres usually ranged between 10^5 - 10^6 p.f.u./ml.

Fifty ml of λ host strain LE392 was grown overnight in LB media containing 10 mM $MgSO_4$ in a 37 ^{O}C waterbath without aeration. The next morning 2 ml of lysate was added to 5 ml of LE392 and incubated for 10 min at room temperature to allow for phage adsorption. This was added to 350 ml of warmed LB (with 10 mM $MgSO_4$) and incubated at 37 ^{O}C (with aeration at 200 rpm) until lysis was observed (usually 6 - 10 h). Ten grams of NaCl (~ 0.5 M) and 1 ml of $CHCl_3$ were added and the culture was incubated for another 10 min under the above conditions. Lysates were transferred into 500 ml polypropylene Nalgene bottles (Fisher Scientific, Ltd., Ottawa, ON) and cell debris was removed by centrifugation at 6000 rpm, 4 ^{O}C for 15 min. Lysates were carefully transferred to clean 500 ml Nalgene bottles and 10 % (w/v) PEG 8000 was added. Lysates were then stored on ice for 1 h after thorough mixing. The phage-containing PEG 8000 pellet was collected by centrifugation (6000 rpm, 4 ^{O}C for 15 min). The supernatant was decanted completely and the phage-containing pellet was resuspended in 5 ml of TM buffer. Lysates were transferred to 15 ml polypropylene tubes (Sarstedt, Newton, NC) and the PEG 8000 was removed by extraction with equal

volumes of $CHCl_3$ several times. DNase (80 µg/ml final concentration) and RNase (200 µg/ml final concentration) were added to remove traces of chromosomal DNA and bacterial RNA. Phage DNA was isolated as previously described (Roy et al., 1995) and quantified spectrophotomerically at OD_{260}. Sequencing of DNA prepared from UV-induced lysates was performed using a 25-mer primer, 5' CCCGAATAATCCAATGTCCTCCCGG 3' located 30 nucleotides from the Mu c end (Roy et al., 1995). DNA sequencing was performed by the MOBIX central facility at McMaster University. Approximately 500 - 1000 ng of DNA was used in each sequencing reaction. All RpoS-dependent sequences were identified by comparison to the Genbank database using the BLAST algorithm (Altschul et al., 1990).

3.2.4 Northern blot analysis and amplification of PCR amplified probes

Growth of bacterial cultures was carried out as described in section 3.2.2 and aliquots were removed as indicated in the figure legends. Total bacterial RNA was extracted using a RNeasy Midi Kit (QIAGEN, Valencia, Ca.), quantified by measuring the absorbency at 260 nm (an absorbency of 1 OD unit at 260 nm corresponds to 40 µg of single stranded RNA per ml), and stored at -80 ^{o}C. Northern analysis was performed on RNA extracted from exponential and stationary phase cultures of wild type, *rpoS* and pMM*katF*(*rpoS*)*3*-complemented *rpoS*$^{-}$ strains using standard methods (Seldon, 1989). RNA was transferred, overnight, onto BIOTRANS Nylon Membranes (ICN, Irvine, Ca.) by the capillary action transfer method described in (Seldon, 1989) and fixed by baking at 80 °C for 2 h. Pre-hybridization and hybridization was carried out at 42 °C with gentle agitation. Control blots were hybridized with a DNA probe specific to an RpoS-

independent gene (*rrnA*) to confirm equivalence of loading and efficacy of transfer.

Oligonucleotide primers (listed in Appendix II, Table 1) were synthesized by the MOBIX central facility to generate specific, Polymerase Chain Reaction (PCR)-amplified probes to detect mRNA transcripts of putative RpoS-dependent genes by Northern blot analysis. All PCR tubes contained 1x PCR buffer (50 mM KCl, 20 mM Tris pH 8.4), 50 pmole of each of the forward and reverse primers, 0.4 mM of each of the four dNTPs, 4 mM $MgCl_2$, ~50 ng of *E. coli* DNA and ~50 U of Taq polymerase in a final volume of 50 µl. Reactions were run for 25 cycles under the following conditions: (1) 96 °C for 30 s; (2) 61 °C for 60 s; (3) 72 °C for 90 s. PCR products were separated on 1.2 % agarose gels to verify the size of each probe. The sequence of each probe was verified by DNA sequencing performed at the MOBIX central facility. PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN, Valencia, CA) and radiolabelled with [α-^{32}P]-dCTP (NEN Life Science Products, Inc. MA) by random-primed labeling.

3.2.5 Protein and DNA sequence analysis

DNA sequence analysis was performed using the BLASTN algorithm (Altschul et al., 1998). Protein sequence alignments were performed using BLASTX (Altschul et al., 1998) and, where appropriate, the CLUSTAL IV multiple sequence alignment (Thompson et al., 1994) (http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html).

3.3 Results

3.3.1 Identification of RpoS-dependent genes

λ DNA was prepared and sequenced from strains carrying RpoS-dependent

promoter-*lacZ* fusions as described in section 3.2.3. RpoS-dependent genes identified in this study are described in Table 2. Some of the RpoS-dependent promoter-*lacZ* fusions identified in this study were isolated in *E. coli* ORFs of unknown function. The nucleic acid and corresponding amino acid sequences of these ORFs were searched against the Genbank database for homology to genes or proteins in other organisms. Figure 1 is a typical result of a homology search and alignment using the CLUSTAL IV program. Amino acid sequence alignment predicts that the RpoS-dependent, *E. coli* ORF *f430*, shows homology at the protein level to a hypothetical *Bacillus subtilis* protein (Fig. 1a) and to a *Synechocystis* sp. signal transduction protein (Fig. 1b). All searches and alignments were performed using the algorithms mentioned in section 3.2.5 and the results of the other searches are described in Table 2. The map positions, locations of the fusion junctions, and position relative to other *E. coli* genes of RpoS-dependent genes identified in this study (and not reported in Chapter 2) are outlined in Figure 2.

3.3.2 Northern blot analysis of RpoS-dependent gene expression in exponential and stationary phase cultures.

A limitation of utilizing the λp*lac*Mu vector system described in Chapter 2 has been the inability to verify the fusion junction at the 5' end of the gene fusion. This problem occurs as a result of a stable stem-loop structure that is present in the Mu S end of the vector (Bremer et al., 1985) which can impair DNA polymerase-read through in a sequencing reaction. To confirm the RpoS-dependence of the genes identified in this study, we tested the mRNA levels of several RpoS-dependent genes in wild type and *rpoS* mutant strains sampled in exponential and stationary phase to verify the RpoS-

Table 2. Characteristics of RpoS-dependent genes identified in this study.

Mutation	Gene	Known or Postulated Function
rsd1022	*narY*	Encodes the β-subunit of the second nitrate reductase, NRZ of *E. coli*, paralogous to NRA (Bonnefoy et al., 1997).
rsd1024	*katE*	Encodes the catalase HPII. Previously shown to be RpoS-dependent (Mulvey et al., 1988; Sak et al., 1989; Schellhorn and Hassan, 1988).
rsd1033	*f430*	The hypothetical protein shows homology to a *Bacillus subtilis* protein (YhcK) and to a response regulator protein in *Synechocystis* sp. (see Fig. 3).
rsd1046	*o190*	This gene is not homologous to any known bacterial gene or protein.
rsd1056	*f60*	This gene is not homologous to any known bacterial gene or protein.
rsd1057 *rsd1058*	*gabD*	Encodes succinate semialdehyde dehydrogenase and is another member of the GABA operon along with *gabP* (and *gabT*) (see Chapter 2).
rsd1082	*aidB*	Encodes a protein that is homologous to several mammalian acyl coenzyme A dehydrogenases and is alkylation damage-inducible (Landini et al., 1994).
rsd1085	*yebF*	This gene is not homologous to any known bacterial gene or protein.
rsd1098	*otsA*	Encodes trehalose synthase and has been previously shown to be RpoS-dependent (Hengge-Aronis et al., 1991).

Figure 1 Sequence alignment analysis of the RpoS-dependent *Escherichia coli* ORF *f430* using the CLUSTAL IV alignment algorithm. The amino acid sequence of the hypothetical F430 protein was subjected to a homology search against the Genbank database. This protein shows homology to a hypothetical *Bacillus subtilis* protein, YhcK **a)** and to a *Synechocystis* sp. signal transducer **b)**. Common amino acids are denoted with an asterix.

a)

```
    E.coli F430   MTWLFCDRIATKIDK NNGISMITHNFNTLD LLTSPVWIVSPFEEQ LIYANSAAKLLMQDL TFSQLRTGPYSVSSQ KELPKYLSDLQNQHD
B.subtilis YhcK   --------------- --------------- --------------- --------------- --------------- ---------------

    E.coli F430   IIEILTVQRKEEETA LSCRLVLRKLTETEP VIIFEGIEAPATLGL KASRSANYQRKKQGF YARFFLTNSAPMLLI DPSRDGQIVDANLAA
B.subtilis YhcK   --------------- --------------- --------------- --------------- --------------- ---------------

    E.coli F430   LNFYGYNHETMCQKH TWEINMLGRRVMPIM HEISHLPGGHKPLNF VHKLADGSTRHVQTY AGPIEIYGDKLMLCI VHDITEQKRLEEQLE
B.subtilis YhcK   --------------- --------------- --------------- ---LIDLHSYELHLY FWIISIAGGMLSLYI IDHETNAHLLFKQYK
                                                                  * *       *    * * * * * *     *   *  *
    E.coli F430   HAAHHDAMTGLLNRR QFYHITEPG-QMQHL AIAQDYSLLLIDTDR FKHINDLYGHSKGDE VLCALARTLESCARK GDLVFRWGGEEFVLL
B.subtilis YhcK   FQAHFDFLTGVYNRR KFEETTKALYQQAAD TPHFQFALIYMDIDH FKTINDQYGHHEGDQ VLKELGLRLKQTIRN TDPAARIGGEEFAVL
                  ** *  **  *** *  *   *          *   *** *  *** *** **  ** *  *   *   *    *****  *
    E.coli F430   LPRTPLDTALSLAET IRVSVAKVSISGLP- ---RFTVSIGVAHHE GN-ESIDELFKRVDD ALYRAKNDGRNRVLA A-
B.subtilis YhcK   LPNCSLDKAARIAER IRSTVSDAPIVLTNG DELSVTISLGAAHYP NNTEQPGSLPILADQ MLYKAKETGRNRV-- -
                  **   ** *   **  **  *   *          * * * **   * *   *   *   ** **  *****
```

