ROLE OF RPOS IN ESCHERICHIA COLI

ROLE OF RPOS IN GLOBAL GENE REGULATION

AND

VIRULENCE IN ESCHERICHIA COLI

By

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ABSTRACT

Bacterial adaptation to changing conditions and to the host environment requires coordinated changes in gene expression that permit more efficient utilization of metabolites and increased survival. An important form of gene control is through the use of alternative sigma factors that direct RNA polymerase to recognize a distinct group of genes. One such sigma factor is RpoS, which is widely present in Proteobacteria including many serious human pathogens. As a key stress response regulator, RpoS plays an important role in adaptation, but its effect on virulence varies in different species. RpoS contributes to virulence through either enhancing survival against host defense systems or directly regulating expression of virulence factors in some pathogens, while RpoS is dispensable, or even inhibitory, to virulence in others. The primary objective of this study is to understand the mechanism of RpoS control in gene expression and pathogenesis of *Escherichia coli*. This thesis first describes the characterization of RpoS regulon in laboratory and pathogenic *E. coli* strains by transcriptome profiling analysis. Comparison of RpoS regulons identifies a core set of RpoS-controlled genes as well as strain-specific groups of genes, including many implicated in virulence. The contribution of RpoS to enteropathogenesis *in vivo* was tested using a *Citrobacter rodentium* (CR)-mouse infection model that is commonly used to simulate *E. coli* infection in human intestine. Mutations in *rpoS* result in reduced colonization and delay in mortality, indicating RpoS is important for full virulence. Clinical and natural *E. coli* isolates exhibit variable abilities in stress resistance and virulence, which is partly attributable to attenuating polymorphisms of *rpoS* commonly found in *E. coli* populations. A possible

mechanism responsible for the occurrence of *rpoS* polymorphisms in pathogenic *E. coli* is addressed. Using a group of representative enterohemorrhagic *E. coli* strains, we report that growth-enhanced mutants can be selected during growth on succinate and other poor carbon sources under both aerobic and anaerobic conditions. The majority of these mutants carry nonsense or missense mutations in *rpoS*. Phenotypic microarray analysis reveals that *rpoS* mutations result in increased utilization of 92 nitrogen and 8 carbon sources. Therefore, the occurrence of *rpoS* polymorphisms may increase the fitness of the population as a whole for better nutrient scavenging. In conclusion, RpoS may be viewed as a transient regulator that orchestrates the temporal expression of a large regulon for better adaptation under specific conditions including natural and host environments. Under conditions not requiring RpoS, its functions can be turned off through decreasing expression, rapid proteolysis, inhibition of RpoS activity, or selection of attenuating mutations. The final part of this thesis reviews the distinct and niche-dependent involvement of RpoS in virulence of many *rpoS*-bearing pathogens.

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PREFACE

This thesis is organized in a sandwich format consisting of a series of published articles, which have been modified to comply with the thesis format regulation. The preface of each chapter states the detail of corresponding publication and the relative contribution of this author to each multiple-authored work.

Chapter I presents a general review of RpoS research, including the importance of RpoS in various functions and the sophisticated mechanism that governs expression and activity of RpoS at multiple levels. Chapters II to IV examine the RpoS-regulated gene expression in laboratory *E. coli* K12 strain and pathogenic *E. coli* O157:H7 strain. The comparison of identified RpoS regulons reveals that RpoS-regulated gene expression varies substantially in different strains and environments. Chapter V describes the effects of RpoS on virulence *in vivo* using *Citrobacter rodentium*-mouse infection model. Chapter VI introduces a working mechanism for the occurrence of *rpoS* polymorphisms that result in variable stress resistance and virulence abilities in pathogenic *E. coli*. Chapter VII summarizes our current understanding of RpoS control in virulence in many different pathogens.

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LIST OF ABBREVIATIONS

AA	Amino acid
BCCDC	British Columbia Centre for Disease Control
cAMP	Cyclic adenosine monophosphate
c-di-AMP	Cyclic diadenosine monophosphate
cDNA	Complementary DNA
CR	*Citrobacter rodentium*
CRP	cAMP receptor protein
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EHEC	Enterohemorrhagic *E. coli*
EPEC	Enteropathogenic *E. coli*
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani broth
LFZ	Laboratory for Foodborne Zoonoses
LI	Log_2 intensity
LMER	Log_2 mean expression ratio
MER	Mean expression ratio
mRNA	Messenger RNA
NCBI	National Center for Biotechnology Information
NLEP	National Laboratory for Enteric Pathogens
ORF	Open reading frame
PCR	Polymerase chain reaction
ppGpp	Guanosine-tetraphosphate
RNA	Ribonucleic acid
sRNA	Small non-coding RNA
VTEC	Verocytotoxin-producing *E. coli*
WT	Wild type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER I

Introduction

from

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Springer, Berlin, Germany, pp. 313-337.

1.1 Preface

The contents of this chapter were contributed primarily by the author of this thesis. The text was written, in its entirety, by Tao Dong. Table 1.1 and Figure 1.2 were prepared by Charlie Joyce. It is adapted from the article published in the book of Bacterial Physiology - A Molecular Approach to fit the thesis format requirements.

1.2 Overview

Bacteria are the most abundant life form on Earth and can be found in nearly every environmental niche in the world, from hot springs to cold deep-sea beds. This wide distribution is largely due to an important feature of bacteria, their extreme adaptability, which allows bacteria to survive adverse environmental conditions in the natural habitat. Two distinct strategies are employed: cellular differentiation into specialized structures, e.g. sporulation in Gram-positive bacteria (reviewed in (Piggot & Hilbert, 2004)), and specific induction of stress-response genes that improves the fitness of the vegetative cell, as found in Gram-negative bacteria. Spores are extremely stable and are able to endure many stresses such as UV light and heat exposure. When they meet favorable growth conditions, spores become active and start proliferation. In non-sporulating bacteria, however, stress response and adaptation depend on the stimulated expression of stress response-governing regulators and their regulated genes.

Stress response regulators can be divided into two types- (i) specific regulators that are induced under particular stress conditions and control genes required for dealing with this specific stress (e.g. OxyR in oxidative stress (Farr & Kogoma, 1991)) and (ii) general stress regulators that are induced in response to multiple environmental signals and activate the expression of large regulons that include genes required not only for the proximal stress condition but also genes for other potential stress conditions. Therefore, general stress regulators likely provide a preventative mechanism that prepares the cell for concurrently-experienced stresses compared with specific regulators that mainly activate genes only for protection against the current stress and repair of damage. One of

the major general stress regulators is RpoS, an alternative sigma factor primarily found in beta- and gamma-proteobacteria.

In the model organism of Gram-negative bacteria *Escherichia coli*, RNA polymerase holoenzyme is composed of five subunits ($\alpha\alpha\beta\beta'\sigma$) (for reviews see (Gross *et al.*, 1998; Helmann & Chamberlin, 1988; Ishihama, 2000)). The $\alpha\alpha\beta\beta'$ subunits are assembled as a core enzyme that, though self-sufficient for transcription elongation, requires a sigma factor to specifically bind to promoter regions and initiate the transcription process. There are seven known sigma factors - RpoD, RpoN, RpoS, RpoH, RpoF, RpoE, and FecI – with each regulating the transcription of particular genes in response to corresponding environmental conditions except for RpoD and RpoS (reviewed in (Ishihama, 2000)). RpoD is the vegetative sigma factor responsible for the transcription of most genes in fast-growing cells, while the alternative RpoS is highly induced during the transition from exponential phase to stationary phase or under stress conditions. RpoS functions as a general stress response regulator, while other alternative sigma factors are specific for certain stress conditions (Table 1.1).

RpoS was originally identified in several independent contexts as a regulator of the expression of phosphatase (Touati *et al.*, 1986) and of catalase (Loewen & Triggs, 1984) and in protection from near-UV light (Sammartano *et al.*, 1986; Tuveson, 1980). It soon became clear that RpoS likely controls the expression of many other genes as well, and these genes, as a group, are expressed primarily in post-exponential phase (Lange & Hengge-Aronis, 1991b). Historically, gene regulation studies have been performed using exponential phase cultures under "balanced" growth conditions, and this probably led to a

delayed appreciation of the importance of genes expressed when the cell is in a slow growth state.

1.3 Distribution of RpoS

The RpoS-controlled stress response mechanism is a conserved function among the gamma and beta proteobacteria. Homologous *rpoS* sequences from 29 genera can be identified by searching *rpoS* against all the annotated genomes in the TIGR database (Figure 1.1). The phylogeny of RpoS is similar to that of the 16S rRNA tree, indicating the conserved nature of RpoS in these species (unpublished data). Although it is still not clear how RpoS arose during evolution, it is likely that RpoS evolved by duplication from RpoD, with which RpoS shares 59% identity in gene sequence. Furthermore, many RpoS-regulated genes can be transcribed by RpoD-associated holoenzyme *in vitro* suggesting a strong functional similarity. In contrast, many RpoS-regulated genes, including *aldB, katE, gabP,* and *osmY,* have likely been laterally transferred between species (unpublished data). This is consistent with the idea that horizontal transfer is more likely to have occurred for operational genes, such as those involved in amino acid synthesis and other housekeeping functions, rather than informational genes that are involved in transcription, translation and regulation processes (Simonson *et al.*, 2005). How genes are recruited into the RpoS regulon remains unclear. One possible scenario is that when an external gene with a promoter region that is poorly recognized by existing sigma factors is introduced into a bacterium's genome, and if the expression of this new gene can confer a growth advantage under selective pressure, either promoter mutations or sigma factor mutations may be selected that enhance the expression of this gene.

Although promoter mutations are more frequently observed under laboratory conditions than structural changes of a sigma factor, and thus are more likely to occur, one major disadvantage of such a mutation is that constant expression of this newly recruited gene may impose an excessive biosynthetic energy cost. Therefore, mutations in sigma factors resulting in specialization may be preferred so that alternative sigma factors such as RpoS are evolved. However, this hypothesis requires further experimental evidence and testing.

1.4 Regulation of RpoS expression and function

RpoS is induced during the transition from exponential phase to stationary phase or in response to stress conditions, followed by activation of RpoS-regulated general stress response machinery that subsequently results in a series of physiological and morphological changes (Hengge-Aronis, 2002a; Lange & Hengge-Aronis, 1991b). Therefore, the expression of RpoS must be strictly regulated as inappropriate expression of this large regulon would likely have deleterious consequences for the cell. Indeed, many regulatory factors have been identified to regulate RpoS expression at the transcriptional, translational and post-translational levels (Figure 1.2 and reviewed in (Hengge-Aronis, 2002a; Ishihama, 2000)).

1.4.1 Transcriptional regulation

The *rpoS* gene shares two promoters with its upstream gene *nlpD*, generating polycistronic *nlpD-rpoS* mRNAs which are independent of environmental stimuli and may provide a low, but constant, level of *rpoS* transcript throughout growth (Lange *et al.*, 1995). However, the major *rpoS* promoter lies inside the *nlpD* gene and this promoter is primarily responsible for *rpoS* induction. Growth rate reduction (Ihssen & Egli, 2004),

guanosine 3',5'-bispyrophosphate (ppGpp) (Gentry *et al.*, 1993), polyphosphate (Shiba *et al.*, 1997), and acetate (Schellhorn & Stones, 1992) positively regulate *rpoS* transcription, while Fis (Hirsch & Elliott, 2005) is a negative regulator that can bind to the *rpoS* promoter region to block transcription. The cAMP-CRP molecule regulates *rpoS* transcription in a growth-phase dependent manner- a negative regulator in exponential phase but a positive regulator in stationary phase (Lange & Hengge-Aronis, 1994). Although expression of *rpoS* is greatly reduced in a ppGpp deficient strain (Gentry *et al.*, 1993), how ppGpp enhances *rpoS* expression is still not clear. It was shown that ppGpp is important for *rpoS* transcriptional elongation (Lange *et al.*, 1995), while another study showed that overproduction of ppGpp has little effect on the abundance of *rpoS* transcripts (Brown *et al.*, 2002). The stimulation of *rpoS* expression by ppGpp is growth-phase independent (Hirsch & Elliott, 2002). Fis, a global transcriptional factor, inhibits *rpoS* transcription by directly binding to the *rpoS* promoter region. Fis levels are growth-phase dependent. At the onset of stationary phase, Fis disappears and the transcription of *rpoS* is induced (Hirsch & Elliott, 2005). Polyphosphate is an important inorganic molecule that is produced by many bacterial species and functions as a phosphate reservoir, a cation chelator and a regulatory factor (for a review see (Kornberg *et al.*, 1999)). Polyphosphate-free mutants are stress sensitive and are impaired in survival in stationary phase (Rao & Kornberg, 1996). Consistently, it has been found that polyphosphate stimulates *rpoS* transcription and this stimulation is likely to be independent of ppGpp (Shiba *et al.*, 1997). High cellular NADH to NAD ratio also attenuates *rpoS* transcription, although the mechanism is not known (Sevcik *et al.*, 2001).

1.4.2 Translational regulation

Translational control of *rpoS* is also important for increased RpoS levels in stationary phase (Lange & Hengge-Aronis, 1994). Upon onset of stationary phase, the *rpoS* mRNA level increases 10-fold, while the RpoS protein level increases 30-fold (Lange & Hengge-Aronis, 1994). Translation of *rpoS* is stimulated by cold shock (Sledjeski *et al.*, 1996), polyamines (Yoshida *et al.*, 2002), high osmolarity (Muffler *et al.*, 1996c), CspC and CspE (Phadtare & Inouye, 2001), DsrA (Majdalani *et al.*, 1998; Sledjeski *et al.*, 1996), RprA (Majdalani *et al.*, 2001; Majdalani *et al.*, 2002), Hfq (Zhang *et al.*, 1998), HU (Balandina *et al.*, 2001), and UDP-glucose (Bohringer *et al.*, 1995).

The major *rpoS* transcript starts in the middle of the upstream gene *nlpD*, generating an untranslated 567nt segment at the 5' end of the mRNA product (Lange *et al.*, 1995; Takayanagi *et al.*, 1994). This leader region folds into a hairpin structure that stabilizes the *rpoS* transcript and blocks ribosome binding to prevent translation initiation (Brown & Elliott, 1997; Cunning *et al.*, 1998). Therefore, the translation of *rpoS* is blocked until positive regulatory factors such as Hfq, DsrA, RprA and HU bind to the 5' end hairpin to expose the translation initiation region (Cunning *et al.*, 1998; Majdalani *et al.*, 1998; Majdalani *et al.*, 2001; Zhang *et al.*, 1998). Hfq is an RNA binding protein which plays an important role in stabilizing small RNA regulators, as discussed below. Binding of Hfq to *rpoS* mRNA may also allow other regulators to be recruited to initiate the translation. Depletion of UDP-glucose stimulates RpoS expression in exponential phase in an Hfq-dependent manner (Bohringer *et al.*, 1995). The cold shock proteins CspC and CspE can also stimulate RpoS expression by stabilizing the *rpoS* mRNA

(Phadtare & Inouye, 2001). Overexpression of CspC or CspE increases the abundance of *rpoS* mRNA transcripts by about 4-fold, while deletion of *cspC* and *cspE* leads to a 4-fold decrease in *rpoS* transcript level (Phadtare *et al.*, 2006). Similarly, HU, a nucleoid protein, stimulates *rpoS* translation, possibly by altering the secondary structure of *rpoS* mRNA (Balandina *et al.*, 2001).

H-NS and OxyS are negative regulators for *rpoS* translation. H-NS is a universal repressor in gene regulation that forms nucleo-protein complexes with target genes (Barth *et al.*, 1995; Yamashino *et al.*, 1995). As H-NS can bind directly to *rpoS* mRNA, it may function as an RNA chaperone to alter the secondary structure of the *rpoS* transcript. H-NS may also inhibit the binding between DsrA and *rpoS* mRNA. By contrast, OxyS competes with DsrA and *rpoS* mRNA for binding to Hfq (Zhang *et al.*, 1998).

Small regulatory RNAs can modulate gene expression by basepairing with target RNAs. More than 60 small RNAs have now been identified in *E. coli* (reviewed in (Majdalani *et al.*, 2005)). Among them, DsrA and RprA are positive regulators of *rpoS,* while OxyS is an inhibitor of *rpoS* transcription. The 87nt long *dsrA* forms a secondary structure with three stem loops, part of which can complementarily bind to the antisense region at the 5' end of *rpoS* mRNA to release the transcription initiation region for ribosome binding (Majdalani *et al.*, 1998; Majdalani *et al.*, 2002). The *dsrA* gene is expressed at temperatures below 30°C (Repoila *et al.*, 2003; Repoila & Gottesman, 2001) and is protected from nuclease degradation by direct interaction with Hfq (Brescia *et al.*, 2003; Moll *et al.*, 2003). It is possible that Hfq alters the structures of both *dsrA* and *rpoS* mRNA to facilitate the binding between DsrA and *rpoS*. There is another binding domain

in DsrA for *hns* mRNA that inhibits *hns* mRNA translation (Lease *et al.*, 1998). The competition for DsrA binding between *rpoS* mRNA and *hns* mRNA may partly explain the negative impact on *rpoS* translation by H-NS. With a similar structure to DsrA, RprA functions by pairing to the same untranslated upstream region of *rpoS* (Majdalani *et al.*, 2005). OxyS is induced under oxidative stress conditions (Altuvia *et al.*, 1997; Zhang *et al.*, 1998) and down-regulates *rpoS* translation by binding to Hfq and blocking Hfq interaction to both *rpoS* and DsrA (Zhang *et al.*, 1998). When RpoS is induced during oxidative stress, negative regulation of *rpoS* by OxyS may provide a fine-tuning system to prevent over-expression of RpoS-regulated genes induced by oxidative stress, such as *katE*, a highly RpoS-dependent gene encoding hydrogen peroxidase II (Altuvia *et al.*, 1997; Zhang *et al.*, 1998).

1.4.3 Post-translational regulation

In addition to the plethora of identified transcriptional and translational modulators, regulated proteolysis of RpoS is a key (and perhaps the most important) mechanism for maintaining low levels of this sigma factor in optimal growth conditions and for rapidly increasing its levels during adaptation to starvation. In exponential phase, the RpoS protein is unstable with a half life of 1.4 min, while during stationary phase, the half-life of RpoS significantly increases to 20 min (Lange & Hengge-Aronis, 1994). This increased stability alone can result in a significant increase in RpoS levels, underscoring the importance of post-translational regulation in RpoS control.

In exponential phase cells, RpoS is specifically targeted for proteolysis by RssB (Muffler et. al., 1996; Schweder et. al., 1996) (also known as SprE (Pratt & Silhavy, 1996)

and MviA (Bearson *et al.*, 1996)) which directs the protein to the ATP-dependent ClpXP protease (Zhou *et al.*, 2001). RssB is a response regulator, whose affinity to RpoS is modulated by phosphorylation at D58 of the conserved N-terminal receiver domain. This affinity, though likely important for targeted degradation, is probably not a sufficient condition as increases in RpoS stability during starvation still occur in strains carrying a mutation eliminating the D58 phosphorylation site (Peterson *et al.*, 2004). Levels of RssB increase as cells enter stationary phase, while levels of the ClpXP are fairly constant. Other factors may also play a key role. In contrast to RssB, DnaK inhibits RpoS proteolysis as RpoS stability substantially decreases in *dnaK* mutants (Rockabrand *et al.*, 1998).

A small recently-identified protein (Bougdour *et al.*, 2006), IraP, inhibits the activity of RssB under low phosphate conditions and can thus stabilize RpoS under some but not all conditions (since it is not effective during glucose starvation). The complex nature of proteolytic control of RpoS thus allows cells during starvation to more quickly increase levels of this regulator in response to specific starvation signals, independent of increases in transcription or stabilization of transcript (see above). The metabolic imperative for evolving this type of control is likely driven by the necessity of increasing the efficiency of *de novo* expression of the large RpoS regulon during starvation. While in the starvation state, the limited availability of precursors and energy sources may reduce the effectiveness of earlier controls at the transcription/translation level.

In view of the large number of factors that participate in the RssB-ClpXP pathway, what is the proximal signal that causes RpoS stabilization? Recently, the accumulation of

oxidized, misfolded proteins has been shown to be an important physiological signal that is indirectly sensed by the RssB-ClpXP pathway (Fredriksson *et al.*, 2007). Accordingly, ribosomal stalling, caused by depletion of the pool of charged tRNAs during starvation, results in a large increase in misfolded protein which, under aerobic conditions, readily oxidize and become substrates for ClpXP. The competition by alternative proteolysis substrates reduces the availability of ClpXP to participate in the RssB-targeted degradation of RpoS. Consistent with this model, experimental perturbation of degradation through inactivation of ClpXP, mutation of the conserved RssB aspartate phosphorylation site (Peterson *et al.*, 2004), decreasing the fidelity of translation (Fredriksson *et al.*, 2007) or increasing the availability of competing misfolded protein substrates for ClpXP (Fredriksson *et al.*, 2007) all result in increased levels of RpoS through stabilization. An appealing aspect of this model is that a single metabolic state, the stalled ribosome, signals both an increase in expression of the RpoS regulon and, through the stringent response (Gourse *et al.*, 1996), attenuation of the major RpoD-dependent operons such as the ribosomal operons.

1.4.4 Sigma factor competition

Transcription of RpoS-regulated genes requires a relatively high number of RNA core polymerase associated with RpoS to initiate the process. However, even in stationary phase, where RpoS is induced, the protein level of RpoS is only about 30% of that of RpoD, and the binding affinity to the RNA polymerase core enzyme of RpoD is 16-fold higher than that of RpoS (Maeda *et al.*, 2000). Furthermore, the concentration of RpoD itself already exceeds the amount of freely-available RNA polymerase core enzyme (not

actively participating in transcription), as shown in both exponential phase (Grigorova *et al.*, 2006; Ishihama, 2000) and stationary phase cells (Ishihama, 2000). Therefore, there is an excess of sigma factors competing for the limited amount of core enzyme. Over-expression of RpoS reduces the expression of RpoD-regulated promoters, while mutations in *rpoS* have the opposite effect (Farewell *et al.*, 1998a).

Given the poor affinity and low cellular concentration of RpoS, even in stationary phase, it is intriguing that RpoS-regulated genes are effectively expressed in this phase. A combination of other factors, including anti-sigma factors, promoter preference, and physiological changes, are involved in this phenomenon (Typas *et al.*, 2007b). Anti-sigma factors like Rsd (regulator of sigma D) and 6S RNA seem to play an important role in the competition between RpoD and RpoS. Rsd, induced at the onset of stationary phase, can directly bind to RpoD causing inhibition of RpoD and leading to enhanced expression of RpoS-regulated genes (Jishage & Ishihama, 1998). 6S RNA can also directly bind to RpoD, reducing its activity (Wassarman & Saecker, 2006; Wassarman & Storz, 2000). Physiological changes during the transition from exponential phase to stationary phase include the accumulation of small metabolites such as glutamate, acetate and trehalose, which may contribute, either directly or indirectly, to an enhanced RpoS competition for core polymerase. The alarmone ppGpp, induced in stationary phase, also plays an important role in the activation of not only RpoS but also RpoE, RpoH and RpoN (Bernardo *et al.*, 2006; Costanzo & Ades, 2006; Jishage *et al.*, 2002; Laurie *et al.*, 2003). However, ppGpp stimulates both the expression and activity of RpoS (Kvint *et al.*, 2000), while increasing the activity only of RpoE, RpoH and RpoN ((Costanzo & Ades,

2006; Sze & Shingler, 1999; VanBogelen & Neidhardt, 1990) and see reviews (Gourse *et al.*, 2006; Nystrom, 2004b)).

Transcription of RpoS-regulated genes may also require additional trans-acting regulators such as Crl, a low temperature induced regulatory protein that binds directly to RpoS-holoenzyme to stimulate RpoS activity (Bougdour *et al.*, 2004; Farewell *et al.*, 1998a; Pratt & Silhavy, 1998). There are 63 proteins that are co-regulated by RpoS and Crl (Lelong *et al.*, 2007).

1.4.5 Summary of regulation

All these regulatory factors clearly support a central role for RpoS as a multi-signal receiver in the stress response circuit. Cell adaptation to growth conditions is a complicated process involving signal receiver factors to sense the environmental changes, signal transducers and processors, and response effectors. As a general adaptation regulator, RpoS may be considered as both a multi-signal receiver that can sense the environmental changes indirectly through these specific regulators and a processor that can transduce these signals into functional effectors to ensure survival or growth fitness. For example, low temperature (below 30°C) triggers *dsrA* induction, which, in turn, stimulates *rpoS* translation, resulting in the expression of many genes including the curli biosynthesis genes, *csgD* and *csgAB,* which are controlled by RpoS (Romling *et al.*, 1998). In this temperature-related regulation route, DsrA plays a thermosensor role to input the cold shock signal into the RpoS regulatory network. RpoS then both receives the cold shock signal and initiates downstream changes for cell adaptation or protection

against this harmful input, thereby protecting the cell from the cold – enhancing the cell's prospect of survival.

1.5 RpoS, a master regulator in stress response and adaptation

Global expression studies, including microarray analysis and mutational screens, have greatly improved our understanding of the RpoS regulon in *E. coli* (Lacour & Landini, 2004; Patten *et al.*, 2004; Vijayakumar *et al.*, 2004; Weber *et al.*, 2005). More than 10% of the *E. coli* genome is controlled by RpoS, most of which is involved in stress response such as nutrient limitation (Notley & Ferenci, 1996), resistance to DNA damage (Khil & Camerini-Otero, 2002), osmotic shock (Cheung *et al.*, 2003), high hydrostatic pressure (Robey *et al.*, 2001), oxidative stress (Schellhorn & Hassan, 1988), ethanol resistance (Farewell *et al.*, 1998b), adaptive mutagenesis (Lombardo *et al.*, 2004), acid stress (Lin *et al.*, 1996) and biofilm formation (Schembri *et al.*, 2003b), underlining the importance of RpoS in cell adaptation.

1.5.1 Acid response

RpoS mutants are very sensitive to low pH challenge (Small *et al.*, 1994), indicating the importance of RpoS in acid resistance. *E. coli* has three known acid resistance (AR) systems, including RpoS-dependent oxidative, arginine- and glutamate-dependent decarboxylase AR systems (Audia *et al.*, 2001; Castanie-Cornet *et al.*, 1999; Price *et al.*, 2000). RpoS is essential for the oxidative system, but only partially required for the other two AR systems (Lin *et al.*, 1996). The arginine AR system, including the arginine decarboxylase (*adiA*) and the regulator CysB, requires the addition of arginine during the acid challenge. The *adiA* gene is positively controlled by RpoS (Vijayakumar

et al., 2004). The glutamate AR system includes two glutamate decarboxylase genes, *gadA* and *gadB*, and is RpoS–dependent in stationary phase induction but RpoS-independent in acid induction (Castanie-Cornet *et al.*, 1999).

1.5.2 Oxidative stress

E. coli may face oxidative stress caused by reactive oxygen species (ROS) including the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\cdot}$). ROS may be generated as endogenous respiratory products or externally secreted by other competing bacteria or host immune systems. Two catalases, HPI and HPII, encoded by *katG* and *katE* respectively, exist in *E. coli*. Both *katE* and *katG* are regulated by RpoS in stationary phase and under some stress conditions (reviewed in (Schellhorn, 1995)). KatG is also regulated by OxyR, an important factor in the oxidative stress response (Christman *et al.*, 1989; Ivanova *et al.*, 1994). KatE is the major catalase in stationary phase and is highly RpoS-dependent (Schellhorn & Hassan, 1988). Another anti-oxidative cell defense system involves glutaredoxin or thioredoxin that can reduce cytosolic disulfide in *E. coli* (Holmgren, 2000; Ritz & Beckwith, 2001). There are three glutaredoxins, encoded by *grxA, grxB* and *grxC*, two thioredoxins, encoded by *trxA* and *trxC*, and an NrdH redoxin that shows a glutaredoxin-like sequence but a thioredoxin-like activity (Holmgren, 1989; Jordan *et al.*, 1997). It has been shown that the *grxB* gene is positively regulated by RpoS and ppGpp in stationary phase, while *trxA* is ppGpp-dependent but RpoS-independent (Potamitou *et al.*, 2002). Expression of *nrdH* is high in early exponential phase and drops significantly during the transition from mid-

exponential phase to stationary phase in rich media (Monje-Casas *et al.*, 2001) and is RpoS-independent throughout growth (Monje-Casas *et al.*, 2001).

1.5.3 Anaerobic growth

In continuous culture, anaerobic growth of *E. coli* leads to 2-fold lower levels of RpoS than under aerobic conditions (King & Ferenci, 2005). Consequently, the expression of RpoS-regulated genes decreases, and cells become more stress sensitive (King & Ferenci, 2005). However, wild type strains can out-compete *rpoS* mutants under anaerobic conditions, indicating that RpoS expression, although low, confers a growth advantage under anaerobiosis (King & Ferenci, 2005). Acid resistance of anaerobically-grown wild type and *rpoS* mutant strains is similar for cells adapted to acidic conditions (Small *et al.*, 1994). However, wild type strains adapted to alkaline pH are more acid resistant than *rpoS* mutants (Small *et al.*, 1994).

1.5.4 Biofilm formation

Many bacteria in the natural environment are found as free-living individual cells (planktonic) or in large sessile communities (biofilms). Biofilm growth requires a significant change in gene expression resulting in the morphological adaptation from planktonic individuals to sessile communities. The highly organized structure of a bacterial biofilm can confer cell resistance against antimicrobial agents and host immune defenses (Nickel *et al.*, 1985; Stewart & Costerton, 2001). RpoS is important in biofilm formation, as mutations in RpoS cause lower biofilm cell density and an alteration in biofilm structure when cells are grown to stationary phase in minimal media (Adams & McLean, 1999). It has been reported that *rpoS* mutants failed to establish a biofilm within

42 hours, and at least 30 RpoS-regulated genes are involved in biofilm formation (Schembri *et al.*, 2003b). However, several other studies show that RpoS negatively regulates biofilm formation in exponential growth in rich complex media (Corona-Izquierdo & Membrillo-Hernandez, 2002; Domka *et al.*, 2006). Therefore, it is likely that RpoS regulation of biofilm formation is growth-phase dependent and involves other factors. Extracellular structures, such as cellulose and curli fimbriae that are important for biofilm formation (Romling, 2005), are positively regulated by RpoS, possibly through regulating the production of a small signaling molecule c-di-GMP (Weber *et al.*, 2006). However, this RpoS-dependent regulation is operant only at low temperatures (Romling, 2005; Weber *et al.*, 2006).

1.5.5 Adaptive mutagenesis

RpoS is important for adaptive point mutations and amplifications in stationary phase (Bjedov *et al.*, 2003; Lombardo *et al.*, 2004). Stress-induced mutation plays an important role in bacterial evolution by increasing the adaptive mutation rate, resulting in a growth advantage in stationary phase or other stress conditions (Bjedov *et al.*, 2003). In stationary phase, RpoS protects DNA from damage caused by oxidative stress and UV irradiation through increased expression of Dps, a non-specific DNA binding protein that can condense the chromosome into a compacted nucleoprotein complex (Nair & Finkel, 2004). However, once the damage occurs in the chromosome, RpoS activates a mechanism to bypass the error without correction through up-regulating the DinB error-prone DNA polymerase (Layton & Foster, 2003) or down-regulating the methyl-directed mismatch repair (MMR) system mediated by MutS, MutL and MutH (Feng *et al.*, 1996) .

DinB, encoding DNA polymerase IV, repairs DNA lesions with reduced fidelity, leading to an increased mutation rate (Kim *et al.*, 1997). DinB is up-regulated by RpoS in stationary phase (Layton & Foster, 2003). Inactivation of MMR can lead to high rates of mutation and recombination (Li *et al.*, 2003), and in stationary phase, the level of MutS protein drops by at least 10 fold compared to that in exponential phase (Feng *et al.*, 1996). Many other RpoS-regulated genes including *xthA* (encoding exonuclease III), *aidB* (methylation damage repair), and *ftsQZ* (cell division) are possibly involved in the point mutation and amplification mechanism.

1.5.6 Pathogenesis

RpoS controls expression of virulence factors in several organisms, including *Salmonella* ser. Typhimurium (Fang *et al.*, 1992), *Yersinia enterolitica* (Iriarte *et al.*, 1995), *Pseudomonas aeruginosa* (Suh *et al.*, 1999), and *Vibrio cholerae* (Yildiz & Schoolnik, 1998). RpoS enhances not only the bacterial defense system, by regulating acid and oxidative resistance, but also the expression of virulence genes. In *Salmonella*, RpoS controls the expression of *spv* virulence genes that are located on a pathogenicity plasmid (Fang *et al.*, 1992). RpoS is an important virulence factor in many, although not all, pathogenic *E. coli* strains. For example, RpoS stimulates the invasion of brain microvascular endothelial cells by some *E. coli* K1 strains (Wang and Kim 1999). RpoS also controls the expression of several genes on the Locus of Enterocyte Effacement (LEE) pathogenicity island, which is responsible for forming the characteristic Attaching and Effacing (AE) lesions (Elliott *et al.*, 1998). LEE carries 41 genes in five polycistronic operons (LEE1 to LEE5) (Nataro & Kaper, 1998), encoding a type III secretion system

(TTSS), an intimin Eae (Nataro & Kaper, 1998) and intimin-receptor Tir (Kenny *et al.*, 1997), and secreted effector proteins (Elliott *et al.*, 1998). RpoS positively controls the expression of the LEE3 operon, encoding part of the structural and regulatory components of the TTSS (Nataro & Kaper, 1998), and Tir, which is important for *E. coli* adherence (Sperandio *et al.*, 1999). Some other genes on LEE, however, are RpoS down-regulated (Iyoda & Watanabe, 2005; Laaberki *et al.*, 2006; Tomoyasu *et al.*, 2005). RpoS is also required for full expression of *csgA* and *csgB* encoding proteins for curli formation, an important cell surface structure implicated in pathogenesis (Uhlich *et al.*, 2002), and *rfaH*, a primary virulence regulator in *Salmonella* and *E. coli* which modulates the biosynthesis of cell surface structures (Bittner *et al.*, 2004; Creeger *et al.*, 1984).

1.5.7 Negative regulation by RpoS

As a sigma factor, RpoS is expected to have a positive regulatory role in transcription. Surprisingly, however, the expression of many genes is higher in *rpoS* mutants, and this negative regulation may help explain why, under some circumstances, there is a strong selection for loss of *rpoS* function (Zambrano *et al.*, 1993). RpoS negatively regulates genes involved in flagellum biosynthesis, the entire TCA cycle and a cluster of genes in the Rac prophage region (Patten *et al.*, 2004). The flagellum genes, including those encoding structural components such as FliC and others specific regulatory factors such as FliA, are required for cell motility. A second group of RpoS down-regulated genes, those in the TCA cycle, are important for active metabolism and energy production (Figure 1.3). Since RpoS is induced in stationary phase, a nutrient-limiting condition, preservation of energy sources by repressing cell motility and energy

consumption pathways may be important for long-term survival. Consistent with this, the viability of *rpoS* mutants is significantly lower than that of wild type strains after long term incubation (Lange & Hengge-Aronis, 1991b).

As a transcription sigma factor, the negative regulatory role of RpoS may result from two possibilities: (i) sigma factor competition for core RNA polymerase; (ii) a negative intermediate regulator in the RpoS regulon. The second explanation is supported by the fact that some repressors are found within the RpoS regulon, such as FNR (Patten *et al.*, 2004).

Non-specific binding of sigma factors and RNA core polymerase to DNA sequences, including not only promoter regions but also within open reading frames, has been described by many studies using both *in vitro* and *in vivo* models (deHaseth *et al.*, 1978; Grigorova *et al.*, 2006; von Hippel *et al.*, 1974). Although these studies mainly focus on mechanisms releasing the core RNA polymerase from non-specific binding sites, this non-specific binding feature may also confer upon sigma factors, in this case RpoS, the ability to block active gene transcription as a repressor. However, this hypothesis requires experimental validation.

1.6 Consensus RpoS-controlled promoter sequence

It is interesting to note that there are only minor differences in consensus promoter sequences recognized by RpoS and RpoD. Promoters preferentially activated by RpoD exhibit a consensus –10 region (TATAAT), while RpoS controls a more degenerate promoter sequence featuring a –10 region (TAYACT), a cytosine at −13, a TG motif at positions −14 to −18, and an A/T-rich region downstream of the −10 region

(Typas *et al.*, 2007b). The cytosine at -13 is considered to be a specific marker for RpoS-regulated promoters and can be found in most RpoS-regulated genes (Weber *et al.*, 2005). The −35 region in RpoS-dependent promoters is also more flexible than that in RpoD-controlled promoters, though the consensus sequence for each -35 region is the same (TTGACA) (Hengge-Aronis, 2002b; Lacour & Landini, 2004). In addition, a random promoter test shows that the strength of RpoS-regulated promoters can be altered by modulating the sequence from –37 to –14 upstream of the −10 region (Miksch *et al.*, 2005).

However, not all known RpoS-dependent promoters have these conserved features. The lack of a single conserved and specific RpoS-dependent promoter sequence may allow RpoS to selectively transcribe genes under specific conditions through the action of other additional regulators. As a result, these other regulators could then modulate the stress response of the cell as needed for the particular stress stimulus.

1.7 Role of polymorphisms of *rpoS* in cell adaptation

Despite the clear importance of RpoS for the survival of cells under stress conditions, *rpoS* polymorphisms, as well as variations in the nearby *mutS-rpoS* region, are common in both laboratory strains and natural isolates of *E. coli* (Herbelin *et al.*, 2000; King *et al.*, 2004). For example, there is significant variation at codon 33 even among K-12 strains; an amber mutation TAG is present in some K-12 strains, while CAG encoding glutamate or GAG encoding glutamine are found in other strains (Atlung *et al.*, 2002). Many clinical isolates of pathogenic *E. coli* are sensitive to acid challenge because they possess *rpoS* null mutations (Bhagwat *et al.*, 2005). One possible explanation for *rpoS*

polymorphisms is that mutations in *rpoS* may confer a growth advantage under carbon source starvation (Farrell & Finkel, 2003). The *rpoS* mutants can out-compete wild type strains and take over the population of cells after prolonged incubation (Zambrano *et al.*, 1993). In addition, growth on succinate and other TCA cycle intermediates can specifically select for *rpoS* mutations in *E. coli* K-12 strains (Chen *et al.*, 2004). The selective pressure may result from the negative regulation of RpoS on the TCA cycle genes, as previously mentioned. The consequence of such selection for *rpoS* mutations is to support better growth on these non-preferred carbon sources, though at the expense of the cell's capability to cope with stress. This mutational switch to turn off a large set of genes may be important for *E. coli* cells to be competitive in a complex environment, such as the mammalian intestine which is host to at least 1,000 different bacterial species (Sears, 2005).

Table 1.1 Sigma factors and their corresponding functions.

This table lists the seven known sigma factors in *Escherichia coli*. For a review on the functions of sigma factors, see Ishihama (2000).

Sigma subunit	Molecular weight	Primary function
RpoD	70 kDa	Growth-related genes
RpoS	38 kDa	Stationary phase and stress response genes
RpoN	54 kDa	Nitrogen regulation and related stress response genes
RpoH	32 kDa	Heat shock and related stress response genes
RpoF	28 kDa	Flagella-chemotaxis genes
RpoE	24 kDa	Extreme heat shock and extracytoplasmic genes
FecI	19 kDa	Ferric citrate transport and extracytoplasmic genes

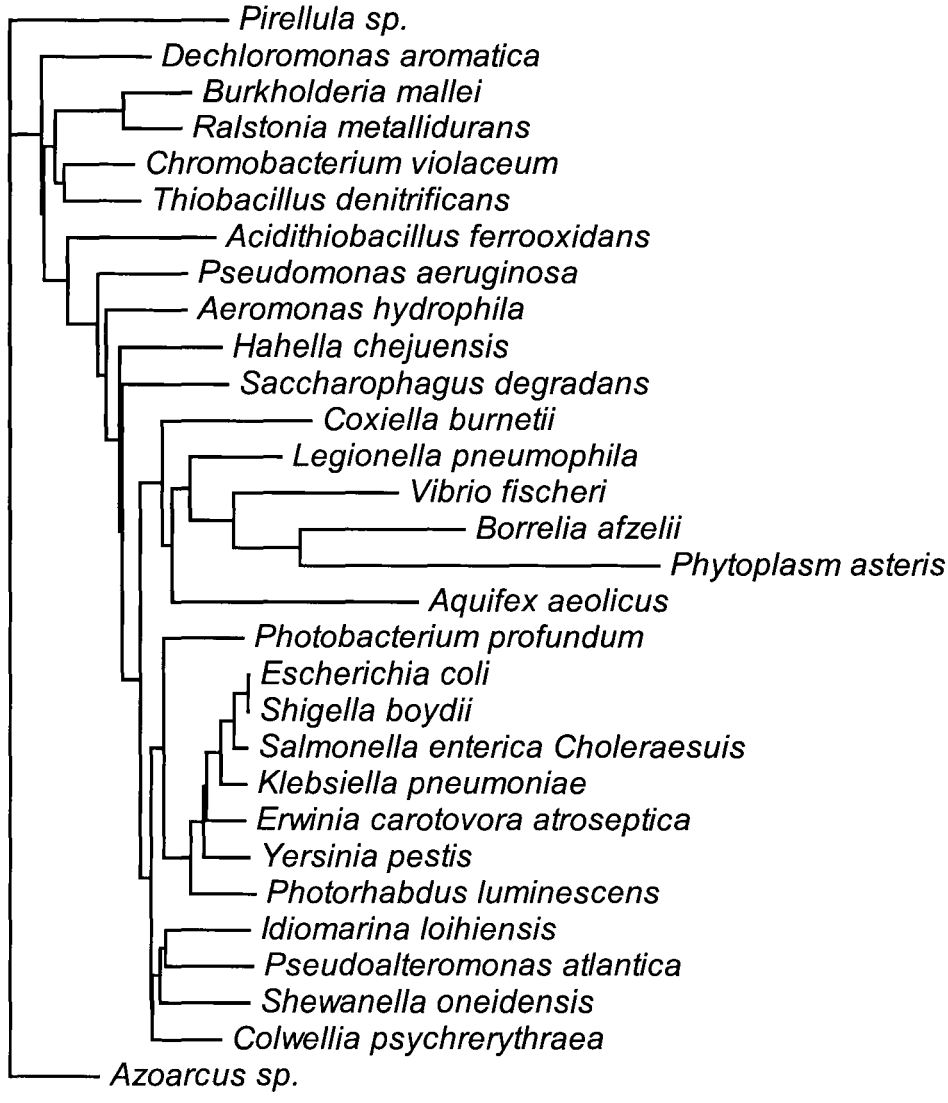

Figure 1.1 Distribution and phylogeny of *rpoS*.

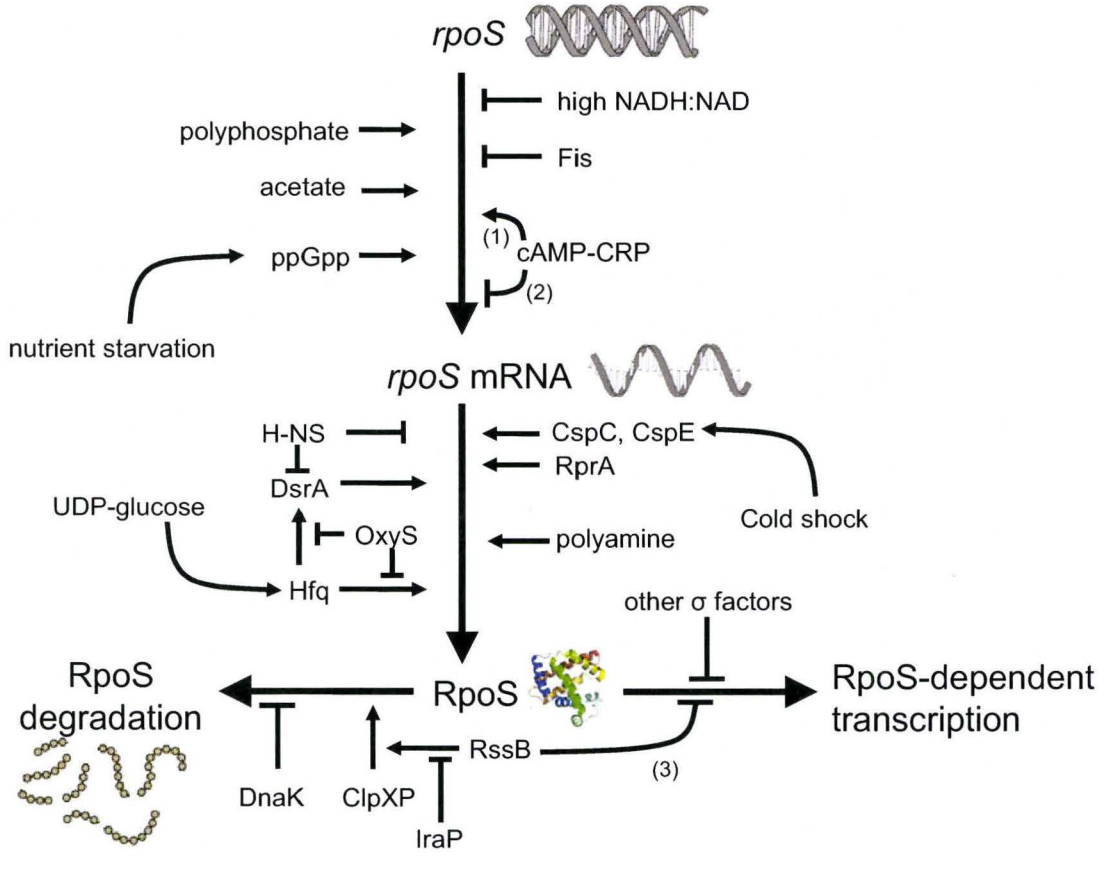

Figure 1.2 Factors involved in regulation of RpoS at various levels.

(1) Active during stationary phase; (2) active during exponential phase; (3) active under protease-limited conditions.

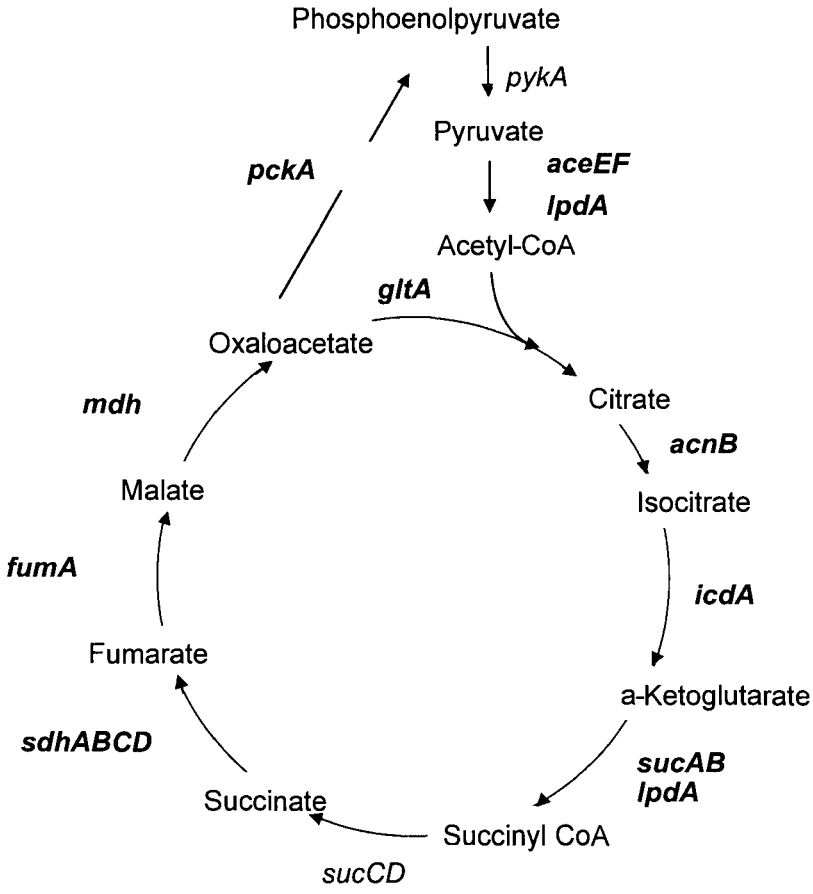

Figure 1.3 RpoS negative regulation of genes involved in the TCA cycle.