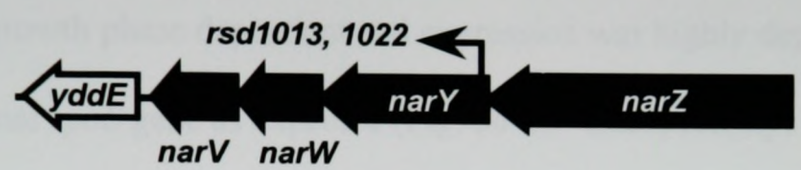
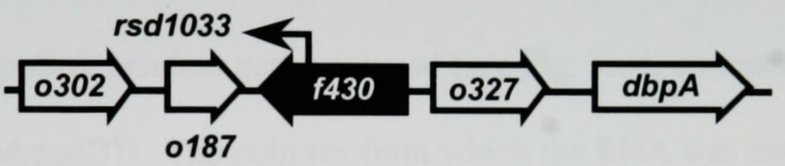
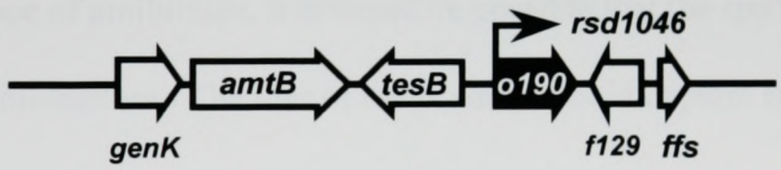
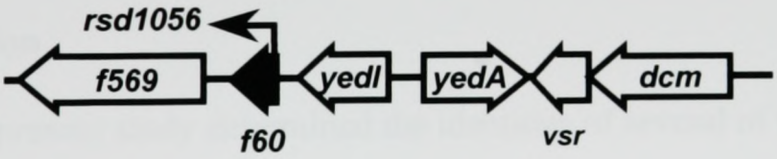
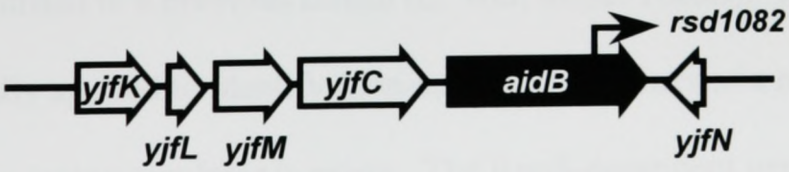
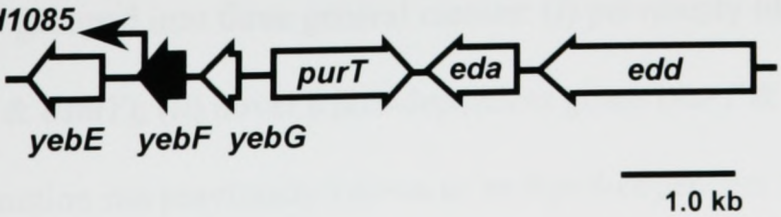
b)

```
    E.coli F430   MTWLFCDRIATKIDK NNGISMITHNFNTLD LLTSPVWIVSPFEEQ LIYANSAAKLLMQDL TFSQLRTGPYSVSSQ KELPKYLSDLQNQHD
Synechocystis sp. --------------- --------------- --------------- --------------- --------------- ---------------

    E.coli F430   IIEILTVQRKEEETA LSCRLVLRKLTETEP VIIFEGIEAPATLGL KASRSANYQRKKQGF YARFFLTNSAPMLLI DPSRDGQIVDANLAA
Synechocystis sp. --------------- --------------- --------------- --------------- --------------- ---------------

    E.coli F430   LNFYGYNHETMCQKH TWEINMLGRRVMPIM HEISHLPGGHKPLNF VHKLADGSTRHVQTY AGPIEIYGDKLMLCI VHDITEQKRLEEQLE
Synechocystis sp. --------------- --------------- --------------- --------------- --------VRDITDR KRMELELQAANQQLQ
                                                                                              *     **
    E.coli F430   HAAHHDAMTGLLNRR QFYHITEPGQMQHLA IAQDYSLLLIDTDRF KHINDLYGHSKGDEV LCALARTLESCARKG -DLVFRWGGEEFVLL
Synechocystis sp. LLANIDGLTHIANRR RFDEYLAQEWQRHCR EQKPLSLILVDIDYF KAYNDLYGHQKGDDC LQKVANTLVDIAKRI TDLVARYGGEEFVIV
                  * * * *** *          *       ** * * * * *  ****** ***   *   * **   *     *** * ******
    E.coli F430   LPRTPLDTALSLAET IRVSVAKVSISG--- --LPRFTVSIGVAHH E--GNESIDELFKRV DDALYRAKNDGRNRV LAA-
Synechocystis sp. LPNTNRQDALAMAEN MLQAIAALAIPHEGS SVSKYVTISIGVSSI IPMPEDTIERIISEA DQALYSAKSQGRNRA IA--
                  ** *    **  **      *   *          * ****          *        * *** **  ****   *
```

Figure 2 RpoS-dependent promoter-*lacZ* fusions isolated in this study. The location of the point of insertion of the fusion, the map position, and location with respect to other genes on the *Escherichia coli* chromosome. Arrows indicate the direction of transcription of the ORFs shown. RpoS-dependent ORFs are shown in black and the position of the fusion is represented by the thin arrows. Other *E. coli* ORFs are shown in white.

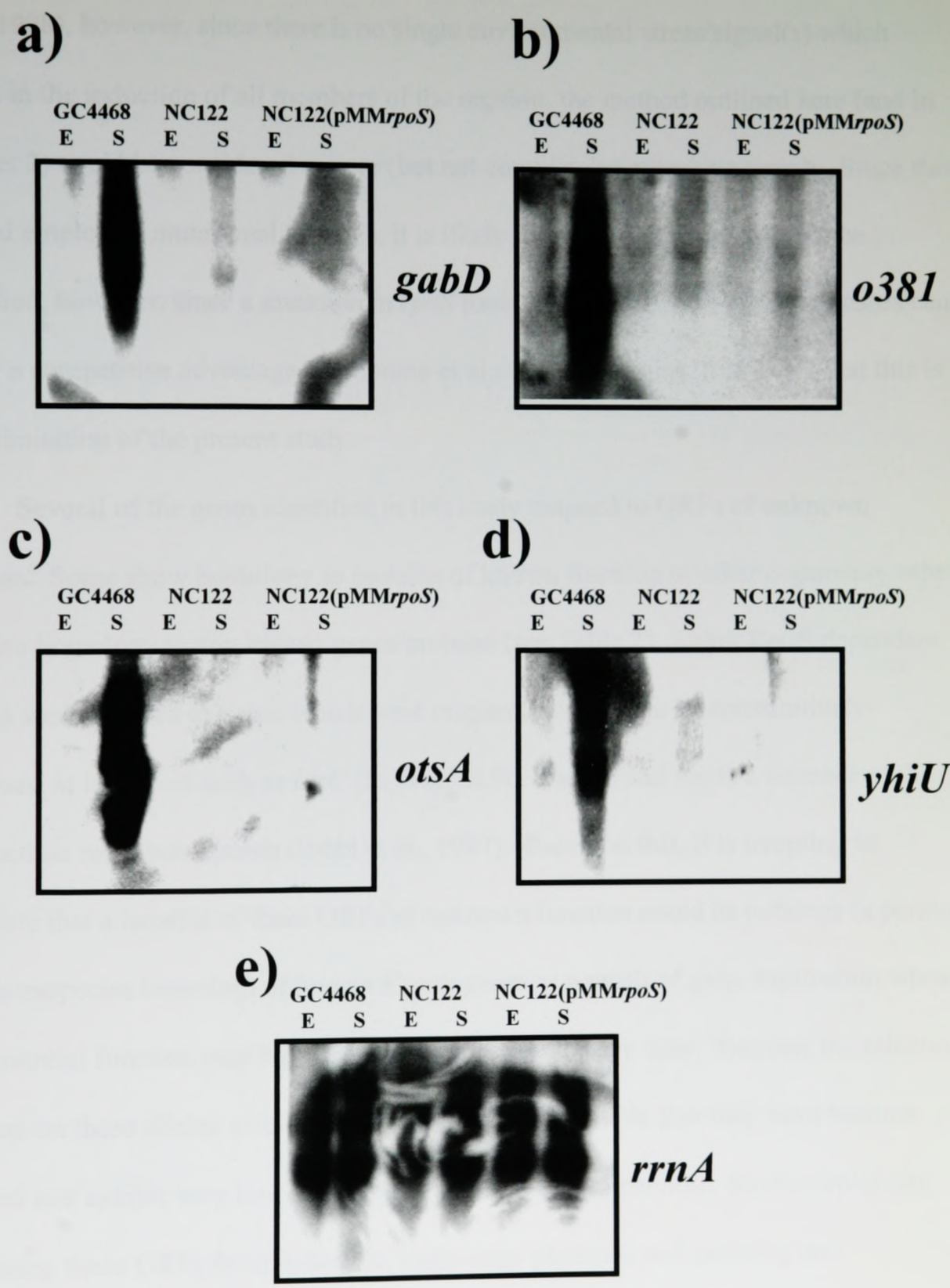
Strain	Position of mutation	Map position
HS1022		33.09
HS1033		30.27
HS1046		10.23
HS1056		43.67
HS1082		95.08
HS1085		41.55

dependence of these genes. Total bacterial RNA was isolated and hybridized to PCR-amplified probes specific to several RpoS-dependent genes. RNA loading and transfer efficacy was verified by probing a control blot with a DNA probe specific to an RpoS-independent gene, *rrnA* (Fig. 3e). The expression of these putative RpoS-dependent genes was growth phase dependent and expression was highly dependent on the presence of a functional *rpoS* gene as expected (Fig. 3a-d). Unexpectedly, an *rpoS* mutant strain transformed with pMM*rpoS3* did not demonstrate complete restoration of expression of any of the RpoS-dependent genes assayed (see Fig. 3a-d, compare GC4468 and NC122(pMM*rpoS*3)). The cultures from which the RNA was extracted were not grown in the presence of antibiotics, it is therefore possible that the *rpoS*-containing plasmid was cured from this culture. The loss of the plasmid would explain the non-complementation result in Figure 3a-d.

3.4 Discussion

The present study determined the identities of several of the RpoS-dependent mutants identified in a previous screen (L. Wei, M.Sc. Thesis). This screen could be more generally applied to identify (non-essential) members of a regulon/stimulon for which a trans-acting regulator is exists. The RpoS-dependent genes identified in this study can be grouped into three general classes: (*i*) previously identified RpoS-dependent genes (*katE* & *osmY*); (*ii*) novel RpoS-dependent genes (*narY* & *gabP*); and (*iii*) ORFs of unknown function not previously known to be RpoS-dependent (*f430*). Other efforts to identify members of the RpoS regulon have employed phenotype-dependent screens (see Chapter1, section 1.2) such as identification on the basis of carbon starvation induction

Figure 3 Expression of RpoS-dependent mRNA transcripts in wild type, *rpoS*, and *rpoS*-complemented strains confirm the RpoS-dependence of genes identified in this study as determined by Northern analysis. Overnight cultures of wild type (GC4468), *rpoS* (NC122), and *rpoS*-complemented (NC122 pMM*rpoS*3) were subcultured into LB media and maintained in exponential phase for eight generations prior to the start of the assay. Cultures were sampled in exponential (E) phase (OD_{600}~0.2) and in stationary (S) phase (OD_{600}~1.6) and total bacterial RNA was isolated. Specific PCR-amplified DNA probes (labeled with [α^{32}P]-dCTP) for the *gabD* **a)**, *o381* **b)**, *otsA* **c)**, and *yhiU* **d)** genes were hybridized to total RNA. To confirm equivalent RNA loading and transfer, a separate blot was hybridized with a control probe specific to the RpoS-independent *rrnA* gene **e)**.


a)
GC4468 NC122 NC122(pMMrpoS)
E S E S E S
gabD
b)
GC4468 NC122 NC122(pMMrpoS)
E S E S E S
o381
c)
GC4468 NC122 NC122(pMMrpoS)
E S E S E S
otsA
d)
GC4468 NC122 NC122(pMMrpoS)
E S E S E S
yhiU
e)
GC4468 NC122 NC122(pMMrpoS)
E S E S E S
rrnA

(Weichart et al., 1993) and identification on the basis of internal acidification (Van Dyk et al., 1998), however, since there is no single environmental stress/signal(s) which results in the induction of all members of the regulon, the method outlined here (and in Chapter 2) could be considered a more (but not completely) extensive search. Since this method employs a mutational analysis, it is likely that essential genes will not be identified, however, since a mutation in *rpoS* itself is non-lethal and in several cases can confer a competitive advantage (Zambrano et al., 1993) to strains, it is likely that this is not a limitation of the present study.

Several of the genes identified in this study mapped to ORFs of unknown function. Some show homology to proteins of known function in other organisms, others show no homology to any known genes/proteins (see Table 2). Other RpoS-dependent fusions were isolated to genes which were originally thought to be constitutively-expressed at low level such as *ldcC* (L. Wei, M.Sc. Thesis) and *narY*, a member of the NRZ nitrate reductase operon (Iobbi et al., 1987). Based on this, it is tempting to speculate that a handful of these ORFs of unknown function could be paralogs (a paralog is an intraspecies homolog) of known *E. coli* genes as a result of gene duplication whose non-essential function may have been lost over evolutionary time. Because the selection pressure on these alleles would be quite low, it is conceivable that they have become mutated and exhibit very low similarity to their functional paralog. Studies involving expressing these ORFs from inducible, multi-copy plasmids and isolating and determining the function (if any) of these hypothetical proteins would be a step in testing this hypothesis.