All genes highlighted in bold are negatively regulated by RpoS. This figure is modified from (Patten *et al.*, 2004).

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CHAPTER II

RpoS regulation of gene expression during exponential growth of *Escherichia coli* K12

from

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2.1 Preface

The contents of this chapter are contributed primarily by the author of this thesis. The RNA samples for microarray were extracted by Mark G. Kirchhof. I conducted all the other experiments and data analyses, and wrote the manuscript in its entirety, with editing from the other authors. This chapter has been published in Molecular Genetics and Genomics.

2.2 Abstract

RpoS is a major regulator of genes required for adaptation to stationary phase in *E. coli*. However, the exponential phase expression of some genes is affected by *rpoS* mutation, suggesting RpoS may also have an important physiological role in growing cells. To test this hypothesis, we examined the regulatory role of RpoS in exponential phase using both genomic and biochemical approaches. Microarray expression data revealed that, in the *rpoS* mutant, the expression of 268 genes was attenuated while the expression of 24 genes was enhanced. Genes responsible for carbon source transport (the *mal* operon for maltose), protein folding (*dnaK* and *mopAB*), and iron acquisition (*fepBD*, *entCBA*, *fecI*, and *exbBD*) were positively-controlled by RpoS. The importance of RpoS-mediated control of iron acquisition was confirmed by cellular metal analysis which revealed that the intracellular iron content of wild type cells was two-fold higher than in *rpoS* mutant cells. Surprisingly, many previously-identified RpoS stationary-phase dependent genes were not controlled by RpoS in exponential phase and several genes were RpoS-regulated only in exponential phase, suggesting the involvement of other regulators. The expression of RpoS-dependent genes *osmY*, *tnaA* and *malK* was controlled by Crl, a transcriptional regulator that modulates RpoS activity. In summary, the identification of a group of exponential phase genes controlled by RpoS reveals a novel aspect of RpoS function.

2.3 Introduction

As an alternative sigma factor and a stress response regulator, RpoS plays an important role in cell adaptation by controlling the expression of a large set of genes in

stationary phase and many stress conditions, including osmotic shock (Cheung *et al.*, 2003), oxidative stress (Sammartano *et al.*, 1986) and near-UV exposure (Sammartano *et al.*, 1986). RpoS is also important for the DNA damage response (Khil & Camerini-Otero, 2002), adaptive mutagenesis (Lombardo *et al.*, 2004) and biofilm formation (Schembri *et al.*, 2003a). About 400 genes are positively regulated by RpoS in stationary phase and in response to stress (Lacour & Landini, 2004; Patten *et al.*, 2004; Weber *et al.*, 2005). RpoS also negatively regulates the expression of many genes including those required for flagellum biosynthesis and for the TCA cycle metabolism in stationary phase (Patten *et al.*, 2004). This negative regulation by RpoS is likely due to competition between RpoS and other sigma factors for limiting RNA core polymerase (Farewell *et al.*, 1998a).

Although much has been learned regarding RpoS in stationary phase, there is little information on physiological functions of RpoS in exponential phase. RpoS protein level is low in exponentially growing cells (Lange & Hengge-Aronis, 1991b; Lange & Hengge-Aronis, 1994; Schweder *et al.*, 1996) (Lange & Hengge-Aronis, 1994) but there are some indications that RpoS is required for full expression of at least some genes. Using promoter-*lacZ* fusions, we have found that a group of genes, including *osmY* and *aidB,* are RpoS-dependent in exponential phase (Schellhorn *et al.*, 1998; Vijayakumar *et al.*, 2004). In addition, RpoS protects exponential phase cells against stress conditions, such as osmotic shock (Hengge-Aronis, 1996) and acid stress (Seputiene *et al.*, 2006). RpoS also protects cells independent of growth phase against toxic electrophiles such as methyglyoxal that are produced *in vivo* as metabolites or present in natural environments (Ferguson *et al.*, 1998b). RpoS positively regulates the expression of 72 genes and

negatively controls genes involved in several metabolic pathways including the TCA cycle and glycolysis in exponential phase (Rahman *et al.*, 2006). All of the above indicate that RpoS may have an important physiological function in exponential phase cells. However, the magnitude of RpoS regulation as well as the composition of the RpoS regulon in fast growing cells is not fully understood.

RpoS regulation often depends on other transcriptional regulators, such as Rsd (Jishage *et al.*, 2002; Jishage & Ishihama, 1998), 6S RNA (Barrick *et al.*, 2005; Wassarman & Storz, 2000), ppGpp (Costanzo & Ades, 2006; Jishage *et al.*, 2002), and Crl (Bougdour *et al.*, 2004; Lelong *et al.*, 2007; Robbe-Saule *et al.*, 2007). While Rsd, 6S RNA and ppGpp increase RpoS-directed transcription indirectly by reducing the effectiveness of sigma factor RpoD, Crl stimulates expression of RpoS-controlled genes directly through direct interaction with RpoS (Bougdour *et al.*, 2004; Robbe-Saule *et al.*, 2007; Typas *et al.*, 2007a). Many, though not all, RpoS-regulated genes have been found to be controlled by Crl (Lelong *et al.*, 2007; Typas *et al.*, 2007a). However, the role of Crl in RpoS regulation in exponential phase has not been examined.

In nature, bacteria likely experience constant shifting growth environments. To understand this dynamic process, it is imperative to examine how RpoS and the RpoS regulon act in exponential phase in comparison to stationary phase when RpoS is a major regulator of gene expression. The role of RpoS in fast-growing cells may be distinct from the known role of RpoS in stationary phase because of the different physiological environment in exponential phase. Consequently, RpoS may control a set of exponential

phase-specific genes. To test this hypothesis, we performed microarray analyses using oligonucleotide arrays to identify RpoS-regulated genes in exponential phase.

2.4 Materials and Methods

Bacterial strains, media, and growth conditions

E. coli K-12 MG1655 and a previously-described precise *rpoS* knockout derivative (Patten *et al.*, 2004) were employed in this study. Cells were grown in Luria Bertani (LB) media at 37°C at 200 rpm. Overnight cultures were inoculated with single, independent colonies in triplicate, incubated aerobically overnight at 37°C, and sub-cultured to a starting OD_{600} of 0.0001. Cultures were harvested at an OD_{600} = 0.3 after being maintained in exponential phase for at least eight generations.

RNA extraction and processing

RNA was isolated using a modified acidic hot-phenol method as described previously (Patten *et al.*, 2004). Exponential phase cultures (10 ml) were immediately mixed with half volume (5 ml) of boiling lysis buffer containing 2% SDS and 16 mM EDTA and equal volume (15 ml) of acidic hot-phenol (pH 4.3, 65°C) to inactivate RNase and thus minimize RNA degradation. RNA quality was determined using a Bioanalyzer 2100 (Agilent Technologies). RNA samples were reverse-transcribed, labeled and hybridized to Affymetrix GeneChip *E. coli* Antisense Genome Arrays as previously described (Patten *et al.*, 2004).

Microarray analysis

Normalization of the probe-level data was performed using the dChip invariant set normalization method (Li & Wong, 2001). Correlation among replicate samples was

assessed by determining the Pearson correlation co-efficient using the BRB ArrayTools software (V3.5.0) (R. Simon and A.P. Lam, Biometric Research Branch, National Cancer Institute).

Gene expression intensities of wild type ($\mathbf{I}^+$) or *rpoS* mutants ($\mathbf{I}^-$) were calculated by the dChip model-based expression analysis using both the perfect match (PM) and the mismatch (MM) probe-sets, and were $\log_2$-transformed (**LI**) for further analysis (Li & Wong, 2001). Changes in gene expression were represented by mean expression ratios (**MER**) ($\mathbf{I}^+_{\text{average}}/\mathbf{I}^-_{\text{average}}$) and $\log_2$ mean expression ratios (**LMER**) (Patten *et al.*, 2004). Student's t-tests were used to test whether the difference in mean expression of probe-sets of wild type and *rpoS* mutants was significant. A given gene was considered to be RpoS-regulated if it fit the following criteria: MER ≥ 2 (positive regulation) or MER ≤ 0.5 (negative regulation) and $P < 0.05$.

In analyses where large numbers of comparisons are made, such as in the comparison of microarrays, there is a high probability of making a type I error (calling a gene differentially expressed when, in fact, it is not). To estimate the number of genes in the dataset that by chance were incorrectly identified as significantly different, FDR (false discovery rate) analysis was performed using the Tusher method (Tusher *et al.*, 2001). Array labels were permuted 1000 times and the same filter criteria (MER ≥ 2 or ≤ 0.5, $P < 0.05$) for RpoS-dependent genes were applied to the resultant datasets. The number of genes identified to be significantly different in each resultant dataset was computed, and FDR was calculated as the median number of significant genes divided by the actual number of identified RpoS-dependent genes (Li & Wong, 2001).

Microarray data of this study is deposited in the Gene Expression Omnibus database at the National Center for Biotechnology Information and the series accession numbers are GSE9814.

Real-time PCR (qPCR)

Expression of representative identified genes from different functional groups was quantified by quantitative real-time PCR (qPCR) using an ABI 7500 Real-Time PCR System (Applied Biosystems). Optimized primers were designed using PerlPrimer software (Marshall, 2004) (length from 19-24 nucleotides and predicted annealing temperatures ranging from 59°C to 61°C) to amplify about 100-300 base pairs at the 3' end of the target genes. First strand cDNA was synthesized using a cDNA synthesis kit (New England BioLabs). PCR conditions were 10 min at 94°C, followed by 40 cycles of heating at 94°C for 30 s and 60°C for 1 min, and final extension at 72°C for 5 min. PCR amplification was detected by SYBR fluorescence dye (Applied Biosystems). A dilution series of genomic DNA was used as PCR template to construct a standard curve to quantify expression levels of tested genes. The *rrsA* gene, encoding the 16S RNA, served as an endogenous control to normalize for differences in total RNA quantity (Kobayashi *et al.*, 2006).

Determination of cellular iron content

Cellular iron content was measured using a Varian Atomic Absorption Spectrometer 220FS (Varian Techtron Pty. Ltd) as described by the manufacturer with modifications (Nunoshiba *et al.*, 1999). Cultures were grown aerobically in LB media to OD_{600} of 0.3. Cultures were then placed immediately on ice followed by addition of

chloramphenicol to a final concentration of 150 μg/ml to stop cell growth. Samples were collected by centrifugation at 4,000 x g at 4 °C for 15 min, washed three times in ddH_2O, and resuspended in 1 ml ddH_2O. The absorbance of iron in the samples was measured at 248.3 nm. Iron content was quantified by comparing sample absorbance to a standard curve that was prepared using a serial dilution of standard $FeCl_3$ solution (0.04 - 0.2 mM).

Construction of a *crl*::*kan* mutant

The *crl* gene in wild type and *rpoS* mutants was replaced by a kanamycin resistance cassette using the Red-recombinase method (Datsenko & Wanner, 2000). Primers used for amplifying the pKD4 template were FP1(TTT GGT AAA ACA GTT GCA TCA CAA CAG GAG ATA GCA GTG TAG GCT GGA GCT GCT TC) and RP1 (AAT ATT GCC GGA TGT GAT GCA TCC GGC ACA TTT CAC CAT ATG AAT ATC CTC CTT AG), and primers used for confirmation of *crl::kan* deletion were FP2 (TCGATTGTCTGGCTGTATG) and RP2 (GTTCAACCACTTCAGTGTCT).

Western blot analysis

Western blot analysis for RpoS expression was performed as described previously (Chen *et al.*, 2004). Samples were harvested at OD_{600} of 0.3 (exponential phase) and 1.5 (stationary phase) and disrupted by sonication (Chen *et al.*, 2004). Protein concentration was determined by Bradford assay (Bradford, 1976). Protein samples (40 μg) were resolved on a 10% SDS-PAGE gel and transferred to a PVDF membrane (Millipore) by electrophoresis. The PVDF membrane was then incubated with an anti-RpoS polyclonal serum (a gift from R. Hengge), followed by incubation with the secondary antibody (goat anti-rabbit serum from Biorad). Signals were detected by incubating the membrane with

the ECL solutions (Amersham Pharmacia Biotech) and exposing to Hyperfilm-ECL (Amersham Pharmacia Biotech).

2.5 Results

Identification of exponential phase RpoS-regulated genes

In exponential phase, the growth of *rpoS* mutants is similar to that of wild type (generation time = 23 min). The similarity of growth is an important prerequisite condition for comparison of gene expression between wild type and *rpoS* mutants, since expression of many genes is growth rate dependent (Pease *et al.*, 2002). Comparisons made when cells grow at the same rate minimize the effect of generation time as a confounding factor on identification of RpoS regulated genes. To examine the role of RpoS in exponential phase, we compared the transcriptome expression of wild type cells with that of *rpoS* mutants using oligonucleotide microarrays. The correlation distance between wild type and *rpoS* mutant groups was much greater than that among the biological replicates in each group, indicating that *rpoS* mutation had a considerable effect on transcriptome expression in exponential phase. Among the 6234 probe-sets (of 7312 probe-sets on each array) that were detected to be present, we identified 268 genes whose expression was at least two-fold higher in wild type cells (MER $\geq$ 2, $P<0.05$), while 24 genes were expressed at least two-fold higher in *rpoS* mutants (MER $\leq$ 0.5, $P<0.05$) (Figure 2.1). The false discovery rate (FDR) was 0.7%. Therefore, the probability of a given identified difference being due to a Type I error was quite low.

RpoS, in general, appears to regulate low expression genes in exponential phase. The average $\log_2$ signal level (LI) of the RpoS up-regulated genes was 8.3, lower than the

average of all genes (the LI for all genes was 9.4) (Figure 2.1). Ribosomal genes, such as *rpsK* and *rpmJ,* were the highest expression genes (LI ≥ 13.2), and none of these were affected by RpoS. There were only 44 RpoS-dependent genes (MER ≥ 2) expressed higher than the average of all genes (Table 2.1).

RpoS-regulated genes in exponential phase were quite different from those regulated by RpoS in stationary phase. Only 12 of the 75 previously-identified highly RpoS-dependent genes (MER ≥ 4) (Patten *et al.*, 2004) were controlled by RpoS (LMER ≥ 1) in exponential phase, and most exponential phase genes identified have not previously been reported to be RpoS dependent under any other conditions (Figure 2.2). We previously found that 29 genes are regulated by RpoS in exponential phase (Vijayakumar *et al.*, 2004), and 17 of these were confirmed by microarray analysis in this study.

To validate the microarray data, the expression of a set of representative genes was further examined by qPCR. We confirmed that all 12 of the genes tested were positively regulated by RpoS (LMER ≥ 1) (Figure 2.3). There was a good correlation between microarray and qPCR profiles, though some genes exhibited higher RpoS-dependence by qPCR than microarray analysis. This is consistent with previous results that microarray data is conservative compared with other measures of gene expression (Patten *et al.*, 2004).

Classes of RpoS-regulated functions in exponential phase

Protein folding

Genes *dnaK, mopA* (*groEL*), *mopB* (*groES*), and *htpG* encoding heat shock proteins were positively regulated by RpoS in exponential phase. Heat shock proteins mediate nascent protein synthesis and folding, disassemble the aggregated proteins, and degrade denatured proteins (Hartl & Hayer-Hartl, 2002). Though DnaK is not essential for cell growth (Kluck *et al.*, 2002), GroEL and its regulator GroES are constitutively expressed and essential for proper protein folding (Horwich *et al.*, 1993) and growth at all temperatures (Fayet *et al.*, 1989). GroEL and DnaK function complementarily in the folding of most proteins (Gragerov *et al.*, 1992). The expression of HtpG is induced by heat shock (Heitzer *et al.*, 1990), acid shock (Heyde & Portalier, 1990) and chemical stress (Mason *et al.*, 1999).

Iron utilization

Ten genes (*fhuA, fhuF, fiu, cirA, entCB, exbD, fecI, fepB* and *fepD*) that are responsible for iron acquisition were positively regulated by RpoS in exponential phase. Efficient utilization of iron is important for many metabolic processes because of the essential role of iron as a redox electron carrier for many enzymes. However, insoluble iron in the natural environment cannot be utilized unless iron is chelated with other molecules such as siderophore and enterobactin. Among the ten iron-acquisition genes, *fhuA* is important for the transport of ferrichrome (Ferguson *et al.*, 1998a; Locher *et al.*, 1998), *cirA, fiu* and *exbD* are responsible for iron-siderophore uptake (Hantke, 1990), and *entCB* and *fepDB* are required for the biosynthesis and transport of enterobactin (Barnard

et al., 2001; Hantash *et al.*, 1997; Shea & McIntosh, 1991). FecI is an alternative sigma factor (sigma19) which stimulates the expression of the ferric citrate transport system encoded by the *fecR* and *fecABCDE* genes under iron-limiting conditions (Van Hove *et al.*, 1990). Both the *fecR* and *fecABCDE* operons were expressed higher in wild type cells in exponential phase (Figure 2.4). Our results confirm and extend previous reports that expression of iron acquisition functions comprises a key component of the RpoS regulon (Lacour & Landini, 2004; Lee *et al.*, 2003).

As a consequence of the positive regulation of iron uptake genes by RpoS in exponential phase, wild type cells may accumulate more iron than *rpoS* mutants. To test this, we quantified the iron content in wild type cells and *rpoS* mutants using spectrometric analysis (Nunoshiba *et al.*, 1999). Iron content in wild type (1.4 ± 0.05 μmole per 10^9 cells) was 2.3-fold higher than that in the *rpoS* mutants (0.6 ± 0.18 μmole per 10^9 cells), which is consistent with the microarray data. Together, these data suggest that RpoS is important for iron acquisition in exponential phase.

Maltose Utilization

Five genes in the *mal* operon, *lamB* and *malEKPS,* responsible for maltose and maltodextrins utilization, were up-regulated by RpoS (MER ≥ 2) in exponential phase. Maltose and maltodextrins are preferred carbon sources utilized by growing cells in LB media (Baev *et al.*, 2006). The maltose transport system is composed of LamB and MalEFGK (Figure 2.5). LamB is an outer membrane porin specific for the diffusion of maltodextrins and other carbohydrates (Boos & Shuman, 1998). Following transport into the periplasm, maltose binds to MalE, a periplasmic maltose-binding protein (MBP)

(Dahl & Manson, 1985), resulting in a conformational change in MalE, which then directly interact with two transmembrane proteins MalF and MalG located on the inner membrane. Both MalF and MalG interact with MalK in the cytoplasm forming two protein complexes. Maltose transport through the inner membrane is coupled with both conformational changes in these two complexes and hydrolysis of ATP to ADP by MalK. The *malP* gene, encoding maltodextrin phosphorylase, is required for maltose and maltodextrin metabolism and is partially dependent on RpoS (Dippel *et al.*, 2005). MalS, a periplasmic amylase, cleaves long maltodextrin molecules to facilitate transport. Note that many other genes including *malQ* (MER = 1.8) and *malT* (MER = 1.5) involved in the maltose/maltodextrin utilization system were also expressed higher in wild type cells (MER $\geq$ 1).

Negative regulation by RpoS in exponential phase

The expression of more than 100 genes is negatively affected by RpoS in stationary phase (Patten *et al.*, 2004). Negative regulation by RpoS also occurs in exponential phase, as we found that expression of 24 genes was two-fold higher in *rpoS* mutants in exponential phase (Table 2.2). Seventeen of these, including genes for succinate utilization (*sdhCDB*), flagella function (*motAB, tar, tap, cheA,* and *fimA*), Rac prophage genes (*ydaC*, *ydaO*, *lar,* and *b1342*), and a global transcriptional regulator FNR, are also down-regulated by RpoS in stationary phase (Patten *et al.*, 2004). Genes that are required for synthesis of flagella and chemotaxis are known to be down-regulated when RpoS is induced under nitrogen or sulfur limiting conditions (Gyaneshwar *et al.*, 2005). FNR controls a large regulon consisting of genes important in respiration,

especially under anaerobiosis (Constantinidou *et al.*, 2006). RpoS may control genes indirectly through the negative regulation of FNR. Indeed, expression of genes such as *tar* and *tap* that are known to be activated by FNR (Constantinidou *et al.*, 2006) were attenuated by RpoS, while expression of genes, including *aldA*, *gadA* and *gadX* that are repressed by FNR (Constantinidou *et al.*, 2006), was higher in the wild type strain in exponential phase. In addition, FNR represses the transcription of *rpoS* promoter (Kang *et al.*, 2005), suggesting an intriguing and complicated feedback relationship between RpoS and FNR.

Effect of Crl on RpoS regulation in exponential phase

Crl has dual antagonistic regulatory effects on RpoS activities. Crl facilitates the formation of RpoS-associated RNA polymerase, promoting RpoS-directed transcription (Gaal *et al.*, 2006; Robbe-Saule *et al.*, 2007; Typas *et al.*, 2007a). However, Crl also stimulates degradation of RpoS through the RssB-ClpXP pathway in stationary phase (Typas *et al.*, 2007a). Of particular relevance to this study, Crl has a stronger positive effect on transcription of RpoS-regulated genes when the cellular RpoS level is low (Robbe-Saule *et al.*, 2007). Given that RpoS level is low in exponential phase, it is plausible that Crl may contribute disproportionately to RpoS modulation in fast-growing cells. To test this, we compared the expression of four RpoS-dependent genes in a series of isogenic strains including wild type, the *rpoS* mutant, the *crl* mutant and the *rpoS crl* double mutant by qPCR (Table 2.3). Deletion of the *crl* gene resulted in decreased expression of *malK, osmY* and *tnaA* but had no effect on expression of *fhuF*, suggesting

that Crl is important for the transcription of some, but not all, of the RpoS regulon in exponential phase.

Western blot analyses of RpoS expression in exponential and stationary phase.

RpoS controls a large regulon in stationary phase when RpoS is highly expressed (Lacour & Landini, 2004; Patten *et al.*, 2004; Weber *et al.*, 2005). Surprisingly, in exponential phase when RpoS is unstable and maintained at a much lower level (Jishage *et al.*, 1996; Lange & Hengge-Aronis, 1994), RpoS also had a substantial effect on transcriptome expression. However, this extensive regulation by RpoS in exponential phase could be the result of an increased level of RpoS caused by some confounding factors under conditions tested (at OD_{600} = 0.3). To test this, we examined RpoS levels in exponential and stationary phase by Western blot analysis. Since Crl stimulates RpoS degradation in stationary phase (Typas *et al.*, 2007a), the effect of Crl on expression of RpoS was also examined in exponential phase (Figure 2.6). Consistent with previous results (Jishage *et al.*, 1996; Jishage & Ishihama, 1995; Lange & Hengge-Aronis, 1994), RpoS was highly expressed in stationary phase but was not detected in exponential phase. Thus, this confirms that RpoS was expressed at a pre-induced level in fast growing cells when we performed the transcriptome expression analysis. In addition, RpoS was not detected in the *crl* mutants in exponential phase, while in stationary phase RpoS was expressed similarly in the *crl* mutants as in wild type cells.

2.6 Discussion

RpoS expression and activity is controlled at multiple levels (Hengge-Aronis, 2002a), which ensures that RpoS effectively directs RNA polymerase to express a large post-exponential phase regulon. The transcriptional induction of *rpoS,* a pre-requisite condition for expression of the stationary phase regulon, commences in mid-exponential phase (OD_{600} = 0.35) in rich media (Schellhorn & Stones, 1992). In examining possible exponential-phase roles for RpoS, it is thus essential to sample cultures at an early stage of growth (OD_{600} = 0.3) prior to induction of *rpoS* (Lange *et al.*, 1995). In this way, RpoS effects in exponential phase can be readily distinguished from those in stationary phase.

Gene modulation by RpoS in exponential phase was found to be extensive. The exponential phase-specific class of genes identified here is larger than the 72 genes previously reported (Rahman *et al.*, 2006), which may be due to differences in methodology. In the previous study, gene expression was quantified using cDNA microarrays (rather than oligonucleotide arrays) in a different strain background (Rahman *et al.*, 2006). However, in both studies, RpoS positively regulates genes responsible for iron uptake and phosphotransferase systems (PTS) for carbohydrate transport and utilization, and negatively regulates TCA cycle genes in exponential phase.

The absence of an observable effect of *rpoS* mutations on growth rate and the known instability of RpoS in exponential phase (resulting in low levels of the regulator) may have contributed to an under-appreciation of RpoS function in fast-growing cells. However, these are not incompatible with a role for RpoS in exponential phase. Even in early stationary phase (OD_{600} = 1.5) when RpoS is fully induced and expressing a large

regulon, *rpoS* mutants grow as well as wild type cells. Although RpoS is far less stable in exponential phase than in stationary phase (Lange & Hengge-Aronis, 1994), the time needed for promoter recognition and transcription initiation by sigma factors is very short (Hansen & McClure, 1980). It is thus plausible that genes can be modulated by a rapidly-recycled RpoS sigma factor. A consequence of RpoS instability, though, may be a decrease in the total amount of RpoS-associated RNA core-polymerase, resulting in the observed low expression of RpoS-controlled genes (this study).

In rich media, there are many nutrients including amino acids and sugars that readily support high growth rates, reducing the cellular need to express anabolic functions. Consistent with this, most RpoS-regulated genes we identified that facilitate carbon source utilization were genes encoding transporters, such as *mal*, *rhaTR* and *rbsC,* rather than genes required for biosynthesis or metabolism. The positive regulation of transporters by RpoS correlates with the predicted availability of sugar molecules. Preferred carbon sources are utilized by *E. coli* in a sequential manner when cells are grown in LB media (Baev *et al.*, 2006). The first carbon substrates utilized are maltose and maltodextrins, followed by a simultaneous assimilation of other sugars including mannose, galactose, fucose, rhamnose, mannitol, trehalose, and arabinose (Baev *et al.*, 2006). Most RpoS-regulated transporters belong to the ATP-binding cassette (ABC) transporter family. In contrast to carbon utilization, only a small number of genes that are responsible for the biosynthesis and metabolism of amino acids and nucleotides were regulated by RpoS in exponential phase.

Although exponential growth in rich media is likely not stressful, some stress response genes, including *osmY* and *gadAB,* were up-regulated by RpoS, suggesting that stress-protective factors may be produced and maintained at a low level prior to stress. This may enable cells to better adapt to changing environments and the potential pre-adaptation role for RpoS and its regulon may be important before the induction of RpoS when cells encounter stress. This is consistent with the known protective role of RpoS in exponential phase cells against osmotic shock (Hengge-Aronis, 1996), toxins (Ferguson *et al.*, 1998b) and acid stress (Seputiene *et al.*, 2006).

Many genes were dependent on RpoS in only one phase of growth, either exponential or stationary, suggesting the likely involvement of other regulatory factors. RpoS regulation often involves other regulatory factors and depends on different growth conditions (Weber *et al.*, 2005). Growth phase specific environmental conditions including nutrient availability and pH may also be relevant for modulating RpoS dependent regulation. Many other transcriptional factors, such as Crp (Lange & Hengge-Aronis, 1994), Fis (Hirsch & Elliott, 2005), Crl (Robbe-Saule *et al.*, 2007; Typas *et al.*, 2007a) and Lrp (Weber *et al.*, 2005), interact with RpoS to control gene expression and these may contribute to growth phase-specific modulation of RpoS regulation. Indeed, we show that Crl regulates three out of four genes tested, indicating that Crl has a substantial effect on RpoS-regulation in exponential phase. The regulatory role of Crl on RpoS activities in exponential phase is consistent with previous reports that Crl functions at low RpoS concentrations and is important for the expression of RpoS-regulated genes (Robbe-Saule *et al.*, 2007; Typas *et al.*, 2007a). Interestingly, in the *crl rpoS* double

mutants, the expression of *malK* and *osmY* was significantly higher than that in the *rpoS* mutants. This inhibitory effect of Crl, in the absence of RpoS, may explain the reported increased expression of many genes, including *gyrB*, *uspA*, and *dps*, in *crl rpoS* double mutants (Lelong *et al.*, 2007). This data suggests that Crl may have RpoS-independent effects on gene regulation.

In exponential phase, RpoS does not have a substantial influence on cell growth under laboratory conditions, which may be due to that most RpoS-regulated genes are low-expression genes and the RpoS-dependence of genes is within a narrow range (from two- to eight-fold). However, when cells grow in a more complex environment, such as the mammalian host, regulation by RpoS may become important as many low expression genes under laboratory conditions are induced in the host environment (e.g., genes on the pathogenic islands in *E. coli*). Similarly, the positive regulation of iron acquisition by RpoS has little effect on growth in LB media, but may become critical in iron-limiting conditions. For example, during urinary tract infection, iron acquisition systems are essential for pathogenic *E. coli* to survive and scavenge iron from urine and the host (Alteri & Mobley, 2007; Torres *et al.*, 2001).

It is clear that RpoS regulates a large set of exponential phase specific genes, supporting the idea that RpoS is important in actively-growing cells. These data also extend previous characterization of the RpoS regulon in *E. coli* MG1655 strain (Patten *et al.*, 2004). As the role of RpoS in pathogenic strains has not yet been fully defined, it is important to examine RpoS controlled functions in these strains as well, which is an ongoing project in our lab to identify RpoS-regulated genes in the model pathogenic *E.*

coli strain O157:H7 EDL933. This study, combined with previous reports of RpoS regulation in stationary phase, will form a basis to evaluate the regulatory effects of RpoS in pathogenic strains.

Table 2.1 RpoS up-regulated genes (MER ≥ 2) with expression higher than the average.

Blattner No.	Gene	Function	Expression (LI)		MER	P-value
			WT	*rpoS*		
b0372	*insF-2*	IS3 element transposition	9.5 ± 0.2	8.3 ± 0.2	2.3	0.008
b1026	*insF-4*	IS3 element transposition	10.2 ± 0.2	8.9 ± 0.3	2.4	0.026
b0014	*dnaK*	Heat shock, protein folding	10.8 ± 0.3	9.5 ± 0.1	2.3	0.047
b0150	*fhuA*	Iron acquisition, porin	9.8 ± 0.3	8.5 ± 0.4	2.5	0.048
b0473	*htpG*	Heat shock protein	9.8 ± 0.0	8.8 ± 0.1	2.0	0.002
b0590	*fepD*	Iron enterobactin transport	9.6 ± 0.1	8.1 ± 0.1	2.8	0.001
b0592	*fepB*	Iron enterobactin binding	9.6 ± 0.1	8.5 ± 0.2	2.1	0.012
b0593	*entC*	Enterobactin biosynthesis	10.3 ± 0.1	9.2 ± 0.0	2.2	0.001
b0595	*entB*	Enterobactin biosynthesis	10.3 ± 0.1	9.0 ± 0.3	2.6	0.029
b0597	*ybdB*	Hypothetical protein	10.4 ± 0.1	9.2 ± 0.2	2.4	0.030
b0805	*fiu*	Iron transport	9.7 ± 0.1	8.6 ± 0.1	2.1	0.008
b1705	*ydiE*	Hypothetical protein	9.4 ± 0.1	8.3 ± 0.1	2.3	0.001
b2155	*cirA*	Colicin I receptor	10.8 ± 0.1	9.6 ± 0.2	2.3	0.014
b2464	*talA*	Transaldolase A	10.1 ± 0.1	9.0 ± 0.2	2.0	0.024
b2673	*nrdH*	Glutaredoxin-like protein	9.9 ± 0.1	8.8 ± 0.1	2.1	0.001
b2675	*nrdE*	Ribonucleotide reductase	9.4 ± 0.1	8.1 ± 0.0	2.4	0.001
b2734	*pphB*	Protein phosphatase 2	9.5 ± 0.2	8.1 ± 0.3	2.7	0.026
b2809	*ygdI*	Hypothetical lipoprotein	9.6 ± 0.1	8.3 ± 0.1	2.4	0.003
b2918	*argK*	Arginine transport	9.8 ± 0.2	8.7 ± 0.1	2.1	0.010
b2990	*hybG*	Hydrogenase-2	9.6 ± 0.1	8.4 ± 0.1	2.3	0.004
b3005	*exbD*	Iron enterobactin transport	11.1 ± 0.0	10.1 ± 0.3	2.1	0.049
b3014	*yqhH*	Hypothetical lipoprotein	9.4 ± 0.2	8.1 ± 0.3	2.4	0.023
b3073	*ygjG*	Putrescine catabolism	10.3 ± 0.2	8.9 ± 0.3	2.6	0.024
b3079	*ygjJ*	Hypothetical protein	9.6 ± 0.3	8.1 ± 0.4	2.9	0.049
b3328	*hofG*	Protein secretion	9.5 ± 0.2	8.5 ± 0.1	2.0	0.011
b3409	*feoB*	Iron transport	10.4 ± 0.0	9.4 ± 0.0	2.0	0.001
b3417	*malP*	Maltodextrin utilization	10.2 ± 0.0	9.0 ± 0.1	2.2	0.009
b3484	*yhhI*	Hypothetical protein	9.4 ± 0.3	7.4 ± 0.1	3.9	0.013
b3534	*yhjQ*	Hypothetical protein	9.7 ± 0.2	8.6 ± 0.3	2.1	0.033
b3571	*malS*	Maltose utilization	9.8 ± 0.2	8.8 ± 0.2	2.1	0.024
b3708	*tnaA*	Tryptophanase	12.0 ± 0.5	9.6 ± 0.1	5.5	0.035
b3897	*frvR*	*frv* operon regulator	9.4 ± 0.1	8.2 ± 0.2	2.3	0.021
b4034	*malE*	Maltose transport	11.1 ± 0.1	9.3 ± 0.3	3.4	0.018
b4035	*malK*	Maltose transport	9.7 ± 0.4	6.7 ± 0.3	7.7	0.004
b4036	*lamb*	Maltose transport	11.5 ± 0.2	9.4 ± 0.4	4.1	0.018
b4060	*yjcB*	Hypothetical protein	9.4 ± 0.2	8.3 ± 0.1	2.2	0.021
b4141	*yjeH*	Putative transporter	9.9 ± 0.2	8.6 ± 0.2	2.5	0.015
b4142	*mopB*	Heat shock, GroES	12.9 ± 0.1	11.8 ± 0.1	2.3	0.001
b4143	*mopA*	Heat shock, GroEL	12.0 ± 0.2	10.4 ± 0.1	3.0	0.002
b4217	*ytfK*	Hypothetical protein	9.6 ± 0.1	8.4 ± 0.0	2.3	0.004

Blattner No.	Gene	Function	Expression (LI)		MER	P-value
			WT	*rpoS*		
b4228	*ytfR*	Putative sugar transport	9.5 ± 0.2	8.2 ± 0.3	2.5	0.023
b4293	*fecI*	Iron utilization	10.2 ± 0.1	9.0 ± 0.0	2.2	0.004
b4367	*fhuF*	Iron hydroxamate transport	10.8 ± 0.1	9.2 ± 0.3	3.1	0.022

Table 2.2 Genes whose expression is enhanced by at least two-fold in *rpoS* mutants in exponential phase.

Blattner No.	Gene	Function	Expression (LI)		1/MER	P-value
			WT	*rpoS*		
b0721	*sdhC*	Succinate dehydrogenase subunit	9.8 ± 0.0	11.3 ± 0.2	2.7	0.018
b0722	*sdhD*	Succinate dehydrogenase subunit	10.1 ± 0.1	11.4 ± 0.2	2.4	0.016
b0724	*sdhB*	Succinate dehydrogenase subunit	9.5 ± 0.1	10.8 ± 0.3	2.4	0.032
b1194	*ycgR*	Hypothetical protein	9.5 ± 0.1	10.9 ± 0.1	2.7	0.001
b1332	*ynaJ*	Hypothetical protein	8.8 ± 0.1	9.9 ± 0.1	2.2	0.003
b1333	*ydaA*	Hypothetical protein	5.1 ± 0.1	8.5 ± 0.1	11.1	0.000
b1334	*fnr*	Regulator in anaerobic respiration	8.5 ± 0.1	11.3 ± 0.1	6.7	0.000
b1342	*b1342*	Hypothetical protein	2.6 ± 0.5	7.5 ± 0.1	33.3	0.007
b1344	*ydaO*	Hypothetical protein	6.9 ± 0.3	9.7 ± 0.0	6.7	0.014
b1345	*b1345*	Rac prophage	6.6 ± 0.1	7.6 ± 0.2	2.1	0.012
b1347	*ydaC*	Rac prophage	4.1 ± 0.4	5.4 ± 0.2	2.6	0.042
b1348	*lar*	Rac prophage	5.1 ± 0.3	6.2 ± 0.2	2.2	0.030
b1566	*flxA*	Qin prophage	9.9 ± 0.1	11.5 ± 0.2	2.9	0.004
b1885	*tap*	Methyl-accepting chemotaxis	9.2 ± 0.2	10.5 ± 0.2	2.5	0.006
b1886	*tar*	Methyl-accepting chemotaxis	10.7 ± 0.1	11.9 ± 0.2	2.3	0.008
b1888	*cheA*	Chemotaxis	10.6 ± 0.3	11.7 ± 0.1	2.1	0.035
b1889	*motB*	Flagellar motor rotation	9.8 ± 0.2	11.1 ± 0.2	2.5	0.012
b1890	*motA*	Flagellar motor rotation	9.8 ± 0.1	10.9 ± 0.1	2.1	0.004
b2579	*yfiD*	Putative formate acetyltransferase	9.0 ± 0.1	10.5 ± 0.2	2.7	0.023
b3525	*yhjH*	Hypothetical protein	10.2 ± 0.1	11.3 ± 0.2	2.2	0.020
b4109	*yjdA*	Hypothetical protein	9.5 ± 0.1	10.6 ± 0.2	2.2	0.034
b4314	*fimA*	Fimbrial protein (pilin)	10.3 ± 0.1	11.6 ± 0.0	2.4	0.006
b4315	*fimI*	Fimbrial protein	8.0 ± 0.3	9.2 ± 0.1	2.3	0.042
b4355	*tsr*	Methyl-accepting chemotaxis	11.1 ± 0.2	12.2 ± 0.1	2.1	0.032

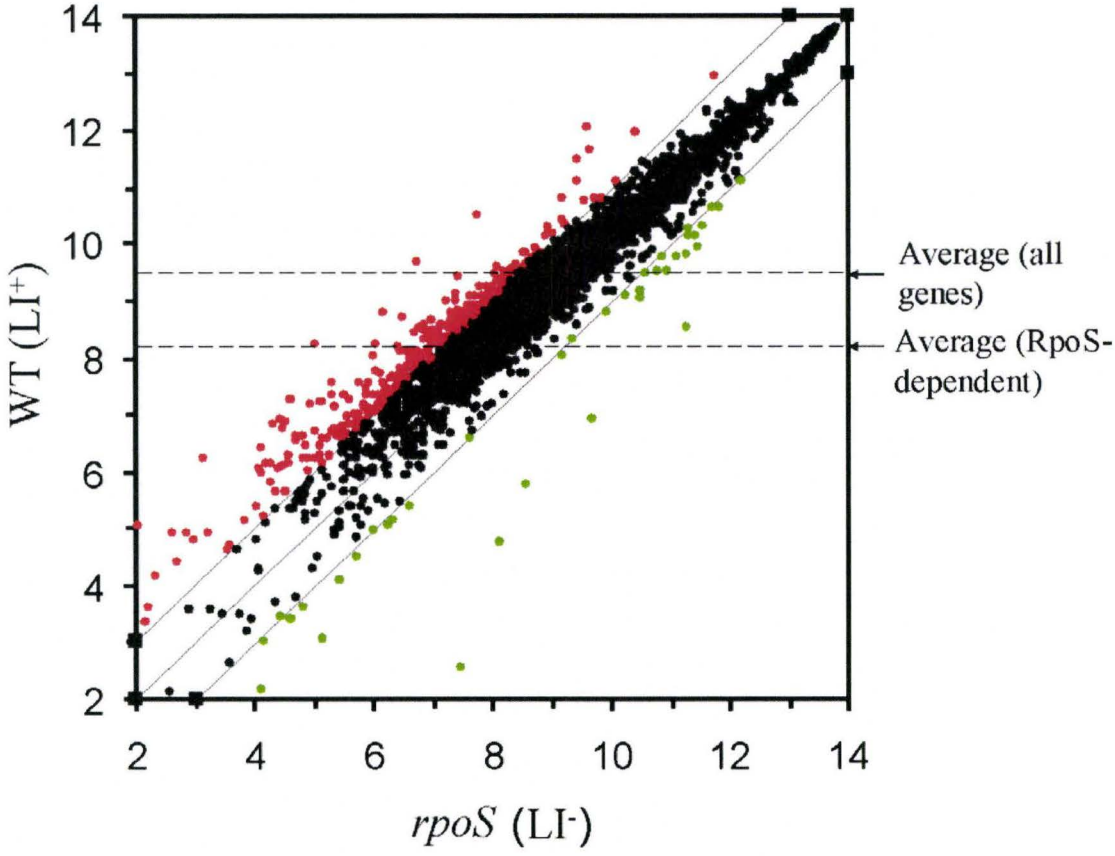


Figure 2.1 Scatter plot of expression of all genes in wild type and *rpoS* mutants of *E. coli*.

Average gene expression intensities (Log_2) of wild type (LI^+) are plotted against LI^- of *rpoS* mutants. Red spots represent genes whose expression is at least two-fold higher in wild type cells (MER $\geq$ 2) while green spots represent genes with MER $\leq$ 0.5. The average expression for all genes and for RpoS-dependent genes is indicated by the dashed lines.

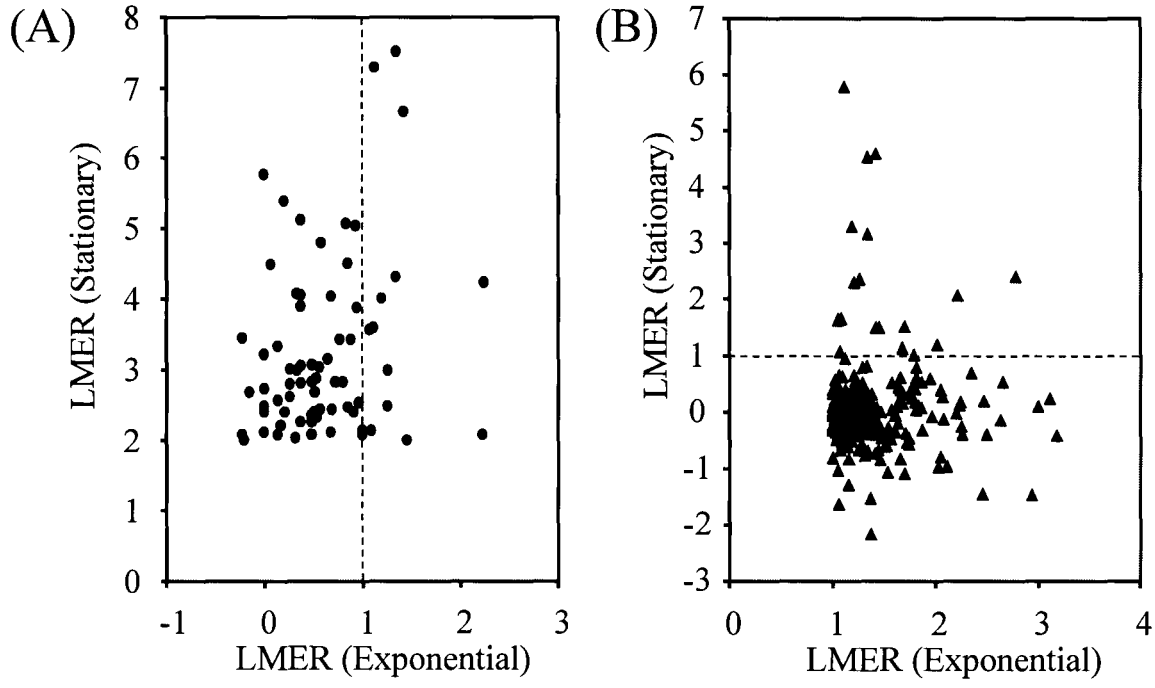


Figure 2.2 Comparison of RpoS regulated genes in exponential phase and stationary phase.

Exponential phase data were compared with previous stationary phase results (Patten *et al.*, 2004). **A.** Log_2 expression ratio (LMER) of previously identified stationary-phase RpoS-dependent genes (≥ 4-fold) in wild type and *rpoS* mutants in stationary and exponential phase. **B.** LMER of newly identified exponential-phase RpoS-dependent genes (≥ 2-fold) in stationary phase and exponential phase. Each circle or triangle represents one gene.

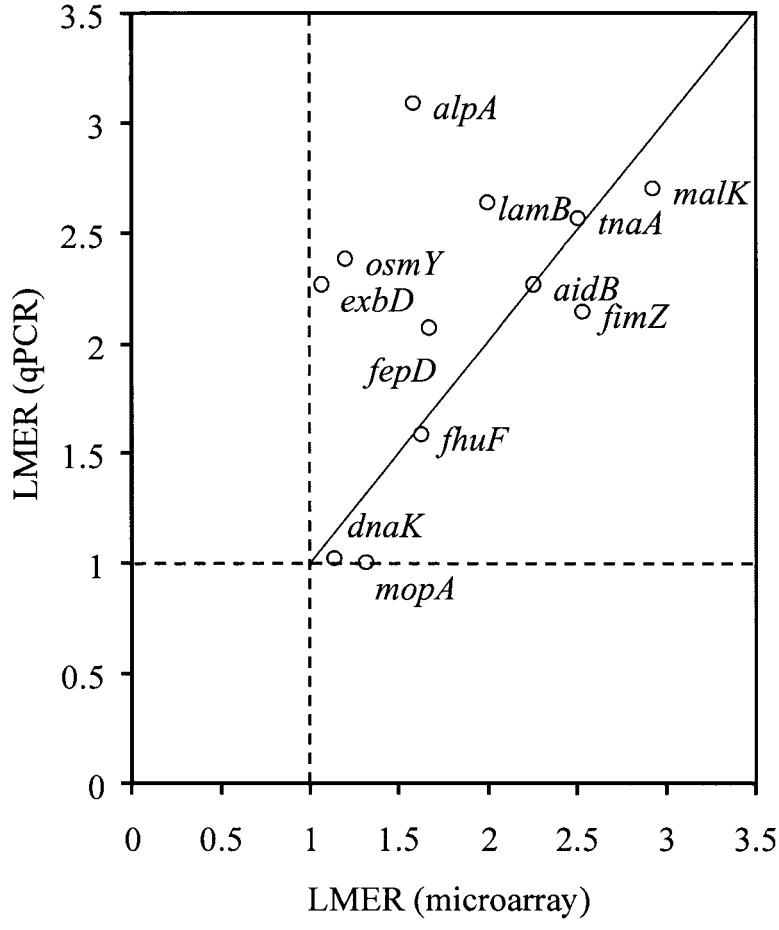
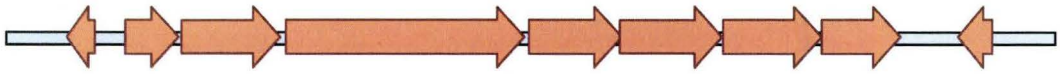


Figure 2.3 Comparison of RpoS dependence (LMER) of genes by microarray and qPCR.