The importance of the RpoS-dependent genes identified in this study is underscored by the idea that this bank of mutants can potentially be utilized as the basis the study of other features of the regulon. Several factors have been identified that co-regulate RpoS-dependent genes such as Fis, cAMP-CRP, and the leucine-responsive protein (Lrp) (see Chapter 1, section 1.3). Utilizing a similar strategy of mating or transducing null alleles of these trans-acting regulators into our bank of RpoS-dependent fusions and scoring for reduced β-galactosidase activity would be amenable to a study of this type. This would provide a quick means of characterizing newly identified members of the regulon.

Although many members of the RpoS regulon have been identified to date, there is a paucity of information about the features that constitute an RpoS-recognized promoter. Computer-based studies have demonstrated that there is a -10 consensus (5'-CTATACT-3') similar to that recognized by the 'housekeeping' sigma factor, RpoD (Espinosa-Urgel et al., 1996). Mutational and *in vitro* studies (Utsumi et al., 1995; Wise et al., 1996) demonstrated that there may be required nucleotides (a CC pair) in the -35 region. Other studies revealed that intrinsic curvature in that region is indicative of an RpoS-recognized promoter region (Espinosa-Urgel and Tormo, 1993). Our identified bank of RpoS-dependent promoter-*lacZ* fusions may provide a basis for further study of these promoter elements. The RpoS-dependence of many of the genes identified in other studies ranges from less than two-fold (e.g. *hmp* (Membrillo-Hernandez et al., 1997)) to about fifty-fold (e.g. *bolA* (Huisman and Kolter, 1994)). RpoS-dependent fusions isolated in the previous study (L. Wei, M.Sc. Thesis) range from two- to over one

hundred-fold. An examination of the putative promoter regions of the group of very highly RpoS-dependent genes reported in Chapter 2 as well as in this section may elucidate the elements which comprise a highly-recognized RpoS-dependent promoter. This method shows potential since our original screen (Chapter 2) identified genes that were highly dependent on RpoS for expression.

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CHAPTER 4.

PREFACE

The following chapter entitled: "The alternative sigma factor, RpoS, is regulated at the level of transcription by BarA, a putative, two-component response regulator" is a continuation of work done by S. Mukhopadhyay (Ph.D. Thesis). This mutation was characterized by S. Mukhopadhyay.

Jonathon P. Audia performed all of the Northern and Western blot experiments presented here.

Jonathon P. Audia, B.Sc. (Hons.)
December, 1998.

CHAPTER 4

4.1 Introduction

The stationary phase expression of many adaptive bacterial proteins is dependent on the conserved, alternative sigma factor, RpoS (for a review see (Loewen and Hengge-Aronis, 1994)). The regulation of RpoS itself is complex and not fully understood, particularly at the level of transcription. Several signal molecules including ppGpp (Gentry et al., 1993; Lange et al., 1995), homoserine lactone (Huisman and Kolter, 1994), and weak acids such as acetate (Schellhorn and Stones, 1992) have been shown to induce *rpoS* at the transcriptional level (also see Chapter 1, section 1.5), however, the exact mechanism of *rpoS* transcriptional induction is still only poorly understood. In this study, we report the identification of BarA, a two-component response regulator, that is required for the exponential phase induction of RpoS. We have determined that the observed hydrogen peroxide sensitivity of a mutant (Mukhopadhyay and Schellhorn, 1997) defective in expression of *barA*, a bacterial virulence factor (Zhang and Normark, 1996), can be explained by a reduction in catalase activity, a RpoS-controlled function in *Escherichia coli* (Mulvey et al., 1988; Sak et al., 1989; Schellhorn and Hassan, 1988). Levels of *katE* mRNA, encoding the major catalase of *Escherichia coli*, were much lower in a *barA* mutant suggesting that BarA is required for expression of this RpoS-regulated gene. Employing Western analyses using anti-RpoS antisera and Northern analyses using probes specific for *rpoS*, we found that BarA is required for the exponential phase induction of RpoS. Promoter-*lacZ* fusion expression studies indicate that BarA itself is transiently expressed in early exponential phase cultures immediately preceding

transcriptional induction of RpoS. These results suggest that regulatory signal(s) that are present in exponentially-growing cultures may play an important role in affecting stationary phase gene expression.

RpoS expression is controlled at many levels. Though many factors regulate RpoS translation (e.g. HF-I (Muffler et al., 1997)) and protein stability (e.g. RssB (Muffler et al., 1996; Pratt and Silhavy, 1996)), the factor(s) that control *rpoS* transcription have not been identified despite the fact that *rpoS* transcription increases thirty-fold during mid-exponential phase (Huisman and Kolter, 1994; Schellhorn and Stones, 1992). In this study, we show that BarA, a conserved, two-component regulator, is required for *rpoS* transcription. The *barA* gene was originally isolated as a multi-copy suppressor of an *envZ* deletion (Nagasawa et al., 1992) but its physiological role is not known. BarA has amino and carboxy termini that are homologous to the family of proteins that contain both the sensor kinase and response regulator domains of two-component response regulatory systems (Nagasawa et al., 1992). This gene was identified as a required virulence factor, *airS*, in uropathogenic *E. coli* and may be involved in a bacterial iron-starvation response (Zhang and Normark, 1996) (also see Chapter 1, section 1.6). BarA homologs have been identified in *Erwinia carotovora* subsp. *carotovora*. The *rfpA* and *expS* loci have been identified as virulence factors required for excretion of protease enzymes responsible for causing 'soft rot' in the *Erwinia* plant host (Eriksson et al., 1998; Frederick et al., 1997).

4.2 Materials and Methods

4.2.1 Bacterial strains

Except where noted, all strains used in this study were derived from the wild type strain, MC4100. HS703 contains a *barA::lacZ* (the point of insertion is located at nucleotide 1010 in the *barA* ORF) transcriptional fusion (Mukhopadhyay and Schellhorn, 1997). HS1600 contains an *rpoS*::Tn*10* insertion. HS143 contains an *rpoS::lacZ* transcriptional fusion (this strain is a derivative of the wild type strain GC4468).

4.2.1 Media and growth conditions

All bacterial strains were grown in Luria Bertani (LB) rich broth (Miller, 1992). Strains were grown overnight in media supplemented with the appropriate antibiotics, diluted to an initial OD_{600}=0.001, grown and sampled as indicated in the figure legends. All cultures were grown at 37 °C with aeration at 200 rpm.

4.2.2 Northern hybridization probe preparation

Oligonucleotide primers were synthesized to generate Polymerase Chain Reaction (PCR)-amplified probes to detect specific mRNA transcripts: *katE* forward 5'-CAAAGCGGATTTCCTCTCAGATC-3', reverse 5'-TGCTCAAATGGCGTCTGACTTAG-3'; *katG* forward 5'-ACTCGCGACTGGTGGCCAAATCAAC-3', reverse 5'-CGTCTACCGCTTCGAACTGGATTGC-3'; *rpoS* forward 5'-AAAGGCCTTAGTAGAACAGGAACCC-3', reverse 5'-GTGAGGCCAATTTCACGACCTA-3'; *barA* forward 5'-CCGACTCTTTGTCACCTCCAATT-3', reverse 5'-

GCTTGAGTTTTTCAGCATTGACTTG-3'; *osmY* forward 5'-CTGCTGGCTGTAATGTTGACCTC-3', reverse 5'-CATCTACCGCTTTGGCGATACTT-3'; and *rrnA* forward 5'-GTGCCCAGATGGGATTAGCTAGTAG-3', reverse 5'-GTCGAGTTGCAGACTCCAATCC-3'. Each PCR tube contained 1x PCR buffer (50 mM KCl, 20 mM Tris pH 8.4), 50 pmole of each of the forward and reverse primers, 0.4 mM of each of the four dNTPs, 4 mM $MgCl_2$, ~50 ng of *E. coli* DNA and ~50 U of Taq polymerase in a final volume of 50 µl. Reactions were run for 25 cycles under the following conditions: (1) 96 °C for 30 s; (2) 61 °C for 60 s; (3) 72 °C for 90 s. PCR products were separated on 1.2 % agarose gels to verify the size of each probe and the identity of each was confirmed by DNA sequencing. PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN, Valencia, CA) and radiolabelled with [α-^{32}P]-dCTP (NEN Life Science Products, Inc. MA) by random-primed labeling.

4.2.3 RNA extraction and Northern blot analysis

Total RNA was extracted from wild type, *rpoS*, and *barA* strains using a RNeasy Midi Kit (QIAGEN, Valencia, CA) and quantified spectrophotomerically at 260 nm. Northern analysis was performed using RNA prepared from samples using standard methods (Seldon, 1989). Total RNA was transferred, overnight, onto BIOTRANS Nylon Membranes (ICN, Irvine, CA) and fixed by baking at 80 °C for 2 h (Seldon, 1989). Pre-hybridization and hybridization was performed at 42 °C with gentle agitation. Blots were stripped by immersing the membrane in boiling in 0.5 % Sodium Dodecyl Sulfate with gentle agitation. This treatment was repeated three times.

4.2.4 Western blot analysis

Cultures were grown and sampled as described in the figure legend. Samples were centrifuged at 5,000 x *g* and resuspended in 50 µl of sample buffer (15 mM Tris/HCl pH 8, 2 % glycerol, 0.5 % SDS, 1.2 % β-mercaptoethanol, 0.0025 % Bromphenol blue). Sample containing 12 µg of protein were separated on 12 % SDS-Polyacrylamide gels (running buffer: 1.4 % Glycine, 0.1 % SDS, 0.3 % Tris base) at 200 V for 45 min. Protein was transferred to an Immobilon-P Transfer Membrane (0.45 µm PVDF, Millipore, Bedford, MA) using a Mini-Protean II transfer system (Bio Rad Laboratories, Mississauga, ON) at 100 V for 1 h (transfer buffer: 0.2 % Tris base, 0.9 % Glycine, 20 % methanol). The blot was then rinsed as follows: Post-Blot Buffer (PBB: 0.9 % NaCl, 10 mM Tris/HCl pH 7.4) twice, 5 min each; PBB + 5 % milk powder (blocking) for 1 h; PBB + 5 % milk powder + 0.5 % NP40 + 5 µl of primary anti-σ^S-antibody (a generous gift from R. Hengge-Aronis, University of Konstanz) for 1 h; PBB + 0.5 % NP40 three times, 10 min each; PBB + milk powder + NP40 + 50 µl of secondary antibody (goat-anti-rabbit conjugated to horseradish peroxidase); Sigma-Aldrich, Oakville, ON) for 30 min; PBB + NP40 twice, 10 min each; and 10 mM Tris/HCl pH 7.4 twice, 10 min each. The blot was stained using an ECL (enhanced chemiluminescence) detection kit (Amersham Life Science, Oakville, ON). Solutions A and B were mixed in a 1:1 ratio and poured directly onto the blot for one minute (without agitation). The solution was removed and the membrane was then exposed to X-ray film (Eastman Kodak Co., Rochester, N.Y.) in a Kodak X-OMAT exposure cassette (Eastman Kodak Co., Rochester, N.Y.) for five to ten minutes.

4.2.5 β-galactosidase assays

Samples were grown and harvested as described in the figure legend. β-galactosidase assays were performed (using whole cells) by measuring the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (Miller, 1992). Activity, expressed as Miller units, was calculated as [(OD_{420} x 1000)/(volume of cells (ml) x culture density (OD_{600}) x time of incubation (min))] (Miller, 1992).