Expression of tested genes was quantified as described in the Materials and Methods. The *rrsA* gene was used as an internal control to normalize differences in total RNA quantity among samples. The threshold for RpoS dependence (LMER $\geq$ 1) is indicated by the dashed lines.



Gene	*fecI*	*fecR*	*fecA*	*fecB*	*fecC*	*fecD*	*fecE*
WT/ *rpoS*	2.2	1.6	1.6	1.7	1.6	1.4	1.6

Figure 2.4 Comparison of the expression of *fecI* and its regulated genes in wild type strain and the *rpoS* mutant.

The expression ratio is indicated.

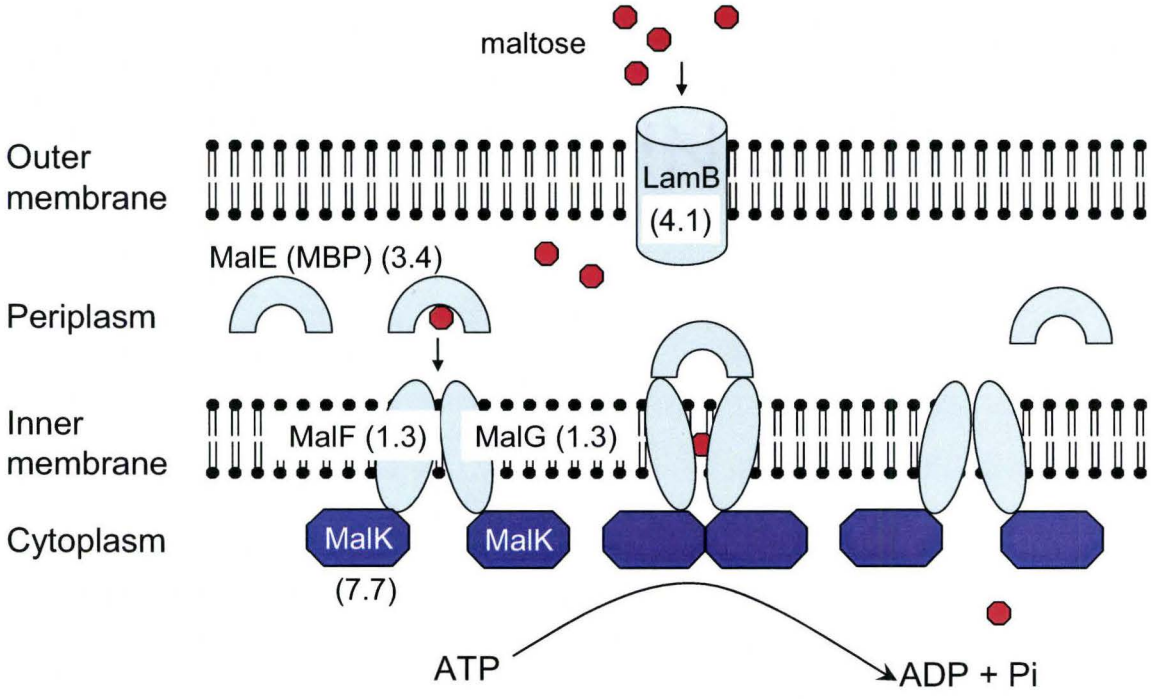


Figure 2.5 The maltose/maltodextrin transport system regulated by RpoS.

The RpoS dependence level (MER) of each gene was indicated in parentheses. For details regarding the maltose utilization system, see the review by Boos and Shuman (1998).

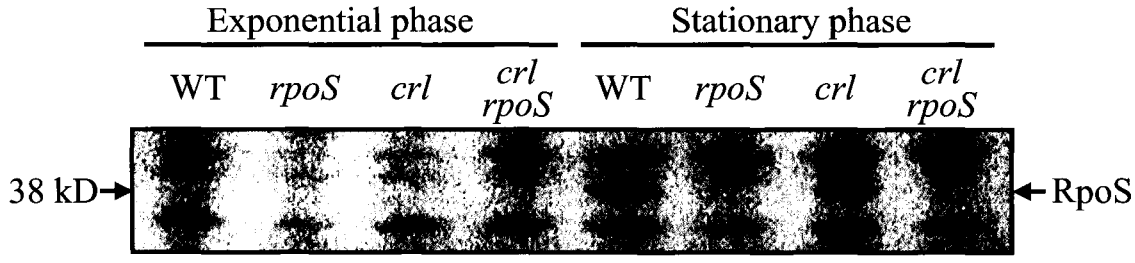


Figure 2.6 Western blot analysis of RpoS in exponential and stationary phase.

Protein samples were isolated from cultures grown to exponential phase (OD_{600} = 0.3) and stationary phase (OD_{600} = 1.5). Proteins (40 μg) were resolved on a 10% SDS-PAGE gel, and RpoS levels were detected using a polyclonal anti-RpoS serum as described in the Materials and Methods.

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CHAPTER III

Control of RpoS in global gene expression of

***Escherichia coli* in minimal media**

from

Dong, T., and Schellhorn, H.E. 2009. Control of RpoS in global gene expression of *Escherichia coli* in minimal media. Molecular Genetics and Genomics 281:19-33

3.1 Preface

The contents of this chapter are contributed primarily by the author of this thesis. The text was written, in its entirety, by Tao Dong. This chapter is adapted from a published article in Molecular Genetics and Genomics to fit the format requirements of the thesis.

3.2 Abstract

RpoS, an alternative sigma factor, is critical for stress response in *Escherichia coli*. The RpoS regulon expression has been well characterized in rich media that support fast growth and high growth yields. In contrast, though RpoS levels are high in minimal media, how RpoS functions under such conditions has not been clearly resolved. In this study, we compared the global transcriptional profiles of wild type and an *rpoS* mutant of *E. coli* grown in glucose minimal media using microarray analyses. The expression of over 200 genes was altered by loss of RpoS in exponential and stationary phases, with only 48 genes common to both conditions. The nature of the RpoS-controlled regulon in minimal media was substantially different from that expressed in rich media. Specifically, the expression of many genes encoding regulatory factors (e.g., *hfq*, *csrA* and *rpoE*) and genes in metabolic pathways (e.g., *lysA*, *lysC* and *hisD*) were regulated by RpoS in minimal media. In early exponential phase, protein levels of RpoS in minimal media were much higher than that in LB media, which may at least partly account for the observed difference in the expression of RpoS-controlled genes. Expression of genes required for flagellar function and chemotaxis was elevated in the *rpoS* mutant. Western blot analyses show that the flagella sigma factor FliA was expressed much higher in *rpoS* mutants than in WT in all phase of growth. Consistent with this, the motility of *rpoS* mutants was enhanced relative to WT. In conclusion, RpoS and its controlled regulators form a complex regulatory network that mediates the expression of a large regulon in minimal media.

3.3 Introduction

Bacteria often experience starvation and many other adverse conditions in nature (Hengge-Aronis, 1993; Matin, 1991). Adaptation and survival under these conditions requires a robust stress response system. In *E. coli* and many other proteobacteria, the general stress response is governed by RpoS, an alternative sigma factor of RNA polymerase that plays a central role in stress response under many stress conditions (see reviews (Hengge-Aronis, 2002a; Ishihama, 2000)). Expression of RpoS is under strict control at transcriptional and post-transcriptional levels (Hengge-Aronis, 2002a). During rapid growth in rich media, RpoS is maintained at very low levels (Lange & Hengge-Aronis, 1994), primarily due to rapid proteolysis by the ClpXP protease (Becker *et al.*, 1999). When cells enter stationary phase, however, RpoS is highly induced due to a combinatorial effect of increase in transcription, translation and protein stability (Lange & Hengge-Aronis, 1994). Concomitant with RpoS induction is the enhanced expression of RpoS-regulated genes. In stationary phase, RpoS modulates the expression of 10% of the *E. coli* genome (Lacour & Landini, 2004; Patten *et al.*, 2004; Weber *et al.*, 2005). Even in exponential phase when RpoS is not induced, RpoS is also required for the full expression of a large number of genes (Dong *et al.*, 2008b; Rahman *et al.*, 2006).

An important characteristic of RpoS is that its control on gene expression largely depends on growth conditions (Dong *et al.*, 2008b; Weber *et al.*, 2005). Though there is a core set of genes that are RpoS-regulated independent of growth, many genes are only controlled by RpoS under a specific condition (Dong *et al.*, 2008b; Weber *et al.*, 2005), which may be due to the modulating effect of other regulators such as Crl (Dong *et al.*,

2008b; Lelong *et al.*, 2007; Typas *et al.*, 2007a) and ppGpp (Bougdour & Gottesman, 2007; Foster, 2007; Traxler *et al.*, 2006).

Most previous work on identification of the RpoS regulon has focused on growth in rich media (Dong *et al.*, 2008b; Lacour & Landini, 2004; Lelong *et al.*, 2007; Patten *et al.*, 2004; Weber *et al.*, 2005). In contrast, our knowledge about RpoS control of gene expression in minimal media is relatively limited. Growth of *E. coli* differs substantially between these two growth conditions. In Luria-Bertani (LB) rich media, there are few fermentable sugars available and cells utilize amino acids as the major carbon source (Sezonov *et al.*, 2007). In glucose minimal media, however, glucose is the carbon source and all essential cellular building blocks, such as nucleotides and amino acids, are synthesized from glucose and inorganic phosphate and nitrogen sources (Tao *et al.*, 1999). As a consequence of the increased anabolic demand on the cell, the growth rate is reduced. Comparisons of transcriptome expression in rich and minimal media reveal substantial alterations in gene expression (Tao *et al.*, 1999). RpoS is likely an important factor contributing to these changes as RpoS expression is sensitive to growth conditions (Hengge-Aronis, 2002a).

To investigate RpoS-regulated gene expression in minimal media, we compared transcriptome expression in *E. coli* K12 MG1655 and an isogenic *rpoS* null mutant. This study, together with previous results (Dong *et al.*, 2008b; Patten *et al.*, 2004), forms a complete expression dataset of RpoS regulon in exponential and stationary phase of rich and minimal media, in which the same microarray platform and strains were used to allow for direct comparison. In addition, since slow growth likely represents an equally

important, if not more (compared with rapid growth in rich media), bacteria lifestyle in nature, studying how RpoS functions under such conditions may also contribute to our current understanding of the adaptive role of RpoS in natural environments.

3.4 Materials and methods

Media and growth conditions

Cultures of *E. coli* K-12 MG1655 and a derivative *rpoS* deletion mutant (Dong *et al.*, 2008b; Patten *et al.*, 2004) were grown aerobically at 37°C in triplicate in M63 minimal media (100 mM of KH_2PO_4; 15 mM of $(NH_4)_2SO_4$; 1.8 μM of $FeSO_4$; 1.0 mM of $MgSO_4$; 1.0 μg/ml of thiamine; pH 7.0) with glucose (0.2%) or glycerol (0.4%), prepared as described (Miller, 1992). Overnight cultures were diluted 1/10,000 to 50 ml of fresh M63 media to an initial OD_{600} = 0.0001, to allow cells to grow for at least 10 generations in exponential phase prior to sampling. Samples were harvested at OD_{600} = 0.3 in exponential phase and 1.5 in stationary phase. For cell motility test, wild type and the *rpoS* mutant were streaked out on LB media and incubated at 37°C overnight. A single colony of each strain was transferred onto an M63 minimal plate (0.3% agar) and incubated for 48 h at 37°C.

RNA extraction and hybridization

RNA extraction and hybridization were performed as previously described (Dong *et al.*, 2008b; Patten *et al.*, 2004). Samples of exponential phase (10 ml) and stationary phase (5 ml) cultures were quickly mixed with one half volume of a boiling lysis buffer (2% SDS and 16mM EDTA, 100°C) and equal volume of hot acidic phenol (pH 4.3, 65°C) (Sigma-Aldrich). The quality of RNA was examined by a Bioanalyzer 2100 (Agilent). Ten μg RNA samples were reverse-transcribed and hybridized to the Affymetrix Antisense *E. coli* Array following the manufacturer's instructions (Affymetrix).

Microarray analysis

Microarray data were analyzed using dChip (Li & Wong, 2001) and BRB Arraytools (Richard Simon *et al.*, 2006). Raw data were normalized and $\log_2$-transformed using the GC-RMA method (Wu & Irizarry, 2004). The mean expression ratio (MER) was determined by the ratio of the average expression intensity of WT (I^+) and *rpoS* mutants (I^-) (Patten *et al.*, 2004). Student's t-tests were performed to evaluate difference in expression. A determined expression difference for a given gene is considered to be significant if the P value of the resulting t-test statistic is less than 0.05 and the MER value is ≥ 2 or ≤ 0.5. MER ≥ 2 indicates positive-regulation by RpoS while MER ≤ 0.5 represents negative-regulation. A minus sign (-) is used to denote negative-regulation by RpoS in Table 3.1 to facilitate comparison.

To estimate type I error effects (calling a difference significant when it is not), FDR (false discovery rate) analysis was employed as previously described (Dong *et al.*, 2008b). Arrays were permuted 1,000 times and the number of RpoS-regulated genes in each permutation was calculated. FDR was then determined as the ratio of the median number of RpoS-dependent genes to the actual number of RpoS-dependent genes (Li & Wong, 2001; Tusher *et al.*, 2001).

Information regarding the functions of genes and their known regulators is from the EcoCyc database (Karp *et al.*, 2002; Karp *et al.*, 2007). Microarray data can be accessed in the Gene Expression Omnibus database at the National Center for Biotechnology Information under the accession number GSE12797.

Quantitative real-time PCR (qPCR)

To verify microarray results, gene expression was examined by qPCR using an Mx3000P QPCR System (Stratagene), as previously-described (Dong *et al.*, 2008b). Primers amplifying 100-300 bp of target genes were designed using PerlPrimer (Marshall, 2004). RNA was reverse-transcribed into first-strand cDNA by a cDNA synthesis kit (New England Biolabs). The SYBR green fluorescence dye (Clontech) was used for detection of PCR amplification. The 16S RNA gene *rrsA* was used as reference for normalization of samples (Kobayashi *et al.*, 2006).

Western blot analyses

Cultures were grown in LB and M63 media at 37°C at 200 rpm and sampled periodically. Samples were centrifuged at 15,000 x g for 2 min, and the cell pellets were flash frozen in liquid nitrogen. For analysis, cells pellets were thawed on ice, resuspended in SDS-PAGE loading buffer to OD_{600} = 1.0, and boiled for 5 min. Ten µl of samples were loaded and resolved on 10% SDS-PAGE gels at 100V for 2h, and transferred to a PVDF membrane (Millipore) at 90V for 1h. After the transfer, the PVDF membrane was blocked with 5% skimmed milk and incubated with mouse monoclonal antibody (Neoclone) to RpoS, RpoD, RpoE and FliA, respectively, followed by incubation with a secondary antibody (Goat anti-mouse serum, Biorad). The membrane was then incubated with ECL solution (Amersham) and exposed to Hyperfilm-ECL (Amersham). Identical SDS-PAGE gels were run in parallel and stained by Coomassie Blue R-250 as a control to confirm equal protein loading.

3.5 Results

Expression of the RpoS regulon in glucose minimal media

Wild type and the *rpoS* mutant grew similarly in glucose (0.2%) minimal media (Figure 3.1). The generation time was approximately 60 min for both strains during exponential growth, and similar cell densities were attained in stationary phase (Figure 3.1). To examine the effect of RpoS on gene expression, transcriptional profiles of WT and *rpoS* mutants in exponential and stationary phase were compared using microarray analyses. Among the 7312 probe-sets on each array, the fraction of probe-sets called "present" by the Affymetrix GCOS software algorithm for each of the 12 chips employed was over 98%. In each biological replicate group, the pairwise correlation coefficient was greater than 0.97, indicating excellent replication. To identify RpoS-dependent genes, the previously-employed criteria (twofold difference, $P < 0.05$) (Dong *et al.*, 2008b) were used. However, it is important to note that fold-difference of gene expression between WT and mutants (e.g. RpoS-dependence in this case) does not yield information regarding the absolute expression levels (Baldi & Hatfield, 2002; Salmon *et al.*, 2003). Therefore, these absolute expression values for each gene under all conditions, together with the calculated RpoS-dependence (MER) and associated *p* values, are provided in a supplemental table in the Gene Expression Omnibus database (GSE12797).

As expected, loss of *rpoS* had a pronounced effect on transcriptome expression in both growth phases (Figure 3.2). In exponential phase, 200 genes were differentially expressed by at least twofold in WT and *rpoS* mutants ($P < 0.05$), with a false discovery rate (FDR) of 1.6%. The expression of 88 genes was positively regulated by RpoS, while

112 genes were under negative regulation. In stationary phase, however, RpoS regulated 225 genes, among which only 22 genes were negatively regulated. The false discovery rate was 0.6%, indicating that, by chance, very few of the identified differences were due to Type I errors (see Materials and methods).

Many RpoS-dependent genes identified belong to large operons, including *hyaABCDEF*, *hycABCDEFGH*, and *dppABCDF* (Table 3.1). The expression of 37 genes in the flagellar operons and 41 genes in the ribosomal operons was mediated by RpoS (Table 3.1). During exponential growth in LB, most genes controlled by RpoS are low expression genes (Dong *et al.*, 2008b). However, this does not appear to be the case in minimal media. The average log expression level (LI) of RpoS-regulated genes was 10.3 in exponential phase and 10.7 in stationary phase, much higher than the average (7.7) of all genes. The *rpoS* transcript levels in exponential and stationary phase were similar, consistent with previous reports that *rpoS* is constitutively expressed in glucose minimal media (Lange & Hengge-Aronis, 1994).

As in LB media where RpoS controls distinct classes of genes in exponential and stationary phase (Dong *et al.*, 2008b), only 48 genes were RpoS-regulated in both growth phase, including 42 genes with known functions (Table 3.1). As a result, RpoS regulates different physiological functions depending on growth phase, as discussed below.

RpoS-regulated functions in exponential phase

RpoS coordinately controlled many genes found within common pathways (Table 3.1). For example, three discontinuous genes, *lysA*, *lysC*, and *dapB*, responsible for L-lysine biosynthesis, were all positively controlled by RpoS. The lysine transporter gene

lysP was also up-regulated. Genes required for glycogen metabolism (*glgCAP* and *glgX*), acetate formation (*pta* and *ackA*), glycolysis (*fbaB* and *pfkB*), and the biosynthesis of arginine (*argB*) and histidine (*hisD*) were up-regulated by RpoS. All genes of the TCA cycle and the glyoxylate shunt pathway were expressed higher in the *rpoS* mutant. RpoS also negatively-regulated the expression of ten genes in chemotaxis (*tap*, *tar*, *trg*, *tsr*, *cheAW*, *cheRBYZ*), as well as 39 genes involved in flagellar functions including *fliA* and *flhDC*.

There are three cytochrome terminal oxidases for respiration in *E. coli*, cytochrome *bd*-I encoded by *cydAB*, cytochrome *bd*-II encoded by *appBC*, and cytochrome *bo* encoded by *cyoABCDE*, which were regulated differently by RpoS in exponential phase. The expression of *appBC* and *cydB* was higher in WT cells while the *cyoDE* genes were expressed higher in the *rpoS* mutants. RpoS also positively regulated three of the four hydrogenase complexes present in *E. coli*, hydrogenase I encoded by *hyaABCDEF*, hydrogenase II by *hybOABC*, and hydrogenase III by *hycBCDEFGH*.

Many genes involved in acid resistance, including *hdeAB*, *hdeD*, *spf*, *dctR*, *gadA*, *gadBC*, *gadX*, *gadW*, *and gadE*, were up-regulated by RpoS. GadX and GadE are transcriptional activators for acid response (Masuda & Church, 2003), while GadW represses the transcription of *gadA* and *gadBC*, probably through repression of GadX (Ma *et al.*, 2002).

RpoS negatively regulated the expression of the *dppABCDF* operon, which encodes a dipeptide ABC transporter. Expression of DppA is required for colonization of

uropathogenic *E. coli* in mouse bladders and kidneys during infection (Haugen *et al.*, 2007).

RpoS-regulated functions in stationary phase

In stationary phase, the majority of differentially-expressed genes (90%) were up-regulated by RpoS and only 22 genes were down-regulated (Table 3.1) (Figure 3.2). Pathways that were expressed higher in the WT include arginine degradation (*astCD*), fatty acid biosynthesis (*fabHDG* and *fabA*), pyrimidine degradation (*deoA* and *deoB*), pentose phosphate pathway (*tktB* and *talA*), acetate production (*ackA* and *poxB*), glycolysis and glyoxylate shunt (*pfkA, fbaB, gapA, pgk, eno, pykF, acnA, aceB* and *pflB*). A group of identified RpoS-regulated genes *adhE*, *eno*, *focA*, *hyaABC*, *pfkA* and *pykF* are also known to be induced under anaerobic conditions (Partridge *et al.*, 2006). In stationary phase, only one gene of the TCA cycle, *fumA*, was expressed higher in the *rpoS* mutant. RpoS also down-regulated four chemotaxis genes (*cheA*, *chew*, *tap* and *tar*) and four genes important for flagella functions (*flhCD* and *motAB*).

A large group of genes (39 genes) encoding ribosomal proteins were expressed higher in the WT than in the *rpoS* mutant in stationary phase (Table 3.1), though the expression of most of these genes was reduced in both WT and *rpoS* mutants compared with that in exponential phase. Many genes involved in protein synthesis, modification and proper folding were also expressed higher in the WT, including those for ribosomal maturation and modulation (*rimM* and *rmf*), for modification of tRNA and rRNA (*trmD*, *fmt*, *ftsJ*, and *miaA*), for translation elongation (*tsf*, *tgt*, *tufB*, and *fusA*), for post-translational modification (*def*), as well as five tRNA genes (*alaT*, *argZ*, *glyT*, *lysW*, and

serV) and genes encoding chaperone proteins (*dnaKJ*, *cbpA*, *clpB*, *mopA* (*groEL*), *hspQ*, and *hchA*).

Expression of protease genes *hslV*, *lon*, and *clpPX* was impaired in the *rpoS* mutant. During starvation in stationary phase, misfolded and unfolded proteins are a major nutrient source that can be utilized as a carbon source or recycled into the cellular amino acid pool through degradation by proteases (Matin, 1991; Nystrom, 2004b). Mutants deficient in protease activities are known to be severely impaired in stationary phase survival, reflecting the importance of phase-specific protein degradation (Weichart *et al.*, 2003). Interestingly, RpoS mutants lose viability much faster than WT in minimal media in late stationary phase (Lange & Hengge-Aronis, 1991b). The up-regulation of these protease genes may, in part, contribute to the better survival ability of WT relative to *rpoS* mutants.