4.3 Results and Discussion

4.3.1 The hydrogen peroxide sensitive phenotype exhibited by a barA *mutant is the result of reduced levels of catalase*

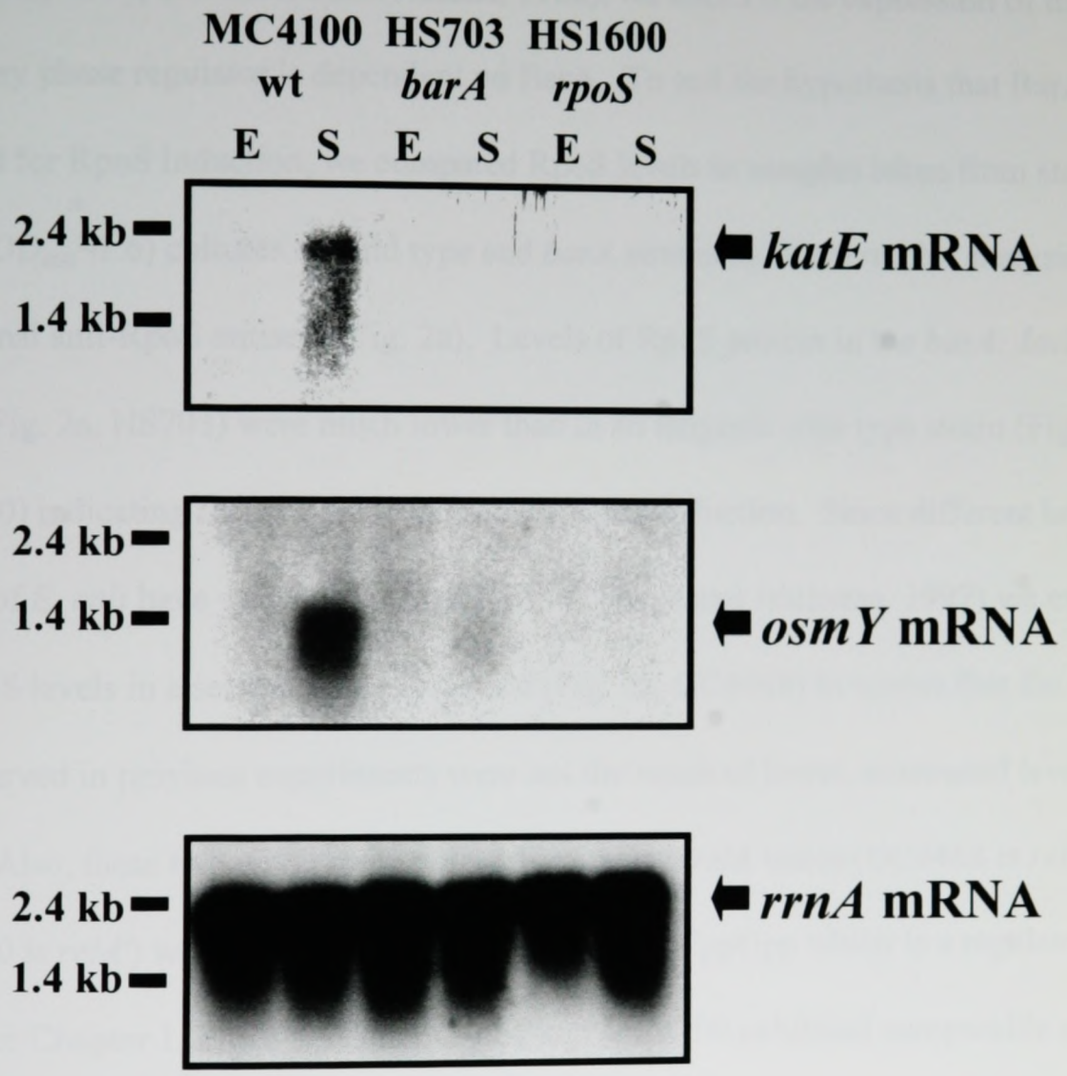
Suman Mukhopadhyay previously identified a chromosomal *barA::lacZ* insertion mutation in strain HS703 that renders cells sensitive to hydrogen peroxide (Mukhopadhyay and Schellhorn, 1997). We hypothesized that BarA may be required for the activity of the *E. coli* HPI (coded for by *katG*) and HPII (coded for by *katE*) catalases which convert hydrogen peroxide into oxygen and water. Consistent with this, results from a catalase test for HPII activity in which colonies grown on LB plates are treated with 30 % hydrogen peroxide (wild type *E. coli* colonies evolve gas bubbles when treated with hydrogen peroxide due to the activity of HPII) revealed that a *barA* mutant exhibited lower activity than an isogenic wild type strain (Schellhorn, unpublished results). Further, the levels of both HPII and HPI have been previously shown to be lower in a *barA* strain relative to an isogenic wild type strain (S. Mukhopadhyay, Ph.D Thesis) indicating that BarA is required for full-functional catalase activity.

4.3.2 The observed reduction in catalase activity in a barA *mutant is due to a reduction of catalase mRNA*

Since the observed decrease in catalase activity demonstrated by *barA* mutant colonies in the catalase test could be attributed to a decrease in the amount of HPII present in the cell, we tested if BarA regulates the expression of the gene which codes for the HPII catalase enzyme, *katE*. This idea seemed plausible in view of the high homology BarA has to the family of two-component transcriptional activators (Nagasawa et al., 1992). To test this, we examined levels of *katE* (encoding HPII) mRNA in isogenic wild type, *barA*, and *rpoS* (a known and well-defined regulator of *katE* expression (Sak et al., 1989; Schellhorn and Hassan, 1988)) strains by probing total bacterial RNA isolated from exponential (OD_{600}~0.2) and stationary phase cultures (OD_{600}~1.6) with a DNA probe specific to *katE*. As expected, the levels of *katE* mRNA were low in exponential phase samples and were high in stationary phase samples in the wild type strain and were absent in an *rpoS* strain (see Fig. 1). Levels of *katE* mRNA in a *barA* mutant were much lower than an isogenic wild type strain indicating that *katE* expression is dependent on BarA.

In toto, these results indicate that the previously-observed hydrogen peroxide sensitivity of the *barA* mutant (Mukhopadhyay and Schellhorn, 1997) can be explained, at least in part, by a decrease in the levels *katE* mRNA which results in an overall decrease in catalase activity.

Figure 1 The mRNA levels of the RpoS-dependent genes, *katE* and *osmY*, is reduced in a *barA::lacZ* mutant strain as determined by Northern blot analysis. Levels of *katE* mRNA and *osmY* mRNA were examined in wild type (MC4100), *barA::lacZ* (HS703), and *rpoS::*Tn10 (HS1600) strains sampled during exponential (OD_{600}~0.2) (E) and stationary (OD_{600}~1.6) (S) phase growth. The blot was hybridized with a probe specific to *katE* and then stripped and re-probed with a probe specific to *osmY*. As a loading and transfer control, the blot was stripped and re-probed for a third time with a probe specific to an RpoS-independent gene, *rrnA*.


MC4100
wt
HS703
barA
HS1600
rpoS
E S E S E S
2.4 kb
1.4 kb
katE mRNA
2.4 kb
1.4 kb
osmY mRNA
2.4 kb
1.4 kb
rrnA mRNA

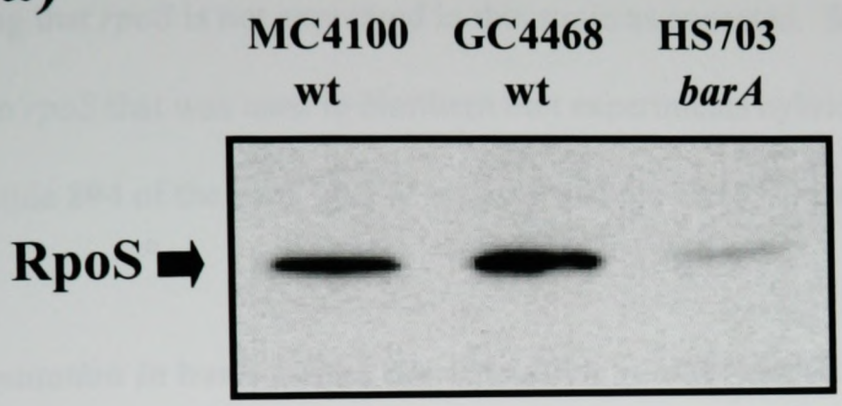
4.3.3 The low levels of katE *expression exhibited by a* barA *mutant is a result of a reduction in expression of the alternative sigma factor,* rpoS

Since both *E. coli* catalase genes are regulated by RpoS (Mukhopadhyay and Schellhorn, 1994; Schellhorn and Hassan, 1988), we asked if the expression of this key stationary phase regulator is dependent on BarA. To test the hypothesis that BarA is required for RpoS induction, we compared RpoS levels in samples taken from stationary phase (OD_{600}~1.6) cultures of wild type and *barA* strains by Western blot analysis using polyclonal anti-RpoS antisera (Fig. 2a). Levels of RpoS protein in the *barA::lacZ* mutant strain (Fig. 2a, HS703) were much lower than in an isogenic wild type strain (Fig. 2a, MC4100) indicating that BarA is required for RpoS induction. Since different laboratory strains of *E. coli* have varying levels of RpoS (Jishage and Ishihama, 1997) we examined the RpoS levels in a second wild type strain (Fig. 2a, GC4468) to ensure that the results we observed in previous experiments were not the result of lower, attenuated levels of RpoS. Also, these two wild type strains differ in their *relA* status (GC4468 is *relA*$^+$ and MC4100 is *relA*$^-$) which is involved in the synthesis of ppGpp which is a regulator of *rpoS* (see Chapter 1, Fig. 1). Both GC4468 and MC4100 exhibited comparable stationary phase levels of RpoS making them both suitable for studies of this type (Fig. 2a).

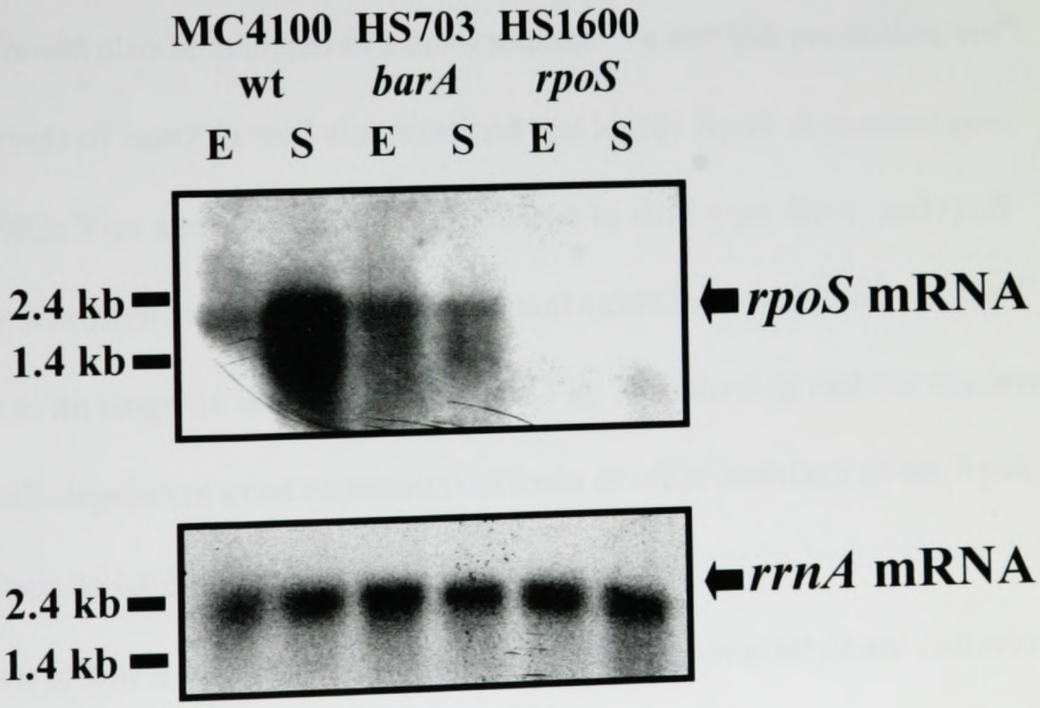
Since RpoS is regulated at the levels of transcription (Lange and Hengge-Aronis, 1994), translation (Muffler et al., 1996; Muffler et al., 1997), and protein stability (Bearson et al., 1996; Muffler et al., 1996; Pratt and Silhavy, 1996; Zhou and Gottesman, 1998), we tested if BarA is a possible regulator of *rpoS* transcription by comparing the levels of *rpoS* mRNA in isogenic wild type, *barA*, and *rpoS* strains. The levels of *rpoS*

Figure 2 A *barA::lacZ* mutant has low levels of RpoS relative to an isogenic wild type strain; a result of reduced levels of *rpoS* mRNA. **a)** Levels of RpoS protein in stationary phase (OD_{600}~1.6) cultures of wild type and *barA::lacZ* strains by Western blot analysis. **b)** Levels of *rpoS* mRNA were examined in wild type (MC4100), *barA::lacZ* (HS703), and *rpoS::*Tn10 (HS1600) strains sampled during exponential (OD_{600}~0.2) (E) and stationary (OD_{600}~1.6) (S) phase growth. As a loading and transfer control, the blot was stripped and re-probed with an *rrnA*-specific probe.

a)


MC4100
wt
GC4468
wt
HS703
barA
RpoS

b)


MC4100
wt
HS703
barA
HS1600
rpoS
E
S
E
S
E
S
2.4 kb
1.4 kb
rpoS mRNA
2.4 kb
1.4 kb
rrnA mRNA

mRNA were substantially reduced in a *barA* strain relative to an isogenic wild type strain (see Fig. 2b) as determined by Northern blot analysis. It is important to note that there is no *rpoS* mRNA present in strain HS1600 which carries an *rpoS*::Tn*10* mutation, confirming that *rpoS* is not expressed in this strain as expected. Since the DNA probe specific to *rpoS* that was used in Northern blot experiments hybridizes from nucleotide 80 to nucleotide 894 of the *rpoS* ORF it is likely that the Tn*10* is located upstream of this site.

4.3.4 A mutation in barA *affects the expression of other members of the RpoS regulon*

If BarA is required for RpoS induction and a *barA* mutant exhibits low levels of expression of an RpoS-dependent gene, *katE*, then the expression of other members of the RpoS regulon should also be reduced in a *barA* mutant. To test this prediction, we examined the levels of *osmY* (a well characterized and highly RpoS-dependent gene (Lange et al., 1993; Yim and Villarejo, 1992)) mRNA in wild type, *barA*, and *rpoS* backgrounds by Northern analysis. The *barA* mutant exhibited lower levels of *osmY* mRNA relative to an isogenic wild type strain (see Fig. 1) indicating that the requirement of BarA for RpoS-dependent gene expression extends to other members of the RpoS regulon in addition to *katE*.

4.3.5 Expression of barA *and* rpoS *is initiated in early exponential phase cultures*

Because RpoS is required for the expression of many stationary phase genes, several studies have focused on identifying post-transcriptional regulatory factors required for RpoS induction in stationary phase cultures. Relatively few studies have investigated initiation and control of *rpoS* transcription that occurs in exponentially

growing *E. coli* cultures. Though several molecules are known to induce *rpoS* at the transcriptional level (see Chapter 1, section 1.5.1), transcriptional regulation of *rpoS* is not fully understood. Studies using transcriptional, promoter-*lacZ* operon fusions to *rpoS* indicate that while transcriptional activity reaches a maximum in mid stationary phase, transcription begins in early exponential phase (at an OD_{600} ~0.2) (Lange and Hengge-Aronis, 1994; Schellhorn and Stones, 1992). The present work examined the expression pattern of a *barA::lacZ* transcriptional gene fusion (S. Mukhopadhyay, Ph.D. Thesis) revealing that this transcriptional regulator of *rpoS* is also expressed in early exponential phase. Transcriptional initiation of the *barA::lacZ* fusion occurred very early during the growth of the culture (OD_{600}<0.02), preceding *rpoS* transcriptional induction (compare Figs. 3 & 4a) and reached a maximum in early stationary phase (see Fig. 3). It is therefore likely that BarA is present in early exponential phase cultures and could initiate transcriptional induction of *rpoS* if the signal(s) required for *rpoS* induction are also present. As mentioned above, transcription of a *rpoS::lacZ* fusion was also initiated in early exponential phase cultures (Fig. 4a) and occurred after the initiation of *barA* transcription. However, since this *rpoS::lacZ* fusion is in another, non-isogenic wild type background we also examined the levels of *rpoS* mRNA in the wild type strain that is isogenic to our *barA::lacZ* mutant, by Northern analysis, to verify the *rpoS::lacZ* fusion data. The overall pattern of expression of *rpoS* mRNA (shown in Fig. 4b) correlated well to the data collected from the *rpoS::lacZ* fusion, as expected. This data forms the basis for a model in which transcription of the regulator, BarA (Fig. 3) precedes, *rpoS* expression (Figs. 4a & b) leading to induction of the RpoS regulon. This model of early

Figure 3 Expression of a *barA::lacZ* promoter-*lacZ* operon transcriptional gene fusion is induced very early during exponential growth (performed by X. Zhou). An exponentially growing culture was inoculated into LB media and samples were removed as indicated. Open circles represent culture density (OD_{600}) and closed circles represent β-galactosidase activity.

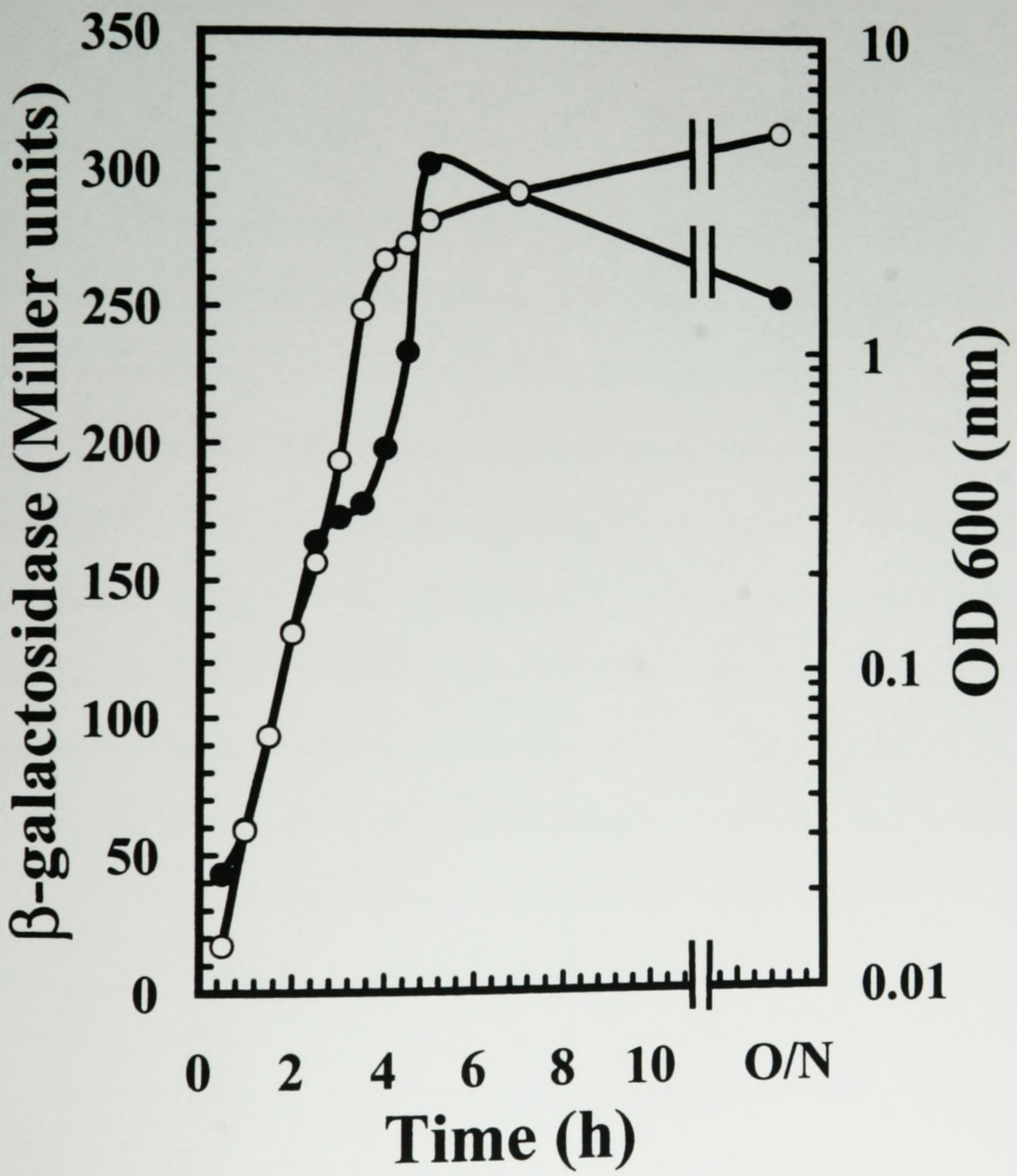
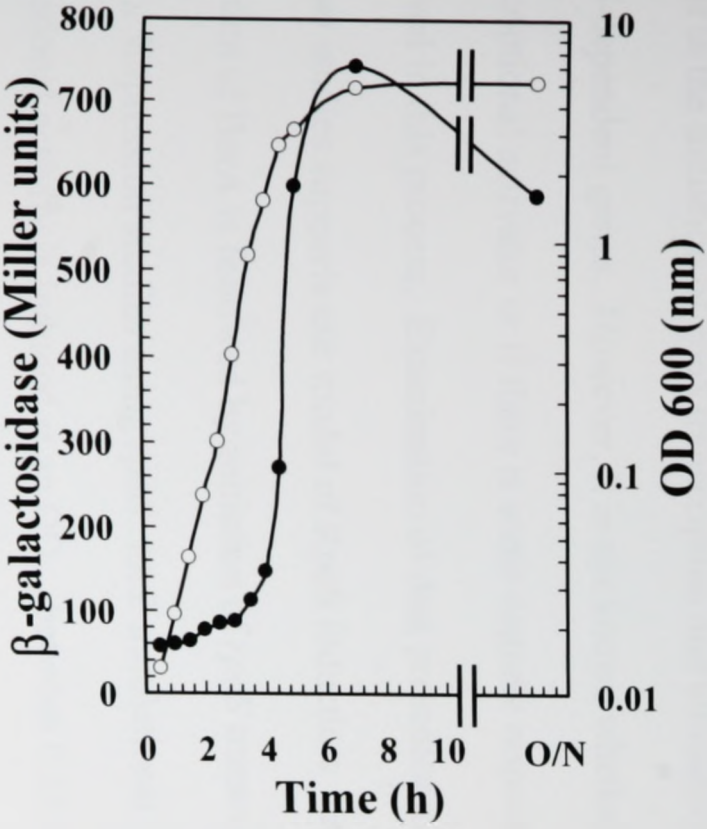
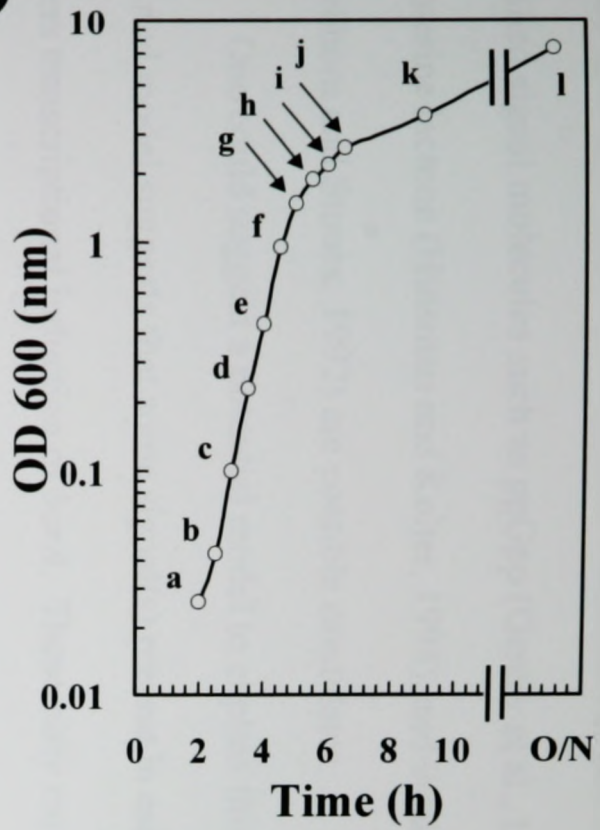

β-galactosidase (Miller units)
350
300
250
200
150
100
50
0
10
1
0.1
0.01
OD 600 (nm)
0
2
4
6
8
10
O/N
Time (h)