RpoS also positively mediated the expression of many transcriptional regulators, including RpoE, GadX, CueR, CsrA, and NsrR, each regulating a subset of genes. RpoE is another alternative sigma factor that is induced during starvation (Costanzo & Ades, 2006), high temperature (Rouviere *et al.*, 1995) and extracytoplasmic stress conditions (Mecsas *et al.*, 1993), and is essential for growth (De Las *et al.*, 1997). Among the RpoS positively-regulated genes identified here, 32 genes have been previously shown to be controlled by RpoE, including 18 of these ribosomal genes (Kabir *et al.*, 2005). RpoE also stimulates the transcription of RpoH (Wang & Kaguni, 1989), the heat shock sigma factor. Consistently, we found that the expression of *rpoH* was 1.6-fold higher ($P = 0.02$) in the WT than in the *rpoS* mutant in stationary phase. RpoE activity is inhibited by an

inner membrane protein encoded by the *rseA* gene that is cotranscribed with *rpoE* (Missiakas *et al.*, 1997). RseA functions as an anti-RpoE factor by direct interaction (Missiakas *et al.*, 1997). This inhibition is necessary to maintain RpoE activity at a proper level, since overexpression of RpoE may lead to cell lysis (Nitta *et al.*, 2000). Under extracytoplasmic stress conditions, inhibition is alleviated through degradation of RseA by proteases DegS (Ades *et al.*, 1999) and YaeL (Kanehara *et al.*, 2002). CsrA, a carbon storage regulator (Romeo, 1998) that controls various functions including glycogen biosynthesis, glycolysis, and biofilm, was up-regulated by RpoS as well.

Four genes *hfq*, *cspC*, and *clpPX*, which regulate RpoS translation and stability, were expressed higher in the WT than in the *rpoS* mutant. During the transition from exponential to stationary phase in minimal media, it is known that RpoS protein levels increase primarily due to post-transcriptional control (Lange & Hengge-Aronis, 1994). Hfq (Muffler *et al.*, 1996b) and CspC (Phadtare & Inouye, 2001) are important for *rpoS* mRNA translation, while ClpXP is the major protease that is responsible for RpoS proteolysis in fast-growing cells (Studemann *et al.*, 2003; Zhou & Gottesman, 1998). This dual regulation of genes for both synthesis and degradation of RpoS suggests a regulatory circuit may be responsible for maintaining RpoS at the proper level.

Verification of microarray results

Many genes identified in this study, such as *osmY*, *talA* and *gadAB*, are known to be RpoS-dependent (Dong *et al.*, 2008b; Lacour & Landini, 2004; Patten *et al.*, 2004; Vijayakumar *et al.*, 2004; Weber *et al.*, 2005). To confirm the microarray results, genes *hdeA*, *fabB*, *wrbA*, *otsB* and *talA* that were found to be regulated by RpoS in both

exponential and stationary phases were further examined by qPCR. All genes tested were expressed higher in the WT than in *rpoS* mutants in both growth phases, consistent with the microarray data (Table 3.2). Array data also indicated a positive effect of RpoS on the expression of *rpoE*, *clpP*, *rmf*, and *sodA*, which has not been previously observed in genomic screening studies. To confirm this, the transcription levels of these genes in WT and *rpoS* mutants were compared using qPCR. The expression of *rpoE* (3.2 ± 0.6 fold), *clpP* (2.6 ± 0.2 fold), *rmf* (3.0 ± 0.3 fold) and *sodA* (3.5 ± 0.7 fold) was significantly higher ($P < 0.05$) in WT than that in *rpoS* mutants in stationary phase, in good agreement with array data. Since a large group of flagella genes were negatively regulated by RpoS, it is possible that *rpoS* mutants possess enhanced motility. To test this, WT and *rpoS* mutants were inoculated on minimal media on soft agar (0.3%), and the colony size was compared after 48h incubation at 37°C (Figure 3.3). As expected, the *rpoS* mutant formed much larger colonies than WT, indicating increased motility. These results are consistent with the microarray analyses.

Effect of RpoS on gene expression in glycerol minimal media

A previous study comparing transcriptome expression on different carbon sources has revealed a striking relation between gene expression and growth rate, that genes are expressed hierarchically and inversely correlated with growth rate (Liu *et al.*, 2005). When comparing cultures grown in preferred carbon sources (e.g., glucose) and those grown in less-preferred or poor carbon sources (e.g., glycerol and proline), genes induced at a high growth rate generally are a subset of induced genes at a low growth rate (Liu *et al.*, 2005). Therefore, to test whether this effect is applicable to RpoS regulation,

we examined the effect of RpoS on gene expression at a lower growth rate by growing cells in glycerol media. The generation time of WT (120 min) and *rpoS* mutants (147 min) was about two-fold higher than that of cells grown in glucose media, consistent with previous results (Liu *et al.*, 2005). Despite the different growth rates, expression of all tested genes, *sodA*, *rmf*, *clpP* and *rpoE*, was significantly higher in WT than in the *rpoS* mutant in stationary phase in glycerol media (Table 3.3), suggesting RpoS may play a similar role in other carbon sources in minimal media.

Expression of RpoS and other sigma factors, RpoD, RpoE and FliA, during growth in M63 and LB media

The observation that more genes were up-regulated by RpoS in stationary phase than in exponential phase may be attributable to increased levels of RpoS in stationary phase. It is known that RpoS protein levels increase when cells enter stationary phase in minimal media. To test whether RpoS is induced under the conditions used in this microarray study, we examined the expression of RpoS by Western blot analyses. Results confirm that the protein level of RpoS was low in early exponential phase and was substantially induced in stationary phase (Figure 3.4). The transcription of two sigma factor genes, *rpoE* and *fliA*, was found to be higher in WT than in *rpoS* mutants. To test whether this is reflected at the protein level, the expression of RpoE and FliA was examined by Western analysis (Figure 3.4). There was a slight increase in the expression of RpoE in WT compared with *rpoS* mutants. The flagella sigma factor FliA was expressed much higher in the *rpoS* mutant than in WT. FliA peaked in early exponential phase in both WT and *rpoS* mutants and then decreased substantially (WT) or gradually

(*rpoS*) during growth into stationary phase, depending on the presence of RpoS. This is consistent with results from microarray (Table 3.1) and motility assays. As expected, the expression of the housekeeping sigma factor RpoD was not affected by the *rpoS* mutation (Figure 3.4).

The observed difference between the RpoS regulon identified in this study and the one previously identified in LB media (Dong *et al.*, 2008b; Patten *et al.*, 2004) (Table 3.1) may also be due to a difference in the expression of RpoS itself. Therefore, RpoS levels were compared in LB and minimal media (Figure 3.5). As expected, RpoS was induced in stationary phase in both LB and minimal media, consistent with previous findings (Lange & Hengge-Aronis, 1994). In early exponential phase, RpoS protein was expressed much higher in minimal media than in LB. The expression of RpoE increased during growth in both LB and minimal media. Interestingly, FliA was differentially expressed in LB and M63. Unlike in M63 where FliA expression was reduced, the expression of FliA increased during growth. However, FliA was negatively regulated by RpoS in LB as well (compare lane 5 with 13 in Figure 3.5). The expression of RpoD was also different between LB and M63. It has been shown that the cellular protein level of RpoD increases from 50 to 80 fmol/μg during exponential and stationary growth in LB media (Jishage & Ishihama, 1995). The observed expression of RpoD in LB is in general consistent with previous findings. In minimal media, however, the expression of RpoD decreased in minimal media when cells enter stationary phase (Figure 3.5), which may need to be further explored by quantitative studies in future.

3.6 Discussion

The natural lifestyle of bacteria has been described as being constantly exposed to feast and famine through successive exposure to nutrient rich and nutrient-limited environments (Hengge-Aronis, 1993; Matin, 1991; Nystrom, 2004b). The feast state is characterized by fast cell growth and rapid proliferation, while during starvation, cells stay in stasis or grow slowly for survival (Hengge-Aronis, 1993; Matin, 1991; Nystrom, 2004b). This high adaptability is achieved by modulating gene expression at the genome level through coordination of global regulators (e.g., RpoS). Examining RpoS regulation in minimal media, a condition used to mimic a slow-growth state that may occur in nature, may provide important information for understanding the role of RpoS in adaptation to natural environment. In this study, we evaluated the effect of RpoS on global gene expression. About 400 genes were RpoS-dependent in minimal media, including many previously unknown RpoS-regulated genes such as *hfq*, *rpoE* and a large group of genes in protein modification and degradation. Results suggest that RpoS may play not only a protective role from exogenous stresses, such as acid stress and osmotic stress, but also a role in nutrient recycling and proper protein folding by mediating the expression of chaperones and proteases for survival during starvation.

During growth in batch culture, cells enter stationary phase because of nutrient limitation. For example, carbon source is the limiting nutrient for cells ceasing growth in LB media (Sezonov *et al.*, 2007). This is likely also the case during growth in glucose minimal media as well, since the addition of glucose to stationary phase cultures, used in this study, supported further growth to higher densities (data not shown). Starvation for

carbon, nitrogen, or phosphate sources all lead to increased RpoS levels through distinct mechanisms (Peterson *et al.*, 2005). During carbon starvation, RpoS expression is elevated due to increased stability at the post-translational level (Mandel & Silhavy, 2005; Pratt & Silhavy, 1996). Phosphate starvation results in enhanced translation of *rpoS* mRNA (Ruiz & Silhavy, 2003). Though RpoS is only induced by twofold upon nitrogen starvation (Mandel & Silhavy, 2005), much less than the induction level under carbon or phosphate starvation, RpoS still plays an important role for survival under such conditions (McCann *et al.*, 1991). The molecular mechanisms responding to these diverse starvation signals for triggering the induction of RpoS, however, remain elusive (Peterson *et al.*, 2005). During growth in batch culture, the dissolved oxygen is depleted in late exponential phase due to respiration, and then increases after entry to stationary phase (Bergholz *et al.*, 2007). Reduced oxygen level in the late exponential phase is unlikely to be the signal factor that triggers RpoS induction, since RpoS expression decreases in anaerobiosis compared with aerobic conditions (King & Ferenci, 2005).

In stationary phase, though the overall activity of translation machinery is attenuated due to nutrient deficiency and the rate of protein misfolding is much higher in stationary phase, the expression of stress-response proteins is due to *de novo* protein synthesis (Groat *et al.*, 1986; Nystrom, 2004b). The higher expression of chaperones and proteases in WT may be required to ensure proper assembling of stationary phase proteins and to recycle unfolded or misfolded proteins as nutrients. An attenuated ability in expressing chaperones and proteases in *rpoS* mutants may be responsible for the

reported increased cell death during late stationary phase (Lange & Hengge-Aronis, 1991b).

Our data further support the notion that the RpoS regulon is quite dynamic (Dong *et al.*, 2008b), in that the composition of this large regulon varies greatly in different growth conditions. By comparing the RpoS regulon in LB exponential phase (Dong *et al.*, 2008b) with that in LB stationary phase (Patten *et al.*, 2004) or with that in stress conditions (Weber *et al.*, 2005), previous studies have shown that, in addition to a core set of genes that are RpoS-controlled under all tested conditions, the RpoS-dependence of many genes is restricted to specific conditions. In glucose minimal media, only 48 genes were regulated by RpoS in both exponential and stationary phases. The RpoS regulon also differs from the one previously characterized in LB. For example, the expression of *rpoE*, *hfq*, and *clpXP* are not controlled by RpoS in LB, while many iron transport genes, up-regulated by RpoS in LB during exponential growth, were not found to be differentially expressed in minimal media (this study). These differences may result from various factors, including changes in nutrients and environmental pH. Cultures of LB turn from neutral to basic (pH 8.8) due to the utilization of degraded peptides as carbon sources (Sezonov *et al.*, 2007). In contrast, cultures grown in M63 minimal media remain neutral (pH 6.8) up to 48h of growth (this study). Despite these differences, there are several conserved RpoS-regulated functions, including positive regulation of stress response genes such as *gadAB* for acid resistance and *osmY* for osmotic shock, and down-regulation of genes in the TCA cycle, chemotaxis and flagella formation.

As a sigma factor, RpoS directly recognizes preferred promoter regions and initiate transcription. However, the RpoS regulation of many genes within the regulon may be indirect resulting from RpoS-dependent expression of intermediate regulators (Figure 3.6). These intermediate regulators, including some known to affect RpoS activity, together with RpoS constitute a hierarchical regulatory network. Metabolites may also play a role in RpoS-mediated gene expression, since a large set of genes involved in metabolic pathways were up-regulated by RpoS, especially those genes involved in acetate and acetyl-phosphate production. Acetate and acetyl-phosphate are signal molecules for cell communication and control the expression of many genes of different functions, including osmoregulation and flagella biosynthesis (Wolfe, 2005).

Gene expression is an intricate process that often involves multiple regulators, which may function synergistically or independently to modulate expression in response to specific environmental signals. Our knowledge of how these regulators interact, though greatly improved, is still limited at the genome scale. Because of its complex structure and organization, the RpoS regulon provides a very good model for studying gene expression control. A cellular model for RpoS regulation in minimal media is summarized in Figure 3.6. RpoS mediates the expression of intermediate regulators, which in turn control the expression of different sets of genes. RpoS may also have a self-regulatory circuit by controlling the expression of genes that regulate RpoS, either positively (e.g., *hfq*) or negatively (e.g., *clpXP*), the existence of which may be important for maintaining a proper level of RpoS. Many genes are regulated by more than one regulator within the RpoS regulon (e.g., *gadAB* controlled by *gadE* and *gadX*). These

regulators may work cooperatively, independently, or may have an opposing effect on gene expression. For example, CsrA, the carbon storage regulator, down-regulates the expression of *glgAC* for glycogen synthesis but positively regulates *flhCD* for flagella formation and *eno* for glycolysis (Romeo, 1998). In contrast, RpoS positively regulated the expression of *csrA, eno,* and *glgAC*, but had an opposing effect on the expression of *flhCD*. This seemingly antagonistic effect may represent an important regulatory mechanism in nature for balanced gene expression.

In summary, RpoS controls a large set of genes in minimal media. Many of these were not previously known to be RpoS-controlled, suggesting that the RpoS-control of these genes may be specific to nutrient-limited conditions. Microarray analysis can be used as a powerful tool for identification of regulons and regulated pathways. However, future work needs to be done to explore the RpoS-regulation of different pathways in detail to fully understand the physiological role of RpoS. Because of the defined nature of minimal media, this array data can also be used as a reference for comparison of the RpoS regulon under different conditions or in different strain backgrounds.

Table 3.1 RpoS-regulated genes in glucose (0.2%) minimal media (twofold, P < 0.05). Only genes with known functions are listed. Genes that have been identified to be RpoS-regulated in LB by our previous studies are underlined. An asterisk mark (*) indicates that some genes in the known operon are not listed because these genes do not fall into our criteria to be RpoS dependent under either exponential or stationary conditions. (-): indication of negative regulation. ND: not-dependent. Information regarding gene function and known regulators is from the EcoCyc database.

Gene	RpoS-dependence (MER)		Function	Major regulator
	Exponential	Stationary		
aceBAK	-2/-4/-4	2/ND/ND	malate synthase A/isocitrate lyase/isocitrate dehydrogenase phosphatase	IclR IHF CRP FruR ArcA
ackA-pta	3/3	2/ND	acetate kinase/phosphate acetyltransferase	Fnr ArcA
acnA	ND	3	aconitase A	SoxS RpoS
acnB	-3	ND	aconitase B	Fis AcrA CRP
adhE	ND	3	acetaldehyde dehydrogenase	Fis RpoS FruR NarL
adhP	ND	3	alcohol dehydrogenase	
aidB	ND	3	isovaleryl CoA dehydrogenase	RpoS Ada Lrp
aldA	-3	ND	aldehyde dehydrogenase A	ArcA CRP DnaA Fnr
aldB	ND	2	aldehyde dehydrogenase B	RpoS Fis CRP
appCBA	3/4/2	3/5/ND	cytochrome bd-II oxidase/acid phosphatase	AppY RpoS ArcA
*argB**	3	ND	acetylglutamate kinase	ArgR RpoS
*astCD**	ND	2/2	arginine degradation	RpoS ArgR RpoN NtrC
bamB	ND	2	protein assembly complex	RpoE
*betB**	-2	ND	betaine aldehyde dehydrogenase	ArcA BetI
bfr	ND	2	bacterioferritin	RhyB
*bioBFC**	-3/-3/-3	ND	biotin biosynthesis I	BirA
*cbpA**	ND	3	co-chaperone of DnaK	RpoS
cfa	ND	3	fatty acid synthesis	RpoS FNR
cheRBYZ	-14/-10/-9/-10	ND	chemotaxis	Fnr FliA
clpB	ND	4	protein disaggregation chaperone	RpoH
clpPX-lon	ND	4/3/3	protease	GadX RpoH RpoE
*csgF**	3	ND	curli production assembly	CRP RpoS CsgD CpxR OmpR RstA
csiD-lhgO-gabD	ND	4/4/3	starvation induced proteins	CRP RpoS CsiR H-NS Lrp
cspC	ND	3	RNA binding regulator	
csrA	ND	3	carbon storage protein	BarA

Gene	RpoS-dependence (MER)		Function	Major regulator
	Exponential	Stationary		
cstA	ND	-3	carbon starvation peptide transporter	CRP CsrA
cueR	ND	2	regulator of copper-responsive genes	
*cydB**	4	ND	cytochrome bd-I terminal oxidase subunit II	ArcA H-NS Fnr
*cyoDE**	-2/-2	ND	cytochrome bo terminal oxidase	GadE CRP AcrA Fnr Fur
*cysD**	ND	5	sulfate adenylyltransferase, subunit 2	CysB
dapB	5	ND	dihydrodipicolinate reductase	
dctA	ND	-2	C4-dicarboxylate transport protein	DcuR CRP ArcA
dcuB	4	ND	anaerobic C4-dicarboxylate transporter	DcuR CRP Fnr NarL
def-fmt	ND	2/2	peptide deformylase / tRNA methylation	
deoAB	ND	ND	thymidine hosphorylase/phosphopentomutase	DeoR Fis CRP CytR ModE
dhaKLM	-10/-3/-2	ND	dihydroxyacetone kinase	DhaR
dnaKJ	ND	6/2	chaperone Hsp70	RpoH
dppABCDF	-2/-3/-4/-3/-2	ND	dipeptide transporter	Fnr IHF
dps	ND	22	stationary phase DNA protein protein	OxyR IHF RpoS
dsbB	-2	ND	oxidoreductase	
*exbB**	ND	-4	uptake of enterochelin and B colicins	
fabA	ND	3	3-hydroxydecanoyl-ACP dehydratase	FadR FabR
fbaB	4	5	fructose-bisphosphate aldolase class I	RpoS
fdhF	2	ND	formate dehydrogenase H	RpoN Fhl NarL Fnr
fhuF	-3	ND	ferric reductase	Fur
fic	ND	3	stationary-phase protein, folate biosynthesis	RpoS
*fimAC**	ND/-2	-3/ND	type 1 fimbriae	IHF Lrp H-NS
flgAMN	-7/-15/-6	ND	flagellar biosynthesis	FlhDC
flgBCDEFGHIJ	-46/-20/-36/-36/-74/-14/-35/-16/-9	ND	flagellar biosynthesis	FlhDC
flgKL	-26/-13	ND	flagellar biosynthesis	FlhDC RpoF
flhBAE	-5/-3/-5	ND	flagellar biosynthesis	FlhDC
flhDC	-410/-172	-160/-50	activator of flagella biosynthesis	CRP Fur H-NS IHF OmpR RcsAB
fliAZY	-37/-61/-8	ND	flagellar biosynthesis	FlhDC ArcA H-NS
fliC	-30	ND	flagellar biosynthesis	H-NS GadE RpoF
fliDST	-12/-23/-5	ND	flagellar biosynthesis	FlhDC
fliFGHIJK	-51/-19/-10/-7/-6	ND	flagellar biosynthesis	RpoF
*fliLMNOPQ**	-20/-144/-14/-9/-6/-9	ND	flagellar biosynthesis	FlhDC
flu	3	3	antigen 43 precursor	OxyR
flxA	-3	ND	flagellar motility	FliA

Gene	RpoS-dependence (MER)		Function	Major regulator
	Exponential	Stationary		
focA-pflB	ND/4	2/3	formate transporter/pyruvate formate lyase I	ArcA NarL Fnr CRP IHF Fis
*folE**	-7	ND	GTP cyclohydrolase I	
*frdC**	2	ND	fumarate reductase subunit C	DcuR Fnr NarL
*ftsJ**	ND	4	23S rRNA methyltransferase	
fumA	-2	-3	fumarate hydratase class I, aerobic	ArcA Fnr CRP
gadAX	10/4	55/2	glutamate dependent acid resistance	RpoS Fis GadEXW CRPH-NSTorR Fnr
gadBC	352/16	556/41	glutamate dependent acid resistance	GadX GadE GadW Fis CRP RpoS
*gadE-mdtE**	6/2	3/ND	glutamate dependent acid resistance	RpoS GadEWX CRPEvgA
gadXW	4/2	2/ND	glutamate dependent acid resistance	RpoS GadEWRutRFnrH-NS
*gapA**	ND	4	glyceraldehyde-3-phosphate dehydrogenase A	FruR
gdhA	2	ND	glutamate dehydrogenase, NADP-specific	Nac CRP
glcC	ND	-2	Glc operon transcriptional activator	Fis CRP GlcC
glgCAP	5/5/2	ND	glycogen biosynthesis	CRP
*glgX**	5	ND	glycogen debranching enzyme	
gltA	-2	ND	citrate synthase	ArcA CRP IHF
*glyT-thrT-tufB**	ND	3/3/2	tRNA, elongtation factor Tu	Fis
gpmA	-2	ND	phosphoglyceromutase 1	Fur
grxD	-3	ND	glutaredoxin 4	
hchA	ND	16	Hsp31 molecular chaperone	H-NS
hdeAB	28/18	31/23	acid-resistance protein	RpoS H-NSGadEX Lrp TorR MarA
hdeD	17	16	acid-resistance membrane protein	GadEX H-NS
hemL	-2	ND	aminotransferase	PhoP PdhR
himA	ND	3	integration host factor alpha subunit	IHF RpoS
*hisD**	2	ND	histidinol dehydrogenase	
holC	ND	2	DNA polymerase III, chi subunit	RpoH
*hslV**	ND	3	protease	RpoH
hspQ	ND	3	hemimethylated heat shock protein	RpoH
hyaABCDEF	22/6/7/6/6/5	3/2/2/2/ND/ND	hydrogenase 1	ArcA RpoS Fis AppY IscR NarL
*hybOAC**	3/3/3	ND	hydrogenase 2	ArcA NarL
*hycABCDEFGH**	2/3/3/3/3/3/3/3	ND	formate hydrogenlyase	RpoN ModE FhlA IHF
*iaaA-gsiA**	2/2	2/ND	asparaginase III/glutathione ABC transporter	
*iadA**	2	ND	isoaspartyl dipeptidase	
icdA	-3	ND	isocitrate dehydrogenase	ArcA Fnr IHF
*ileT-alaT**	ND	2/2	tRNA	
*ileU**	ND	2	tRNA	