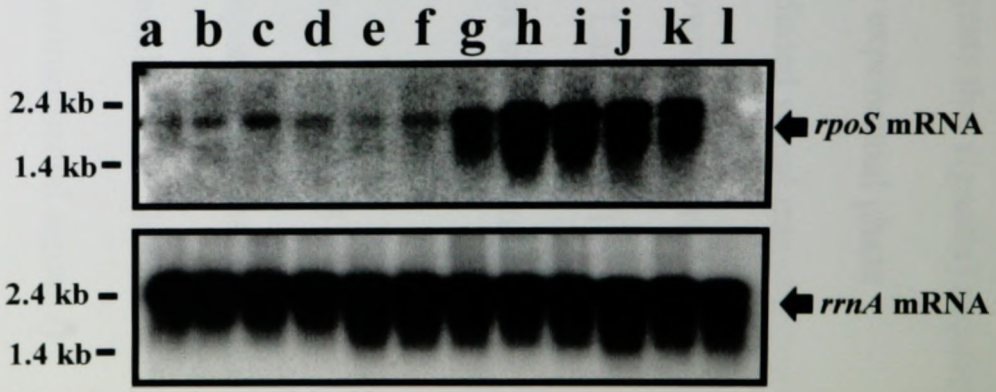
Figure 4 Expression of *rpoS* is controlled at the transcriptional level during exponential growth. **a)** Expression of an *rpoS::lacZ* promoter-*lacZ* operon transcriptional gene fusion is induced during exponential growth (performed by X. Zhou). An exponentially growing culture was inoculated into LB media and samples were removed as indicated. Open circles represent culture density (OD_{600}) and closed circles represent β-galactosidase activity. **b)** Expression of *rpoS* mRNA during growth as determined by Northern blot analysis. An overnight culture of MC4100 was diluted to an initial OD_{600} of 0.001 in LB media and incubated at 37 ^{o}C. Cultures were sampled at the times and culture densities indicated. Open circles represent culture density (OD_{600}). As a loading and transfer control, the blot was stripped and re-probed with an *rrnA*-specific probe.

a)


β-galactosidase (Miller units)
800
700
600
500
400
300
200
100
0
10
1
0.1
0.01
OD 600 (nm)
0
2
4
6
8
10
O/N
Time (h)

b)

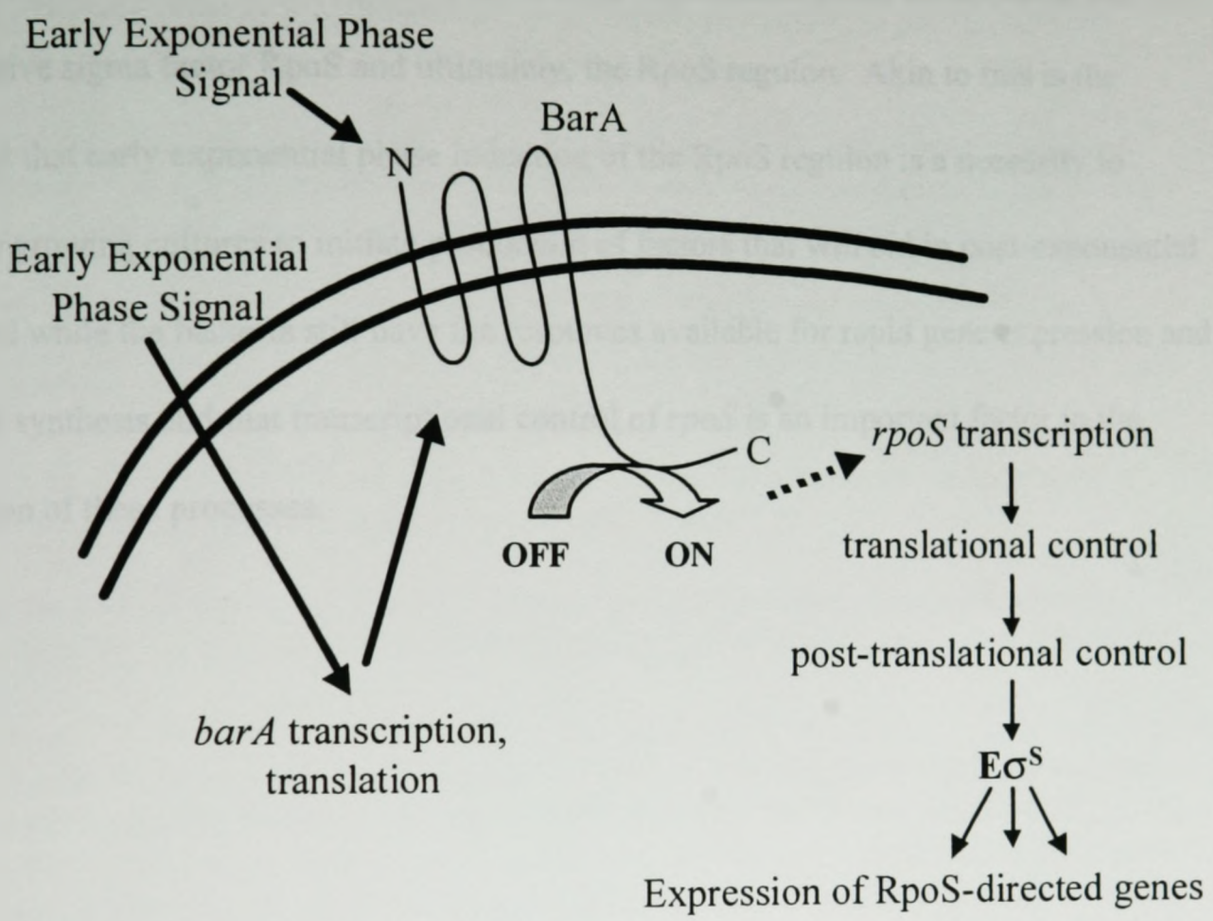

10
1
0.1
0.01
OD 600 (nm)
a
b
c
d
e
f
g
h
i
j
k
l
0
2
4
6
8
10
O/N
Time (h)


a b c d e f g h i j k l
2.4 kb
1.4 kb
rpoS mRNA
2.4 kb
1.4 kb
rrnA mRNA

exponential phase induction of *rpoS* and the RpoS regulon suggests that signal(s) required for induction of *rpoS* and the RpoS-regulon are present in early exponential phase cultures. The signal molecules that may interact in a BarA-mediated signal transduction cascade leading to *rpoS* transcriptional activation remain unknown, though previously-identified signal molecules such as ppGpp (Gentry et al., 1993; Lange et al., 1995), homoserine lactone (Huisman and Kolter, 1994), and weak acids such as acetate (Schellhorn and Stones, 1992) are possible candidates.

One could suggest a potential model to explain this system (see Fig. 5). This proposed model suggests that some signal(s) present in early exponential phase cultures triggers transcriptional induction of *barA*. These early exponential phase cells now contain membrane-bound BarA protein which can 'sense' an environmental signal(s) resulting in its subsequent activation. This triggers the signal transduction cascade that results in the initiation of *rpoS* transcription and ultimately leads to the induction of RpoS-dependent genes. However, it is not known whether BarA itself acts as a transcriptional activator or if there is some cognate response regulator that may be involved in this process. Examination of data presented in this study and data presented in other studies supports our model of RpoS induction. Early exponential phase induction of BarA is followed by initiation of *rpoS* transcription at an OD_{600} of ~0.2 (this study). Expression studies using *rpoS::lacZ* translational fusions demonstrate that *rpoS* translational activity is induced at an OD_{600} between 0.25 and 0.3 (Zhang et al., 1998), following transcriptional induction. Further, RpoS protein stability increases as cells make the transition to stationary phase growth (Lange and Hengge-Aronis, 1994). Close

Figure 5 A proposed model of BarA-mediated exponential phase induction of RpoS.


Early Exponential Phase Signal
BarA
N
Early Exponential Phase Signal
C
OFF
ON
rpoS transcription
translational control
post-translational control
Eσ^S
barA transcription, translation
Expression of RpoS-directed genes

examination of these results may be the first step in delineating the complex RpoS regulatory pathway.

In summary, this proposed model predicts that BarA may be involved in a signal transduction cascade that is required for the early exponential phase induction of the alternative sigma factor RpoS and ultimately, the RpoS regulon. Akin to this is the concept that early exponential phase induction of the RpoS regulon is a necessity to rapidly growing cultures to initiate production of factors that will aid in post-exponential survival while the bacteria still have the resources available for rapid gene expression and protein synthesis and that transcriptional control of *rpoS* is an important factor in the initiation of these processes.

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CHAPTER 5

5.1 General Discussion and Conclusions

The present study is a continuation of two separate projects in the laboratory utilizing a previously-generated bank of random promoter-*lacZ* insertional mutants to: (*i*) identify novel members of a stationary phase-inducible regulon regulated by the alternative sigma factor RpoS and (*ii*) to attempt to determine the mechanism by which a mutation in the putative response regulator BarA confers a hydrogen peroxide sensitive phenotype to *Escherichia coli* cultures. It was determined that BarA is required for the early exponential phase induction of *rpoS*.

Induction of RpoS is complex and involves regulation at the levels of transcription, translation, and protein stability (see Chapter 1, section 1.5). In this work, we have determined that BarA, a probable two-component regulator, is required for the transcriptional regulation of *rpoS*. Transcription of a *barA::lacZ* fusion begins in very early (OD_{600}<0.01) exponential phase cultures (this study) and is followed by transcriptional induction of *rpoS* when the culture reaches an optical density (OD_{600}) of 0.2 (this study,(Lange and Hengge-Aronis, 1994; Schellhorn and Stones, 1992). Translational fusions to *rpoS* are induced in exponential phase at an optical density of 0.25-0.3 (Zhang et al., 1998) and RpoS protein stability increases as cells enter stationary phase (Lange and Hengge-Aronis, 1994). The highly-RpoS-dependent genes reported in

the manuscript in Chapter 2 (see Fig. 3) all exhibit transcriptional induction in exponential phase (OD_{600}=0.3). These data extend the model presented in the previous chapter (Chapter 4, Fig. 6) which predicts that some signal(s) may be present in early exponential phase cultures which lead to induction of RpoS and subsequently, the RpoS regulon. The identity of these potential signals and the manner in which they stimulate BarA-mediated induction of *rpoS* is not known. Akin to these ideas is the question of the holoenzyme specificity switch from $E\sigma^D$ to $E\sigma^S$ that must occur in order to allow for the transcription of RpoS-dependent genes. Many RpoS-dependent genes are also osmotically induced and it has been suggested that the ionic conditions of the cell may confer selectivity (Ding et al., 1995). In response to osmotic stress, *E. coli* accumulates high intracellular levels of potassium which can lead to RpoS-mediated induction of RpoS-dependent, osmotically regulated promoters (Ding et al., 1995). This suggests that the cell must somehow be able to 'sense' its internal environment and alter holoenzyme specificity accordingly. Other potential signal molecules include those previously identified as transcriptional inducers of *rpoS* such as ppGpp (Gentry et al., 1993; Lange et al., 1995), weak acids (Schellhorn and Stones, 1992), and homoserine lactone (Huisman and Kolter, 1994). It is also possible that changes in the metabolite pool ("metabolome") which occur in slow-growing cultures (Tweeddale et al., 1998) could also produce candidate 'signal' molecules. Finally, it is possible that the 'switch' is a consequence of competition between σ^D and σ^S for a limited number of RNA polymerase molecules. Studies have demonstrated that over-expression of *rpoS* can result in down-regulation of RpoD-dependent genes (and conversely, over-expression of *rpoD* can result in down-

regulation of RpoS-dependent genes) (Farewell et al., 1998). Future studies utilizing an inducible vector to vary the expression of *rpoS* at different times during growth may help resolve the timing involved in the 'switch' in sigma factor specificity and help determine the importance of the observed exponential phase induction of the RpoS regulon.

In conclusion, this study identified several RpoS-dependent genes that belong to three groups: (*i*) previously-identified RpoS-dependent genes; (*ii*) novel RpoS-dependent genes of known function (such as *narY*); and (*iii*) *Escherichia coli* open reading frames (ORFs) of unknown function that were not previously known to required RpoS for transcriptional induction. Also, the putative response regulator, BarA, is required for the transcriptional induction of *rpoS* (and subsequently RpoS-dependent genes) in early exponential phase cultures.