Gene	RpoS-dependence (MER)		Function	Major regulator
	Exponential	Stationary		
lysA	6	ND	diaminopimelate decarboxylase, PLP-binding	lysR
lysC	17	ND	aspartate kinase III	
lysP	3	ND	Lysine-specific permease	
*lysW**	ND	2	tRNA	
*manY**	ND	2	mannose-specific enzyme IIC component	CRP DgsA NagC
*map**	-2	ND	methionine aminopeptidase	
mdh	-4	ND	malate dehydrogenase	CRP FlhDC ArcA
mhpR	ND	-2	DNA-binding transcriptional activator	
miaA-hfq	ND	4/3	tRNA-adenosine transferase/RNA binding regulator	RpoH
*mopA**	ND	3	heat shock chaperone GroEL Hsp60	RpoH
motAB-cheAW	-596/-910/-1314/-690	-16/-18/-124/-113	chemotaxis	CpxR FliA
mscS	2	ND	mechanosensitive channel MscS	
msrB	-4	ND	methionine sulfoxide reductase B	
msyB	ND	2	suppresses heat sensitivity of secY mutants	RpoS
mtfA	ND	-2	Mlc titration factor	
nlpI	ND	3	lipoprotein involved in cell division	
nmpC	-11	-5	outer membrane porin protein	CRP OmpR IHF
*nrdHI**	ND	2/3	ribonucleotide reduction	RpoH Fur NrdR
*nsrR**	ND	3	transcriptional repressor	
*nusA**	ND	2	transcription termination/antitermination factor	Fis CRP ArgR
ompA	ND	5	outer membrane protein A	CRP micA and rseX
ompC	3	5	outer membrane porin protein C	LrP EnvY CpxR OmpR IHF
ompF	ND	-5	outer membrane protein F precursor	CpxR OmpR IHF CRP RstA Lrp EnvY Fur
ompT	-5	ND	outer membrane protease VII	EvgA/EvgS
*oppA**	2	-3	oligopeptide transporter subunit	Lrp ModE AcrA
osmC	ND	2	stress-inducible membrane protein	H-NS Lrp NhaR RcsB RpoS
osmY	ND	6	stress-inducible periplasmic protein	RpoS Fis CRP IHF Lrp
otsB	2	5	trehalose-phosphatase	RpoS
pat	ND	2	putrescine aminotransferase / diamine transaminase	RpoN NtrC
pfkA	ND	3	6-phosphofructokinase	FruR
pfkB	2	ND	6-phosphofructokinase II	RpoS
pgk	ND	4	phosphoglycerate kinase	CRP FruR
*pheT**	ND	3	phenylalanine tRNA synthetase, beta subunit	
*poxB**	3	ND	pyruvate dehydrogenase	SoxS MarA RpoS
*prpB**	ND	3	2-methylisocitrate lyase	PrpR CRP RpoN

Gene	RpoS-dependence (MER)		Function	Major regulator
	Exponential	Stationary		
ptsG	-2	ND	PTS system, glucose-specific IIBC component	Fis CRP SoxS ArcADgsA
ptsHI-crr	ND	6/3/2	sugar-non-specific	CRP FruR DgsA Fnr
pykF	ND	4	pyruvate kinase I	FruR
pyrG-eno	ND	11/5	CTP synthatase/enolase	
*rimM-trmD**	ND	2/3	translation	Fnr
rmf	ND	2	ribosome modulation factor	ppGpp
*rplKAJL-rpoB**	ND	3/3/3/2/2	ribosomal protein/RNA polymerase beta subunit	
rplM-rpsI	ND	3/2	ribosomal protein	Fnr
rplNXE-rpsNH-rplFR-rpsE-rplO-prlA-rpmJ	ND	2/2/2/3/3/3/3/ 3/3/3/3	ribosomal protein	
*rplU**	ND	2	50S ribosomal subunit protein L21	
rplY	ND	2	50S ribosomal protein L25	
*rpmB**	ND	3	50S ribosomal subunit protein L28	Fnr
*rpmF-fabHDG**	ND	4/3/2/2	50S ribosomal protein, fatty acid biosynthesis	
*rpoE-rseA**	ND	3/3	sigma24 and its antisigma factor	CpxR RpoE ppGpp
*rpsA**	ND	3	30S ribosomal subunit protein S1	Fnr
rpsB-tsf	ND	13/3	translation	
*rpsF-priB-rpsR**	ND	3/3/2	translation and DNA replication	
*rpsG-fusA**	ND	4/3	translation	
*rpsJ-rplCDW-rpsS-rplV-rpsC-rpmC-rpsQ**	ND	4/4/3/3/3/3/3/11/2	ribosomal protein	Fnr ArcA
rpsMKD-rpoA-rplQ	ND	3/4/4/3/3	ribosomal protein/RNA polymerase alpha subunit	
rpsV	4	4	30S ribosomal protein S22	
*sdaC**	-3	ND	serine transporter	
sdhCDAB	-16/-9/-8/-10	ND	succinate dehydrogenase	ArcA CRPFur Fnr
*secB**	ND	2	protein export chaperone	
secE-nusG	ND	2/3	protein secretion/transcription termination	
*serC**	ND	3	aminotransferase	Lrp CRP
*serV-argVZQ**	ND/-3/-4/-3	3/ND/3/ND	tRNA	
slp-dctR	6/3	3/ND	resistance to metabolic end products	MarA GadXW RpoS
slyB	2	2	outer membrane lipoprotein	PhoP
sodA	-3	3	superoxide dismutase (Mn)	CRP IHF SoxS MarA Rob ArcA Fur
sodB	-3	ND	superoxide dismutase (Fe)	IHF CRP Fur H-NS
*speD**	-2	ND	adenosylmethionine decarboxylase	
spf	4	ND	antisense regulator of *galK* translation	CRP
sucABCD	-6/-8/-5/-6	ND	2-oxoglutarate dehydrogenase,succinyl-CoA synthetase	ArcA Fnr IHF
*sufABCDS**	ND	3/4/4/4/4	iron-sulfur cluster assembly	OxyR IscR IHF Fur

Gene	RpoS-dependence (MER)		Function	Major regulator
	Exponential	Stationary		
talA	3	6	transaldolase A	RpoS
tar-tap	-302/-969	-11/-80	chemotaxis	Fnr FliA
tgt	ND	2	tRNA-guanine transglycosylase	
tktB	ND	3	transketolase 2	RpoS
tolB-pal	ND	3/2	subunits of the Tol-Pal cell envelope complex	
tonB	-3	ND	energy transduction	Fur
trg	-2	ND	chemotaxis	Fis RpoF
tsr	-7	ND	chemotaxis	CpxR RpoF
uspB	ND	4	universal stress protein	IHF RpoS
wrbA	6	14	NAD(P)H:quinone oxidoreductase	RpoS
ybaS	4	4	glutaminase	GadX
yeaG	ND	13	stationary-phase-induced protein kinase	

Table 3.2 RpoS-regulated gene expression in M63 minimal media (0.2% glucose) by qPCR.

RNA samples were prepared as for microarray analyses. Expression of genes was quantified by qPCR using SYBR green, as previously described (Dong *et al.*, 2008b). Briefly, gene transcription levels were determined using a standard curve constructed by a serial dilution of genomic DNA with known concentrations. The expression intensity was log2-transformed (LI), and the RpoS dependence of genes was represented by the mean expression ratio (MER: WT/*rpoS*). The average expression of three replicates of each strain and the standard error are indicated.

Blattner No.	Gene	Function	Exponential phase			Stationary phase		
			WT (LI)	*rpoS* (LI)	MER	WT (LI)	*rpoS* (LI)	MER
b3510	*hdeA*	acid-resistance protein	18.5 ± 0.2	13.3 ± 0.1	36.8	21.6 ± 0.2	17.9 ± 0.3	11.3
b2097	*fbaB*	fructose-biphosphate aldolase class I	14.5 ± 0.3	12.6 ± 0.2	3.7	18.1 ± 0.1	13.3 ± 0.1	27.9
b1004	*wrbA*	NAD(P)H: quinone oxidoreductase	13.9 ± 0.4	12.2 ± 0.1	3.2	17.2 ± 0.0	12.8 ± 0.1	21.1
b1897	*otsB*	trehalose-phosphatase	15.4 ± 0.4	12.0 ± 0.2	10.6	17.2 ± 0.4	13.4 ± 0.1	13.9
b2464	*talA*	transaldolase A	16.7 ± 0.2	12.8 ± 0.1	14.9	17.4 ± 0.2	13.7 ± 0.3	13.0

Table 3.3 Effect of RpoS on gene expression in minimal media with glycerol as the sole carbon source.

Cultures were grown in triplicate in M63 minimal media supplemented with 0.4% glycerol to exponential phase (OD_{600} = 0.3) and stationary phase (OD_{600} = 1.5). RNA samples were extracted using hot acidic phenol. Gene expression was quantified by qPCR using the SYBR green standard curve method. The expression intensity was log2-transformed (LI), and the RpoS dependence of genes was represented by the mean expression ratio (MER: WT/*rpoS*). The average of three replicates and the standard error are indicated.

Blattner No.	Gene	Function	Exponential phase			Stationary phase		
			WT (LI)	*rpoS* (LI)	MER	WT (LI)	*rpoS* (LI)	MER
b3908	*sodA*	Superoxide dismutase, Mn	16.1 ± 0.4	16.4 ± 0.5	0.8	17.1 ± 0.3	15.6 ± 0.2	2.8
b0953	*rmf*	Ribosome modulation factor	21.2 ± 0.6	24.5 ± 1.2	0.1	22.1 ± 1.0	18.8 ± 0.2	9.7
b0437	*clpP*	ATP-dependent Clp protease proteolytic subunit	16.0 ± 0.2	16.1 ± 0.3	0.9	16.6 ± 0.3	15.2 ± 0.3	2.0
b2573	*rpoE*	RNA polymerase sigma 24	16.9 ± 0.2	17.6 ± 0.4	0.6	17.7 ± 0.4	16.3 ± 0.2	2.1

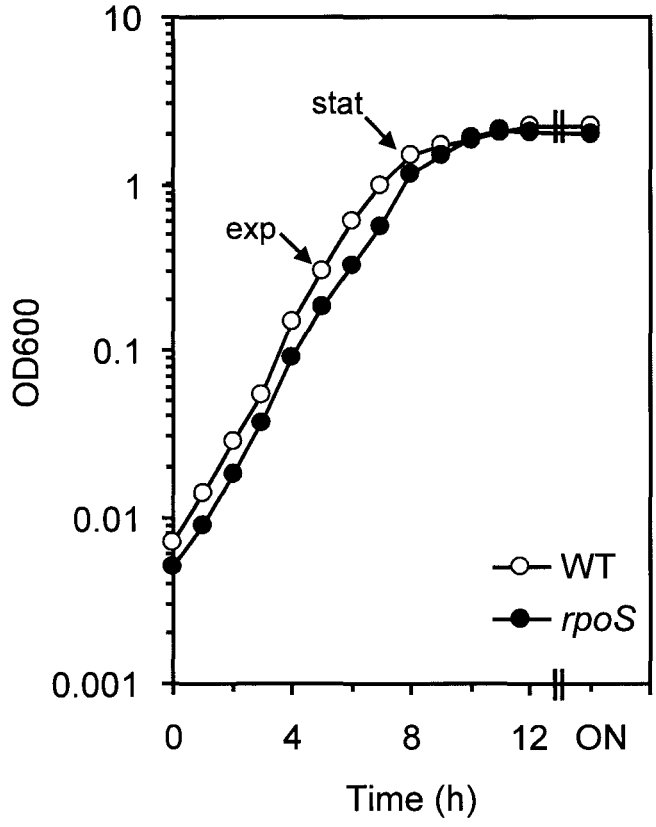

Figure 3.1 Growth of WT and *rpoS* mutant in M63 glucose (0.2%) minimal media. Cultures were inoculated from overnight cultures to a starting OD_{600} = 0.0001 and incubated aerobically at 37 °C at 200 rpm. After recovery from the lag phase and several generations of exponential phase growth, cultures were monitored periodically starting from a set time point (time 0h) when the culture densities were high enough to obtain reliable readings. RNA samples were isolated at OD_{600} = 0.3 and 1.5 as indicated.

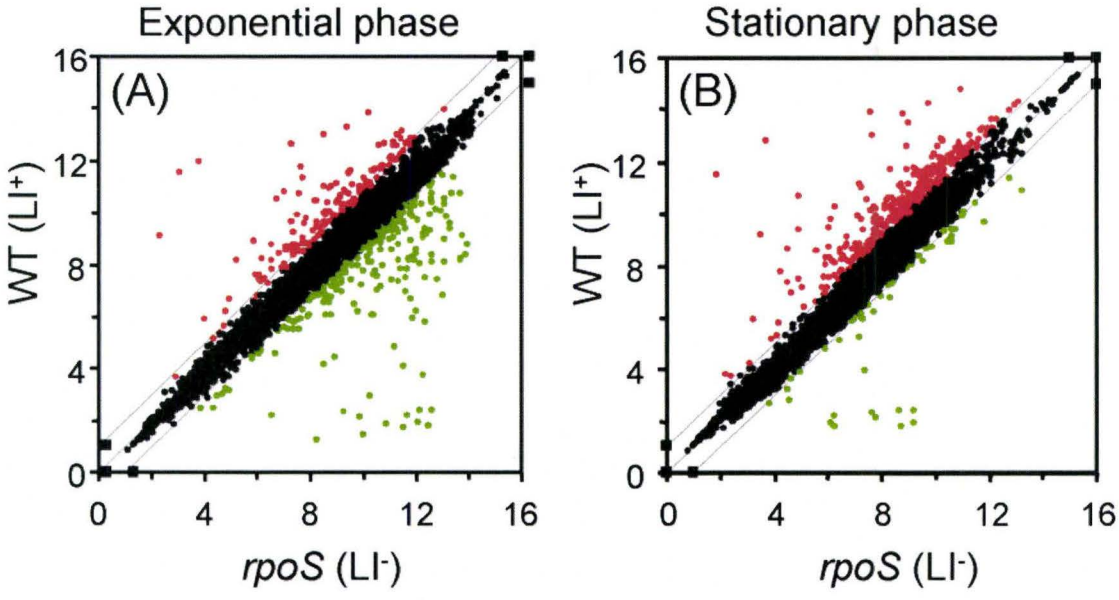

Figure 3.2 Transcriptome overview of WT and *rpoS* mutants.

A scatterplot was used to examine the effects of RpoS on gene expression in exponential (A) and stationary (B) phase. Probe-sets (including genes and intergenic regions) are outlined by two parallel lines dividing the dataset into three different groups: probe-sets expressed at least twofold higher in the WT (red), those expressed with less than twofold difference between WT and the *rpoS* mutant (black), and those expressed at least twofold higher in the *rpoS* mutant (green). Among these probe-sets, 88 and 112 genes were expressed higher in wild type or the *rpoS* mutants in exponential phase, respectively. In stationary phase, 203 genes were expressed higher in WT and 22 genes expressed higher in the *rpoS* mutant. LI: $\log_2$-transformed expression intensity.

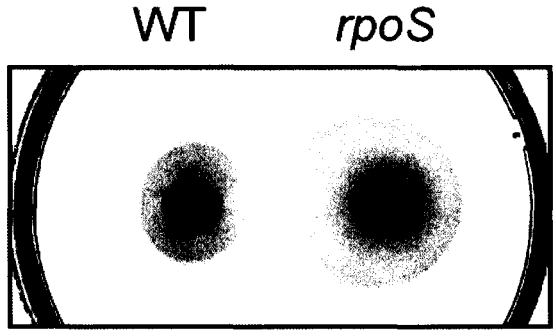

Figure 3.3 Motility of WT and *rpoS* mutants.

Cells were grown on M63 agar (0.3%) at 37°C for 48h.

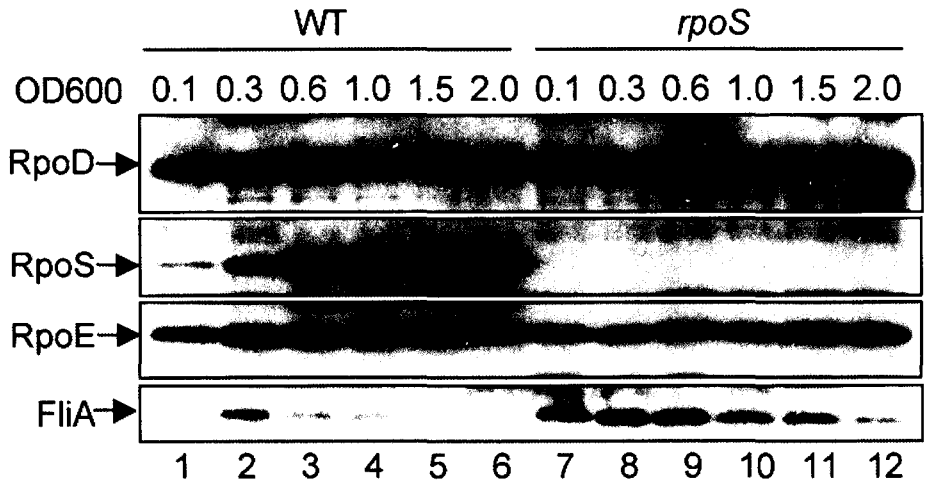

Figure 3.4 Expression of RpoS, RpoE and FliA in WT and *rpoS* mutants in minimal media.

Western blot analyses of the expression of sigma factors were performed using monoclonal antibody to RpoD, RpoS, RpoE, and FliA, respectively. Cultures were centrifuged, and resultant cell pellets were resuspended in SDS loading buffer to a final OD_{600} = 1, followed by boiling in water for 5 min. Ten µl of each sample were loaded and resolved by 10% SDS-PAGE. Identical gels were run in parallel and stained with Coomassie blue to confirm equal loading.

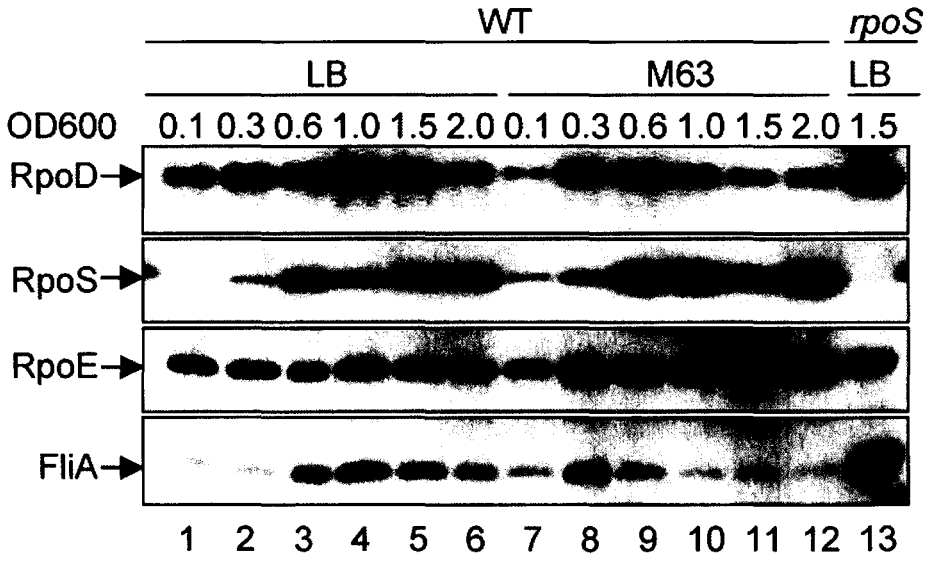

Figure 3.5 Expression comparison of RpoS, RpoE and FliA during growth in LB and M63 media.

The *rpoS* mutant sample (lane 13) included as negative control for RpoS expression was prepared using cells grown in LB at OD_{600} = 1.5. Samples were prepared as described in Materials and method. Ten µl of each sample were loaded and resolved by 10% SDS-PAGE. Equal protein loading was confirmed by Coomassie blue staining of identical gels run in parallel.

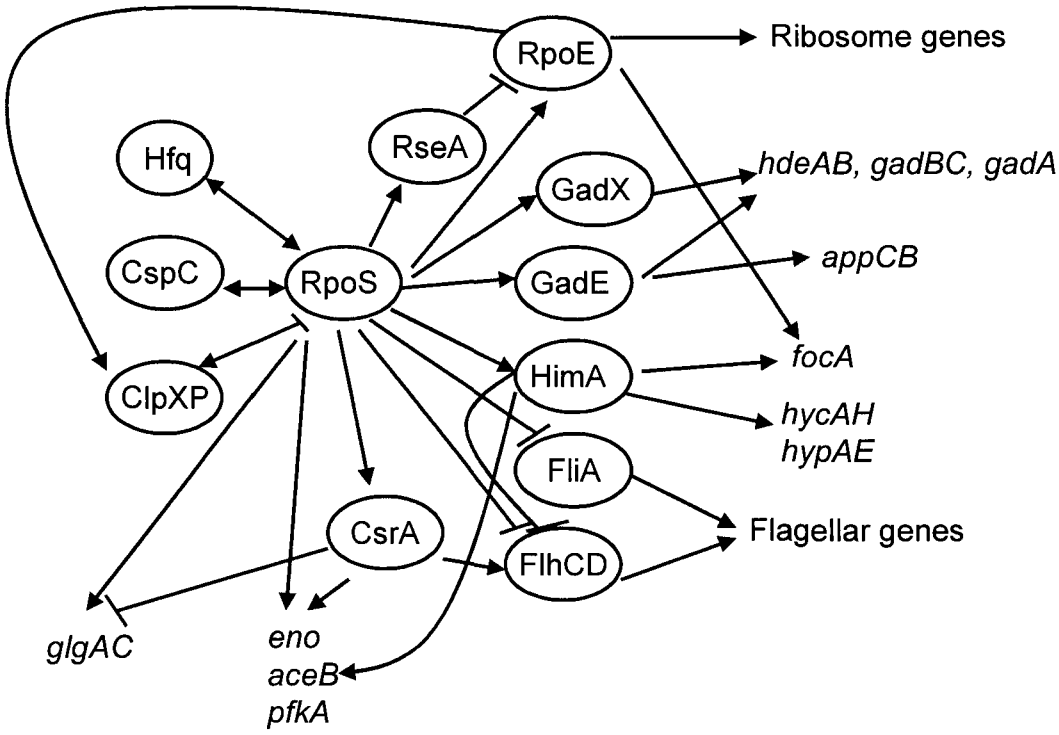

Figure 3.6 Regulatory network controlled by RpoS in minimal media.

Arrows indicate a positive effect on expression while bars indicate a negative regulatory effect.

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CHAPTER IV

Global effect of RpoS on gene expression in pathogenic *Escherichia coli* O157:H7 strain EDL933

from

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4.1 Preface

The contents of this chapter were contributed primarily by the author of this thesis (Tao Dong). The text was written, in its entirety, by Tao Dong. This chapter is adapted from a published article in BMC Genomics to fit the format requirements of the thesis.

4.2 Abstract

RpoS is a conserved stress regulator that plays a critical role in survival under stress conditions in *Escherichia coli* and other γ-proteobacteria. RpoS is also involved in virulence of many pathogens including *Salmonella* and *Vibrio* species. Though well characterized in non-pathogenic *E. coli* K12 strains, the effect of RpoS on transcriptome expression has not been examined in pathogenic isolates. *E. coli* O157:H7 is a serious human enteropathogen, possessing a genome 20% larger than that of *E. coli* K12, and many of the additional genes are required for virulence. The genomic difference may result in substantial changes in RpoS-regulated gene expression. To test this, we compared the transcriptional profile of wild type and *rpoS* mutants of the *E. coli* O157:H7 EDL933 type strain. The *rpoS* mutation had a pronounced effect on gene expression in stationary phase, and more than 1,000 genes were differentially expressed (twofold, $P < 0.05$). By contrast, we found 11 genes expressed differently in exponential phase. Western blot analysis revealed that, as expected, RpoS level was low in exponential phase and substantially increased in stationary phase. The defect in *rpoS* resulted in impaired expression of genes responsible for stress response (e.g., *gadA*, *katE* and *osmY*), arginine degradation (*astCADBE*), putrescine degradation (*puuABCD*), fatty acid oxidation (*fadBA* and *fadE*), and virulence (*ler*, *espI* and *cesF*). For EDL933-specific genes on O-islands, we found 50 genes expressed higher in wild type EDL933 and 49 genes expressed higher in the *rpoS* mutants. The protein levels of Tir and EspA, two LEE-encoded virulence factors, were elevated in the *rpoS* mutants under LEE induction conditions. Our results show that RpoS has a profound effect on global gene expression

in the pathogenic strain O157:H7 EDL933, and the identified RpoS regulon, including many EDL933-specific genes, differs substantially from that of laboratory K12 strains.