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APPENDIX I

Background

Many RpoS-dependent genes in *Escherichia coli* are also co-regulated by factors such as Fis, the cAMP-CRP complex, and the leucine-responsive protein (Lrp) (see Chapter 1, section 1.3). The cAMP-CRP complex is known to regulate several carbon starvation inducible genes such as *osmY* (Weichart et al., 1993) and is required for the de-repression of operons required for the metabolism of less preferred carbon sources (compared to glucose) such as acetate and lactose (reviewed in (Brock and Madigan, 1996)). We tested the top ten RpoS-dependent promoter-*lacZ* fusions identified in Chapter 2 for dependence on the cAMP-CRP complex by introducing these fusions (by P1*vir*-mediated transduction) into *cya* and *crp* mutant strains and scoring for changes in β-galactosidase activity on LB plates containing the indicator dye X-gal.

Materials and Methods

Bacterial strains, phage and growth conditions

All bacterial strains and phage are listed in Table 1.

Unless otherwise indicated cultures were grown in Luria Bertani (LB) rich broth or on LB plates. Cultures were grown overnight in LB media supplemented with the appropriate antibiotics, subcultured the following morning and maintained in exponential phase (OD_{600}<0.2) for at least eight generation prior to sampling. For replica plating

Table 1. E. coli *strains and phage.*

Strains	Genotype	Source/Reference
A) Strains		
MC4100	*araD*139 Δ(*argF-lac*)*U*169 *rpsL*150 *relA* *deoC*1 *ptsF*25 *rbsR flb*5301	laboratory collection
GC4468	Δ*lacU*169 *rpsL*	laboratory collection
SH205	HfrC *phoA*8 *glpD*3 *glpR*2 *relA*1 *tonA*22 (λ) *zah*-735::Tn*10* Δ(*argF-lac*)*U*169	(Schweizer and Boos, 1983)
W3110	λ⁻ *thyA*36 IN(*rrnD-rrnE*)1 *rpsD*1 *deoC*2	M. Berlyn (C.G.S.G.#: 4916)
AB1157	*thr*-1 *ara*-14 *leuB*6 Δ(*gpt-proA*)62 *lacY1 tsx-33 qsr'*-0 *glnV*44(AS) *galK*2(Oc) λ⁻ Rac-0 *hisG*4(Oc) *rfbD*1 *mgl*-51 *rpoS*396(Am) *rpsL*31(strR) *kdgK*51 *xylA*5 *mtl*-1 *argE*3(Oc) *thi*-1	M. Berlyn (C.G.S.G.#: 1157)
CA8000	*thi*1 *relA*1 *spoT*1 λ⁻	(Shah and Peterkofsky, 1991)
CA8306	as CA8000 but Δ(*cya*854)	(Shah and Peterkofsky, 1991)
CA8445	as CA8000 but Δ(*crp*45)	(Shah and Peterkofsky, 1991)
HS1014	as GC4468 but Φ (*rsd014-lacZ*⁺)	L.Wei, M.Sc. Thesis
HS1035	as GC4468 but Φ (*rsd035-lacZ*⁺)	L.Wei, M.Sc. Thesis
HS1047	as GC4468 but Φ (*rsd047-lacZ*⁺)	L.Wei, M.Sc. Thesis
HS1054	as GC4468 but Φ (*rsd054-lacZ*⁺)	L.Wei, M.Sc. Thesis
HS1069	as GC4468 but Φ (*rsd069-lacZ*⁺)	L.Wei, M.Sc. Thesis
HS1081	as GC4468 but Φ (*rsd081-lacZ*⁺)	L.Wei, M.Sc. Thesis
HS1091	as GC4468 but Φ (*rsd091-lacZ*⁺)	L.Wei, M.Sc. Thesis
HS1092	as GC4468 but Φ (*rsd092-lacZ*⁺)	L.Wei, M.Sc. Thesis
HS1093	as GC4468 but Φ (*rsd093-lacZ*⁺)	L.Wei, M.Sc. Thesis
HS1096	as GC4468 but Φ (*rsd096-lacZ*⁺)	L.Wei, M.Sc. Thesis
B)Phage		
P1*vir*	generalized transducing phage	laboratory stock

experiments, cultures were grown in microtiter wells containing 200 µl of LB media overnight at 37 ^{o}C without aeration and plated the following morning. Antibiotics were added to growth media at the following concentration: kanamycin, 50 µg/ml; tetracycline, 15 µg/ml; and streptomycin, 100 µg/ml. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added at concentrations of 15, 25, and 50 µg/ml, respectively.

Strain construction and genetic methods

Using a generalized P1*vir* transducing phage, lysates were made on the RpoS-dependent promoter-*lacZ* fusion carrying strains and the *Δlac*-Tn*10* harboring strain, SH205 (the *lac* deletion is almost 100% linked to the Tn*10*, (Schweizer and Boos, 1983)) listed in Table 1 by the method described in Miller (1992). The P1(*Δlac*) lysate was used to construct Lac^{-} derivatives of CA8000 (the wild type parental strain), CA8306 (*cya* mutant), CA8445 (*crp* mutant) and two other wild type strains, W3110 and AB1157 using the method described in (Miller, 1992). The P1(*rsd-lacZ*) lysates were then utilized to introduce these RpoS-dependent fusions into the CA-series strains to test for cAMP-CRP-dependence. The RpoS-dependent fusions were also moved (by transduction) into the newly constructed, Δ*lac* derivatives of the other wild type backgrounds, W3110, AB1157, and also into MC4100 (the original wild type parental strain as a control). Transductants were inoculated into microtiter wells as described above and replica plated onto LB plates containing X-gal to test for cAMP-CRP-dependence.

Western blot analysis

Cultures of MC4100, GC4468 (the two commonly-used wild type strains in our laboratory), the Δ*lac* derivatives of W3110, AB1157, and CA8000 were grown as described above. Samples from each of the cultures were removed in stationary phase cultures (OD_{600}~1.6) were stored in sample buffer at -80 ^{o}C (see Chapter 4, section 4.2.4). Western analysis of these samples was performed as described in Chapter 4, section 4.2.4.

Results

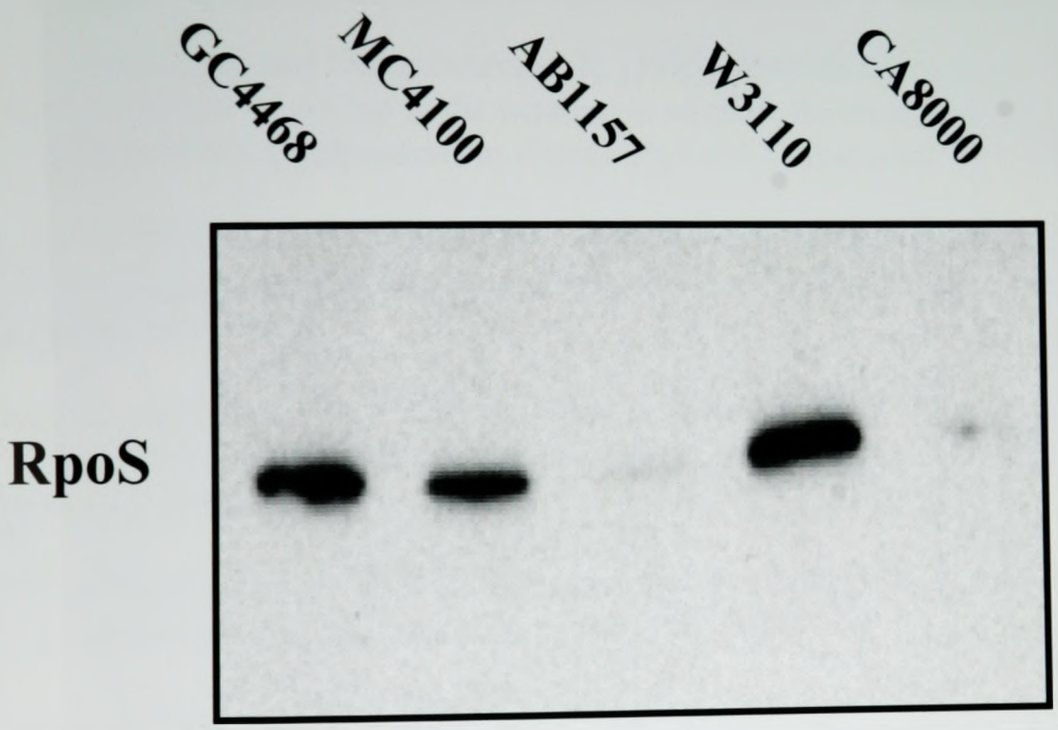
The effects of introduced* cya *and* crp *mutations on the activity of the top ten RpoS-dependent promoter-*lacZ *fusions

The relative β-galactosidase activity of each of the top ten RpoS-dependent fusions in (all of) the wild type, *cya* and *crp* mutant strains grown on LB plates were compared after one and two days of growth at 37 ^{o}C. After one day of incubation it appeared that *rsd014*, *rsd069*, *rsd081*, *rsd091*, and *rsd093* exhibited increased activity in the *cya* and *crp* mutant background compared to the isogenic wild type parental strain. The *rsd035*, *rsd047*, *rsd054*, *rsd092*, and *rsd096* fusions exhibited very little differences in activity after one day of growth. Interestingly, examination of the strains after two days of growth revealed that the activity of **all** of the fusions were similar in both the mutant and wild type strains.

Also, there were clear differences in activity of these promoter-*lacZ* fusions in the different wild type backgrounds. In all cases the fusions demonstrated similar differences in activity as follows, in order of highest activity to lowest: MC4100, W3110, CA8000, and AB1157. It was proposed that these differences may be due to differences in RpoS

levels in these different wild type strains since this property of different *E. coli* wild type strains has been previously demonstrated (Jishage et al., 1996). Jishage *et al.* (1996) demonstrated that some laboratory stocks of a commonly used wild type strain, W3110, contained lower, attenuated levels of RpoS (Jishage et al., 1996). In order to determine if a similar phenomenon was responsible for the differences exhibited by our RpoS-dependent promoter-*lacZ* fusions in the different wild type backgrounds, the stationary phase levels of RpoS were assayed in the different wild type strains by Western blot analysis using anti-RpoS antisera (a generous gift from R. Hengge-Aronis, University of Konstanz). It is clear that the observed differences in activity of the top ten RpoS-dependent fusions can be attributed to differences in RpoS levels in the various wild type strains (see Fig. 1). It is likely that the observed reduced levels of RpoS in CA8000 could be attributed to the lack of ppGpp, a known regulator of *rpoS* (Gentry et al., 1993; Lange et al., 1995), in this background. Since the parental strain of the *cya* and *crp* mutants exhibited attenuated levels of RpoS, it made these strains unsuitable for further study of the effects of the cAMP-CRP complex on the top ten RpoS-dependent fusions. Further study of these strains was not pursued.

Figure 1. Western blot analysis of RpoS levels in different strains of *Escherichia coli*. Overnight cultures were subcultured into LB media and maintained in early exponential phase for at least eight generations prior to sampling (37 ^{0}C with aeration at 200 rpm). Samples were removed from stationary phase cultures (at an OD_{600} of ~1.5), pelleted, resuspended in lysis buffer and boiled for 10 min. 15 μg of total protein was separated by polyacrylamide gel electrophoresis and transferred to a PVDF membrane as described in Chapter 4, section 4.2.4. RpoS protein was detected using polyclonal anti-RpoS antisera and a horseradish-peroxidase-conjugated secondary antibody. The blot was stained using an ECL (enhanced chemiluminescence) detection kit.


GC4468
MC4100
AB1157
W3110
CA8000
RpoS

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APPENDIX II

Table 1. PCR Primers Generated for this Study

E. coli ORF	Primer Number	Sequence	Tm[a] (°C)	Location[b]
rrnA	AB12176 (Forward)	5'-GTGCCCAGATGGGATTAGCTAGTAG-3'	63	-20 °C
	AB12177 (Reverse)	5'-GTCGAGTTGCAGACTCCAATCC-3'	63	-20 °C
rpoS	AB12178 (Forward)	5'-AAAGGCCTTAGTAGAACAGGAACCC-3'	63	-20 °C
	AB12179 (Reverse)	5'-GTGAGGCCAATTTCACGACCTA-3'	63	-20 °C
osmY	AB12166 (Forward)	5'-CTGCTGGCTGTAATGTTGACCTC-3'	63	-20 °C
	AB12167 (Reverse)	5'-CATCTACCGCTTTGGCGATACTT-3'	63	-20 °C
o381	AB12168 (Forward)	5'-CAGCATGACAATAATGACCGCTC-3'	63	-20 °C
	AB12169 (Reverse)	5'-AACTTGCCCCCTTCTGCTATAGG-3'	63	-20 °C
yhiU	AB12170 (Forward)	5'-GTCGGTGTCGTCACACTCTCC-3'	63	-20 °C
	AB12171 (Reverse)	5'-CTGGAGGAAATTGCTCGTGCT-3'	63	-20 °C
o215	AB12172 (Forward)	5'-AGCTGGATTATCGCTTGATTAAGGTAG-3'	63	-20 °C
	AB12173 (Reverse)	5'-CAATATCCGCAATGCTGTAGTTCTC-3'	63	-20 °C
otsA	AB12174 (Forward)	5'-AAAAGGTAACATTACGTGGGCCTC-3'	63	-20 °C
	AB12175 (Reverse)	5'-CGCGGAACTATCTGCTTTAGGTC-3'	63	-20 °C
narY	AB11791 (Forward)	5'-CCTTCGACTACGAACATTTGCATAG-3'	63	-20 °C
	AB11792 (Reverse)	5'-GAACAGGTTGAATTTACTGTCCGAAC-3'	63	-20 °C

narZ	AB12184 (Forward)	5'-GTGCCTGAGCTACAAACAAGTGC-3'	63	-20 ^{O}C
	AB12185 (Reverse)	5'-GGTGTATCCCGCGTTATAGGAGAC-3'	64	-20 ^{O}C
narW	AB12186 (Forward)	5'-CGGCCTGTTGATGGAGTATCC-3'	63	-20 ^{O}C
	AB12187 (Reverse)	5'-TCACCTGTTCCTCTTCCCACAC-3'	63	-20 ^{O}C
gabP	AB12182 (Forward)	5'-CCCATCTGGTTATTTTCCCTCG-3'	63	-20 ^{O}C
	AB12183 (Reverse)	5'-GGTAATAAAGCCGATGACTAGCCAG-3'	63	-20 ^{O}C
gabD	AB12180 (Forward)	5'-GAAAGGCGAAATCAGCTACGC-3'	63	-20 ^{O}C
	AB12181 (Reverse)	5'-CTTCGATGCCATACTTCGAACCT-3'	63	-20 ^{O}C
accA/ldcC	AB9510 (Forward)	5'-CGGAACAAATTGAACAATCCTACG-3'	63	-20 ^{O}C
	AB9511 (Reverse)	5'-ACCACTTTTACCCTGGTAGATCGG-3'	63	-20 ^{O}C
katE	AB11789 (Forward)	5'-CAAAGCGGATTTCCTCTCAGATC-3'	63	-20 ^{O}C
	AB11790 (Reverse)	5'-TGCTCAAATGGCGTCTGACTTAG-3'	63	-20 ^{O}C
sugE	AB11793 (Forward)	5'-GCAACGGTACGACAGCTGTGT-3'	63	-20 ^{O}C
	AB11794 (Reverse)	5'-AATACGATTAACGCCAGACTCGC-3'	63	-20 ^{O}C
barA	AB12656 (Forward)	5'-CCGACTCTTTGTCACCTCCAATT-3'	63	-20 ^{O}C
	AB12657 (Reverse)	5'-GCTTGAGTTTTTCAGCATTGACTTG-3'	63	-20 ^{O}C
katG	AB12788 (Forward)	5'-ACTCGCGACTGGTGGCCAAATCAAC-3'	72	-20 ^{O}C
	AB12789 (Reverse)	5'-CGTCTACCGCTTCGAACTGGATTGC-3'	70	-20 ^{O}C
Mu S	AB10928	5'-CGCATTTATCGTGAAACGCTTTCGC-3'	70	-20 ^{O}C

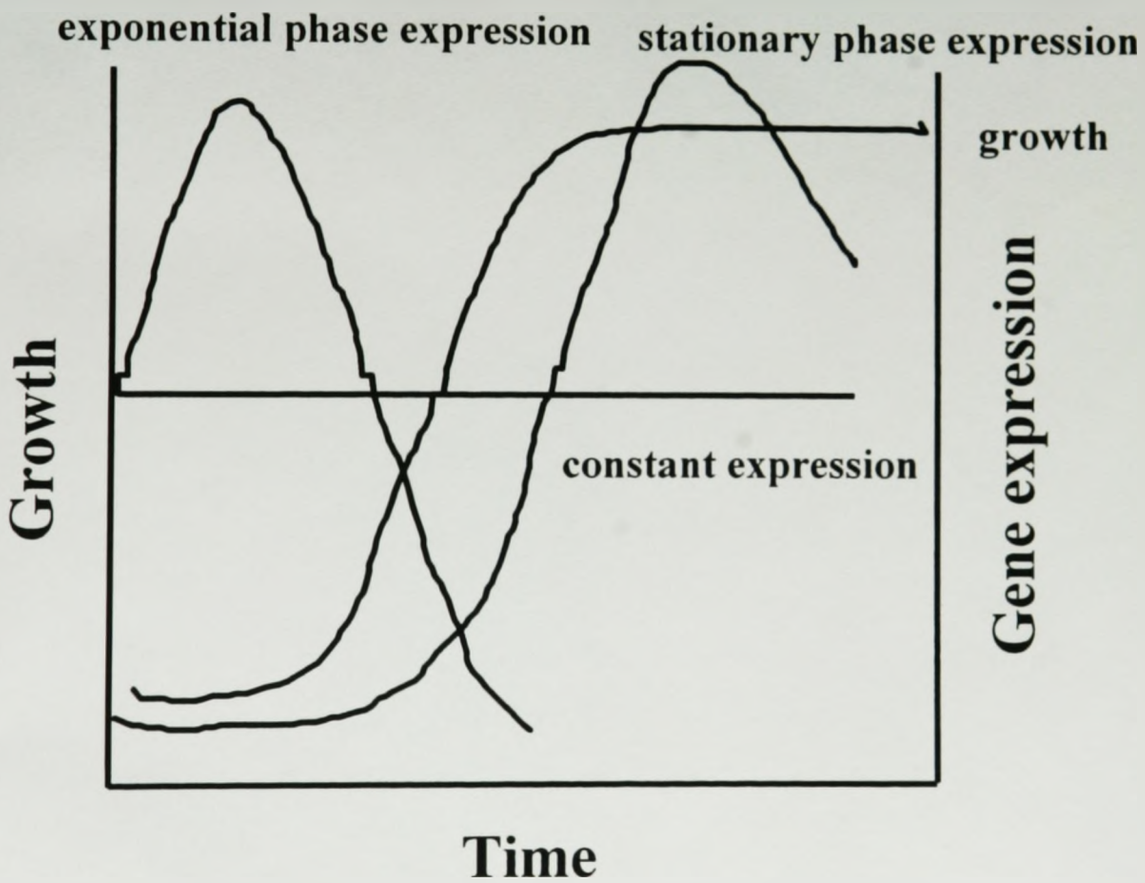
[a] Primer melting temperatures (Tm) were calculated using the **Primers!** calculator (www.williamstone.com/primers/calculator/calculator.cgi).

[b] Refers to the location of the primer stock in the freezer located in the Life Sciences Bldg, Rm 503.

APPENDIX III

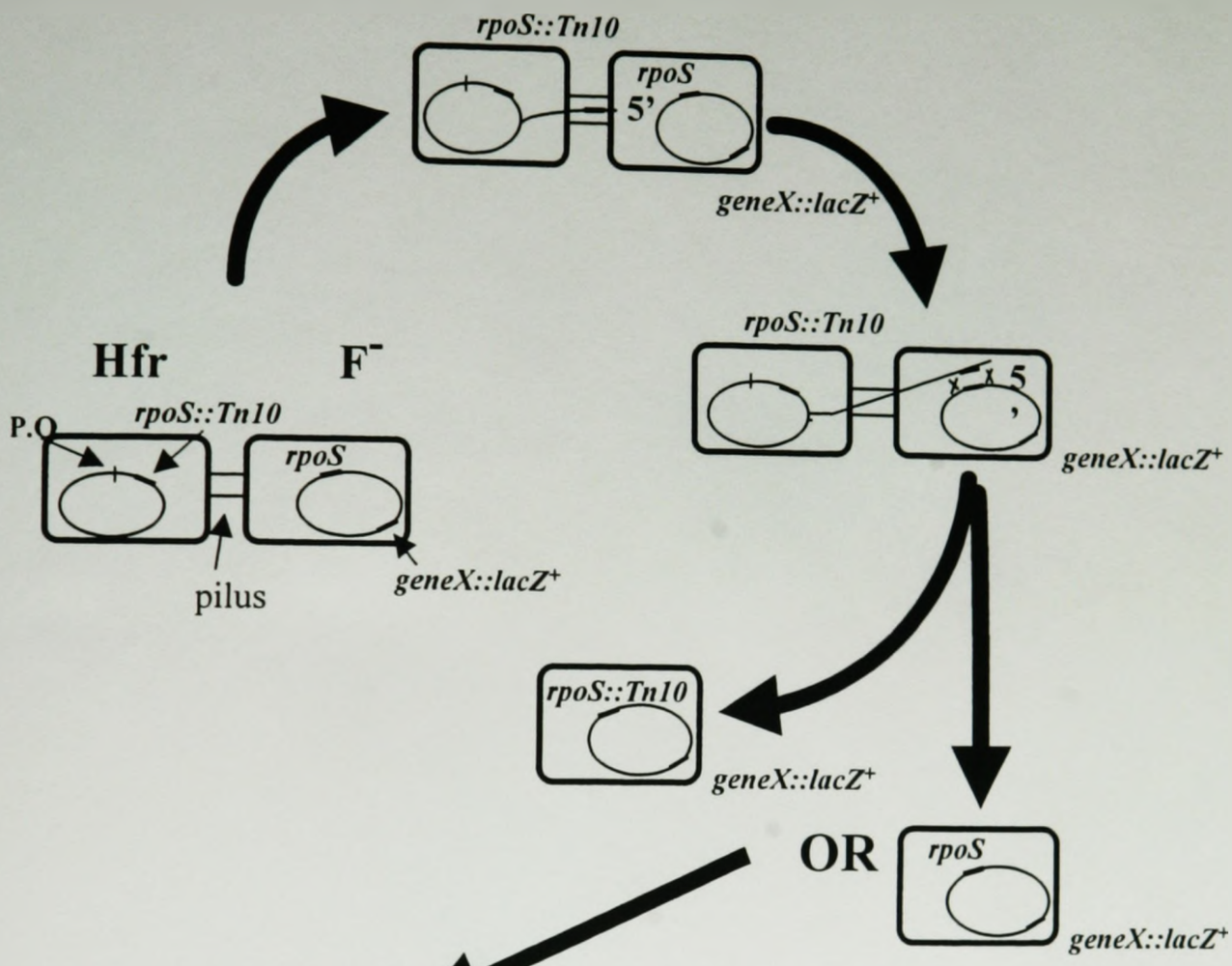
The following are figures presented during the oral defense of the thesis that do no appear anywhere else in the thesis.

A typical growth curve



- Stationary phase gene expression is dependent on RpoS
- helps confer resistance to a variety of environmental stresses

Objective: To attempt to delineate members of the RpoS regulon



LacZ+ Phenotype:
RpoS-independent fusion

LacZ- Phenotype:
RpoS-dependent fusion
rename *geneX::lacZ+*-->*rsd::lacZ+*
(*RpoS*-dependent)

Result: Identification of 105 RpoS-dependent promoter-*lacZ* fusions