4.3 Introduction

Enterohemorrhagic *Escherichia coli* O157:H7 is a serious human pathogen that is responsible for many food-borne epidemic outbreaks, and the infection of *E. coli* O157:H7 can cause bloody diarrhea, hemorrhagic colitis and the hemolytic uremic syndrome (Karmali, 1989; Paton & Paton, 1998). The pathogenesis caused by *E. coli* O157:H7 is a complex process that requires a coordinated expression of virulence factors and regulators (Paton & Paton, 1998). Known virulence factors in *E. coli* include the type III secretion factors encoded on the LEE pathogenicity island (McDaniel *et al.*, 1995) and Shiga toxins (StxI and StxII) (reviewed in (O'Brien & Holmes, 1987)). Many regulators are involved in mediating expression of these virulence factors. For example, genes on the LEE island are under control of H-NS (Barba *et al.*, 2005), IHF (Barba *et al.*, 2005), ClpXP (Tomoyasu *et al.*, 2005) and three LEE-encoded regulators Ler, GrlA, and GrlR (Deng *et al.*, 2004).

In *E. coli* and many other gamma-proteobacteria, the global stress response is controlled by the stationary phase sigma factor RpoS (Dong *et al.*, 2008a; Hengge-Aronis, 2000). RpoS is induced in many stress conditions, including near-UV exposure (Sammartano *et al.*, 1986), acid shock (Small *et al.*, 1994), heat shock (Hengge-Aronis *et al.*, 1991), oxidative stress (Sammartano *et al.*, 1986), and starvation (Lange & Hengge-Aronis, 1991b), many of which *E. coli* may experience during growth and survival in natural environments. RpoS controls a large regulon consisting of 10% of the genome in

E. coli K12 strains in stationary phase and stress conditions (Dong & Schellhorn, 2009a; Lacour & Landini, 2004; Patten *et al.*, 2004; Weber *et al.*, 2005). Even in exponential phase when RpoS is expressed at low levels, mutation in *rpoS* affects the expression of a large set of genes as well (Dong *et al.*, 2008b; Rahman *et al.*, 2006), and RpoS is important for DNA damage response in early exponential phase cells (Merrikh *et al.*, 2009). Though there is an identifiable core set of RpoS-regulated genes, the RpoS-dependence of many genes within the RpoS regulon varies depending on experimental conditions and strain backgrounds (Dong *et al.*, 2008b; Rahman *et al.*, 2006; Weber *et al.*, 2005).

The effect of RpoS on virulence has been examined in many pathogens, and results differ depending on species. RpoS is critical for virulence of *Salmonella* (Fang *et al.*, 1992) and *Vibrio cholerae* (Merrell *et al.*, 2000). By contrast, RpoS does not appear to be required for virulence in *P. aeruginosa* (Suh *et al.*, 1999) and *Y. enterocolitica* (Iriarte *et al.*, 1995). How RpoS is involved in enteropathogenesis of *E. coli* remains elusive, primarily because of the lack of a proper animal model since mice are not susceptible to infection of *E. coli* pathogens (Mundy *et al.*, 2005). To overcome this problem, a model of using *Citrobacter rodentium*, a natural mouse enteropathogen closely related to *E. coli* has been widely used to simulate *E. coli* infection (Mundy *et al.*, 2005). We have found that RpoS is important for full virulence of *C. rodentium* (Dong *et al.*, 2009b), suggesting an important role of RpoS in *E. coli* infection. Consistently, there are a few virulence traits regulated by RpoS. For example, curli production, important for virulence of *Salmonella* and *E. coli,* is positively regulated by RpoS (Dong *et al.*, 2009b;

Romling *et al.*, 1998; Romling, 2005; Uhlich *et al.*, 2006). The effect of RpoS on expression of the LEE virulence genes appears to vary depending on strain backgrounds and experimental conditions. For example, Sperandio et al. (1999) reported that the LEE3 operon and *tir* are positively regulated by RpoS in EHEC strain 86-24 (Sperandio *et al.*, 1999). However, in EHEC O157:H7 Sakai strain, LEE expression is enhanced in *rpoS* mutants (Iyoda & Watanabe, 2005; Tomoyasu *et al.*, 2005). It is likely that the expression of LEE genes is modulated differently depending on strain backgrounds. Surprisingly, expression of LEE genes appears to differ between O157:H7 Sakai and EDL933 strains as well (see Fig. 1 in (Laaberki *et al.*, 2006)). The role of RpoS in strain EDL933 has not been tested. Furthermore, there has been no genomic profiling specifically investigating the involvement of RpoS in regulation of virulence genes in enteropathogenic *E. coli* and other related pathogens.

The genomes of *E. coli* K12 reference strain MG1655 and O157:H7 strain EDL933 differ considerably (Perna *et al.*, 2001). EDL933 and MG1655 possess 5.5 Mb and 4.6 Mb genome sizes, respectively, sharing 4.1 Mb backbone DNA (Perna *et al.*, 2001). DNA segments that are unique to one or the other strain and scattered within each genome are termed "O-islands" in O157:H7 and "K-islands" in K12 (Perna *et al.*, 2001). O-islands consist of 1.34 Mb DNA sequence encoding 26% of all EDL933 genes, while K-islands consist of 0.53 Mb harboring 12% of the genes in MG1655 genome (Perna *et al.*, 2001). Many genes on the O-islands are important in pathogenicity (e.g., genes on the LEE islands) (Perna *et al.*, 2001). In addition, gene polymorphisms on the backbone are common, since 75% of the backbone genes encode proteins that differ by at least one

amino acid in these two strains (Perna *et al.*, 2001). Some genes are extremely divergent. In the case of *yadC*, the protein sequence in K12 and O157:H7 is only 34% identical (Perna *et al.*, 2001). The genome divergence between O157:H7 and K12 may have a substantial effect on gene regulation.

E. coli O157:H7 diverged from K12 strain about 4.5 million years ago (Reid *et al.*, 2000), and genes on O-islands have been acquired through horizontal gene transfer (Hayashi *et al.*, 2001; Perna *et al.*, 2001; Reid *et al.*, 2000). How O-island genes are integrated into preexisting regulatory circuits controlled by RpoS is still unknown. Given that RpoS is known to regulate genes of nonessential functions (Dong *et al.*, 2008a; Hengge-Aronis, 2000; Patten *et al.*, 2004; Weber *et al.*, 2005), it is possible these O-island genes are preferentially under control of RpoS rather than RpoD, the housekeeping sigma factor. This has yet to be tested.

To examine RpoS-regulated gene expression in a pathogenic strain, we employed the *E. coli* O157:H7 strain EDL933 since this strain can cause serious human health problems and its genome is fully sequenced (Perna *et al.*, 2001). To compare with our previous results (Dong *et al.*, 2008b; Patten *et al.*, 2004), we sampled wild type and isogenic *rpoS* mutants of EDL933 under the same growth conditions and compared their transcriptome expression in exponential phase (OD_{600}=0.3) and early stationary phase (OD_{600}=1.5). Herein we report that *rpoS* mutation had a profound effect on transcriptome expression. Genes under control of RpoS included many EDL933-specific genes on the O-islands. Besides stress response genes, RpoS also regulated the expression of genes involved in metabolic pathways, transcription, and virulence.

4.4 Materials and Methods

Strains, media and growth conditions

E. coli strain O157:H7 EDL933 and its *rpoS* mutant derivative were employed in this study. Cultures were grown aerobically at 37°C with shaking at 200 rpm in Luria-Bertani media, and growth was monitored spectrophotometrically at OD_{600}. Antibiotics were used at the following concentrations: ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml).

Construction of EDL933 *rpoS* deletion mutant

An *rpoS* non-polar deletion mutant was constructed by homologous recombination as described previously (Datsenko & Wanner, 2000). Briefly, a linear DNA fragment, harboring the chloramphenicol resistant gene *cat* and homologous *rpoS*-flanking sequences, was amplified using pKD3 plasmid (template) and primers FP1 (CCTCGCTTGAGACTGGCCTTTCTGACAGTGCTTACGTGTAGGCTGGAGCTGC TTC) and RP1 (ATGTTCCGTCAAGGGATCACGGGTAGGAGCCACCTTCATAT GAATATCCTCCTTAG) and introduced into EDL933 pKD46 competent cells by electroporation. Transformants were selected on LB chloramphenicol plates. The *cat* gene was further removed by recombination with the FLP recombinase. The loss of *rpoS* was confirmed by PCR using flanking primers and by sequencing.

RNA preparation

RNA samples were prepared as previously described (Dong *et al.*, 2008b). Overnight cultures were diluted into fresh media at a starting OD_{600} of 0.0001 to allow cells to grow at least ten generations prior to RNA isolation in exponential phase.

Cultures grown in triplicate were sampled at OD_{600}=0.3 (exponential phase) and OD_{600}=1.5 (stationary phase), conditions used in our previous studies for comparison (Dong *et al.*, 2008b; Patten *et al.*, 2004). RNA samples were prepared using hot acidic phenol (pH 4.3, Sigma-Aldrich), and the quality of RNA was examined using a Bioanalyzer 2100 (Agilent Technologies).

Microarray analysis

The Affymetrix GeneChip *E. coli* Genome 2.0 Array was employed in this study. This array chip contains more than 10,000 probe sets that cover all genes in the genomes of four type *E. coli* strains, K12 MG1655, O157:H7 EDL933, O157:H7 Sakai, and the uropathogen, CFT073. A gene that is present in all genomes with high similarity in sequence is represented by a single probe set. Although this is an effective approach to minimize the total number of probe sets used to cover all four genomes, some homologous genes with low sequence similarity in the four strains may be represented by more than one probe set. For example, there are two probe sets in the array representing *rpoS* (probe set IDs: 1761030_s_at and 1767783_s_at) because the *rpoS* sequence in the strain CFT073 harbors an internal mutation that results in two truncated genes, c3306 (519bp probing to 3' end of *rpoS*) and c3307 (435bp probing to 5' end of *rpoS*). Both probe sets hybridized to *rpoS* transcripts and the resultant signals in wild type samples were 4,939 and 7,643 time higher than those in the knockout *rpoS* mutants, respectively (this study). Though both probe sets are representative of *rpoS*, this leads to duplication. To avoid this problem, microarray data were curated to remove redundant probe sets in our analysis. Microarray samples were analyzed using dChip (Li & Wong, 2001) and

BRB Arraytools (Simon *et al.*, 2007), as described previously (Dong & Schellhorn, 2009a). Samples were $\log_2$ transformed and normalized using the GCRMA method (Wu & Irizarry, 2004). RpoS dependence of genes is represented by the mean expression ratio (MER) of WT and *rpoS* mutants. The significance of expression difference was tested using Student's t-tests. Genes with MER value ≥ 2 or ≤ 0.5 and P value <0.05 were considered to be controlled by RpoS (Dong & Schellhorn, 2009a). The false discovery rate (FDR) was estimated by 1,000 time random permutations as previously described (Dong & Schellhorn, 2009a). Microarray data can be accessed in the Gene Expression Omnibus database at the National Center for Biotechnology Information under the accession number GSE17420.

Quantitative real-time PCR (qPCR)

To confirm microarray results, we tested gene transcription by qPCR as previously described (Dong & Schellhorn, 2009a). Primers were designed using the PerlPrimer program (Marshall, 2004) and synthesized by the MOBIX laboratory at McMaster University. RNA samples were prepared as for microarray analysis. First strand cDNA was synthesized using a cDNA synthesis kit (New England Biolabs). Gene amplification was detected using SYBR green (Clontech) in a MX3000P qPCR system (Stratagene). The expression level of genes was determined by constructing a standard curve using serial dilutions of EDL933 genome DNA with known concentrations. The 16S RNA gene, *rrsA,* was used as a reference control to normalize differences in total RNA quantity among samples (Kobayashi *et al.*, 2006).

Western blot analyses

Cultures were grown in LB media aerobically at 37°C and sampled periodically. Samples were immediately mixed with chloramphenicol (150μg/ml) and placed on ice to stop protein synthesis, followed by centrifugation at 15,000 xg for 2 min. Cell pellets were flash frozen in liquid nitrogen prior to use. Cell pellets were thawed on ice, resuspended to OD_{600}=1.0 with SDS loading buffer, and boiled for 5 min. Samples of 10 μl were resolved on 10% SDS-PAGE and transferred to PVDF membrane (Dong & Schellhorn, 2009a). The PVDF membrane was then blocked with 5% milk solution, incubated with mouse monoclonal antibodies for RpoS (NeoClone, Madison, WI), Tir or EspA (a gift from B. Coombes), and HRP-conjugated Goat anti-mouse secondary antibody (Bio-Rad, Hercules, CA). The signal was detected using the ECL solution (Amersham, Pittsburgh, PA) and Hyperfilm-ECL film (Amersham, Pittsburgh, PA). To ensure that equal amounts of protein were loaded, another SDS-PAGE gel was run in parallel and stained with Coomassie Blue R-250.

Survival of mutants upon exposure to stress conditions

Stationary phase cultures were washed and diluted in 0.9% NaCl before exposure to stress. A total number of 1.0 x 10^8 cells were exposed to 1 ml of acidic LB (pH2.5, adjusted with HCl) and 15mM H_2O_2, respectively, while 5.0 x 10^3 cells were treated at 55°C for heat exposure. Viable cells were enumerated by serial plating on LB medium, and survival expressed as a percentage determined by dividing the number of viable cells by the number of cells before treatment.

4.5 Results

Expression of RpoS during growth in LB media

Although RpoS controls the expression of a large set of genes, mutation of *rpoS* has little effect on growth rate of *E. coli* K12 strain MG1655 (Dong *et al.*, 2008b; Dong & Schellhorn, 2009a). To test whether this is applicable to pathogenic *E. coli* EDL933, we compared the growth of *rpoS* mutants with wild type EDL933 grown in LB. Both the growth rate and the time to enter stationary phase were similar between wild type and *rpoS* mutants of EDL933 (Figure 4.1). The generation time in exponential phase was approximately 26 min. This equivalence is important for comparison of genomic expression since the expression of many genes is affected by growth rate (Tao *et al.*, 1999). As expected, the protein level of RpoS was found to be low in early exponential phase, followed by a substantial increase during entry of stationary phase (Figure 4.1).

Expression of genes under control of RpoS

The mutation in *rpoS* had a pronounced effect on genomic expression of EDL933 in stationary phase but a minor effect in exponential phase (Figure 4.2). In exponential phase when RpoS protein level was low, we found that 11 genes were differentially expressed in the *rpoS* mutants (Table 4.1), while in stationary phase, more than 1,000 genes were expressed differently as a result of *rpoS* mutation (twofold, $P < 0.05$) (Table 4.2). The false discovery rate was 1.4%. Among these stationary phase genes, 596 genes were expressed higher in the wild type EDL933, including 105 previously known RpoS-dependent genes in K12 strains. In addition, a mutation in *rpoS* led to increased expression of 536 genes (Table 4.3), indicating that the negative effect of RpoS on gene

expression is also extensive. For genes on O-islands that are specific to EDL933, 50 genes showed higher expression in wild type and the expression of 49 genes was elevated in the *rpoS* mutants.

RpoS-regulated functions in exponential phase

The expression of 11 genes was impaired in *rpoS* mutants in exponential phase (Table 4.1). Three genes, *motAB* and *yhjH,* are involved in the motor function of flagella. The gene *yciF*, encoding a putative structural protein, is RpoS-dependent in K12 strains (Weber *et al.*, 2005). There were seven EDL933-specific unknown genes under control of RpoS, two of which, Z3023 and Z3026, encode putative secreted proteins and play a role in colonization of *E. coli* O157:H7 in the bovine GI tract (Dziva *et al.*, 2004). By contrast, the *rpoS* mutation had a much larger impact on gene expression in stationary phase. We thus focused on the analysis of the RpoS regulon in stationary phase.

RpoS-regulated functions in stationary phase

Stress response

As expected, many of the identified RpoS up-regulated genes were those that are important for stress response. For example, the *rpoS* mutation resulted in decreased expression of stress response genes *yhiO* (*uspB*), *yhbO, gadAXW, gadB, gadE, osmY, csiD,* and *katE* that are known be RpoS-dependent in K12 strains (Karp *et al.*, 2007). The genes *gadAXW*, *gadB*, and *gadE* are important for acid resistance (Ma *et al.*, 2003), *osmY* for hyperosmotic resistance (Yim & Villarejo, 1992), *yhiO* (*uspB*) for ethanol tolerance (Farewell *et al.*, 1998b), *katE* for oxidative response (Imlay & Linn, 1987; Sak *et al.*, 1989), and *yhbO* for survival under oxidative, heat, UV, and pH stresses (Abdallah *et al.*,

2007; Weber *et al.*, 2005). Consistently, survival of *rpoS* mutants under low pH, oxidative stress, and heat exposure was severely impaired in comparison with wild type EDL933 strain (Figure 4.3).

Two starvation-induced genes, *csiD* (for carbon) and *psiF* (for phosphate) were also expressed higher in EDL933 wild type than in the *rpoS* mutants. Unlike in K12, the genes that encode universal stress proteins *uspA*, *yecG* (*uspC*), *yiiT* (*uspD*), *ydaA* (*uspE*) showed attenuated expression in *rpoS* mutants (this study) while their expression is not dependent on RpoS in K12 (Farewell *et al.*, 1998a; Gustavsson *et al.*, 2002).

Transporter and Membrane proteins

The expression of many genes for nutrient transport was affected by the *rpoS* mutation (Figure 4.4). Most of these genes encode proteins belonging to the ATP-Binding Cassette (ABC) transporter family. RpoS positively regulated ABC transporter genes included those for transport of oligopeptide (encoded by *oppABCDF*), dipeptide (*dppABDF*), putrescine (*potFGH*), maltose (*malEFGK*), glutamate/aspartate (*gltIJKL*), D-xylose (*xylFHG*) and *sn*-glycerol-3-P (*ugpABCE*). The expression of genes *yehWXYZ*, encoding a predicted ABC transporter, was also highly dependent on RpoS. Transporter genes expressed higher in the *rpoS* mutants included those for spermidine/putrescine (*potABCD*), glycine/proline (*proWXY*), and Zinc (*znuABC*). Besides ABC transporters, the *tnaB* gene encoding a tryptophan transporter and the *dcuB* gene encoding a transporter for C4-dicarboxylates (e.g., fumarate and malate) uptake were expressed at a lower level in the *rpoS* mutants compared with that in wild type EDL933. The gene *cstA,* encoding a peptide transporter that is induced under carbon starvation, has been shown to

be negatively regulated by RpoS in a K12 strain (Dubey *et al.*, 2003), while we found that the expression of *cstA* was attenuated in the *rpoS* mutants of EDL933.

Metabolism

RpoS had a substantial effect on expression of metabolic genes, primarily for utilization of amino acids and carbohydrates (Figure 4.5). LB medium is rich in amino acids that can be utilized by *E. coli* as nutrient sources (Sezonov *et al.*, 2007). We found that the expression of genes for utilization of serine (*tdcB*), proline (*putA*), glutamine (*ybaS*), aspartate (*asnB*), arginine (*astCABDE*), tryptophan (*tnaA*), threonine (*ilvBCDEMG*), and alanine (*dadAX*) was expressed higher in the wild type EDL933 than in the *rpoS* mutants. The genes *yneH* and *alr*, encoding isoenzymes of YbaS and DadX, respectively, were expressed higher in the *rpoS* mutants (Figure 4.5). Pyruvate and glutamate appeared to be two common intermediate metabolites in RpoS-regulated amino acid utilization (Figure 4.5). For carbohydrate utilization, genes whose expression is positively regulated by RpoS included those encoding for putrescine degradation (*puuABCD*), fatty acid beta-oxidation (*fadBA*, *fadD*, *fadE*, and *fadIJ*), fucose utilization (*fucAO, fucIK, lldD,* and *aldA*), glucarate degradation (*garDLR*), glyoxylate cycle (*aceBA*, *acnA*, and *gltA*), and synthesis of trehalose (*otsBA*) and glycogen (*glgABC*) (Figure 4.5). The *cdd* and *udp* genes for pyrimidine degradation were reduced in expression in the *rpoS* mutant, while the expression of genes *udk*, *cmk*, *upp*, and *codA* that are involved in the pyrimidine biosynthesis pathway was enhanced.

Some of these metabolic genes may play an important role in colonization and pathogenesis of *E. coli in vivo* in host environments. For example, the expression of

fucAO is important for colonization of *E. coli* in mouse intestine (Autieri *et al.*, 2007). Mutants defected in metabolism of maltose and glycogen are also impaired in colonization of EDL933 in mouse intestine (Jones *et al.*, 2008).

Transcription Regulation

The expression of 29 genes encoding known transcriptional regulators was affected by the *rpoS* mutation. Sixteen genes (*lsrR, mhpR, prpR, putA, lldR, hcaR, galS, gadXWE, fucR, dgsA, csgD, cdaR, bolA*, and *xylR*) were expressed higher in the wild type EDL933 while 13 genes (*dicA, deoR, birA, uhpA, marR, metJ, pdhR, purR, rcsA, arsR, asnC, cspA,* and *fis*) were expressed higher in the *rpoS* mutants. The observed differential expression of many genes in the *rpoS* mutants may be an indirect effect of RpoS through these intermediate regulators. Some regulatory genes are known to be RpoS-controlled, such as *bloA* (Lange & Hengge-Aronis, 1991a), *gadE* (Ma *et al.*, 2003), and *csgD* (Romling *et al.*, 1998). Expression of the *hcaR* gene, encoding the hydrocinnamic acid regulator, is stationary phase dependent but RpoS-independent in *E. coli* K12 strain (Turlin *et al.*, 2001). Here we found that expression of *hcaR* was induced in stationary phase in both wild type EDL933 and *rpoS* mutants. However, the induction level was significantly higher in wild type, indicating that RpoS is important for full expression of *hcaR*.

Virulence and O-island genes

We found that 10% of the identified RpoS-regulated genes are located on O-islands. Among them, 50 genes were expressed higher in wild type EDL933 in stationary phase (Table 4.4) while 49 genes expressed higher in the *rpoS* mutants (Table 4.5). The

functions of most of these genes are still unknown. On the LEE island (located on the O-island 148), three genes, *ler*, *cesF* and Z5139, were expressed significantly higher in wild type EDL933 than in the *rpoS* mutants (Table 4.4), while the *eae* gene, encoding the outer membrane intimin protein essential for colonization and virulence, was expressed twofold higher in *rpoS* mutants (Table 4.5). The expression of other genes on the LEE islands was not significantly affected by RpoS. The *espI* gene, though not located on the LEE island, encodes a secreted protein whose secretion requires the LEE-encoded type III secretion system (Mundy *et al.*, 2004). The expression of *espI* was 78 fold higher in the wild type EDL933. The *nlpA* gene, encoding an inner membrane protein that is required for virulence in *Haemophilus influenzae* (Chanyangam *et al.*, 1991), was impaired in its expression in the *rpoS* mutants. The *dppA* operon, required for colonization by uropathogenic *E. coli* (Haugen *et al.*, 2007), was expressed much higher in the wild type EDL933 than *rpoS* mutants.

Western blot analysis of LEE proteins under LEE-induction conditions

Growth condition plays a considerable effect on LEE gene expression (Abe *et al.*, 2002; Kenny *et al.*, 1997). The expression of LEE genes is low in LB media and is induced in LB supplemented with sodium bicarbonate or DMEM media in 5% CO_2 (Abe *et al.*, 2002; Kenny *et al.*, 1997). To determine whether the expression of LEE genes was controlled by RpoS under these LEE-induction conditions, we examined the expression of one gene from each of the five LEE islands by qPCR using cultures grown in LB supplemented with 44mM sodium bicarbonate media (Abe *et al.*, 2002). All genes tested were expressed higher in the *rpoS* mutants. The ratio of expression in *rpoS* mutants verse

wild type EDL933 for *ler* (LEE1), *sepZ* (LEE2), *escV* (LEE3), *tir* (LEE4), *sepL* (LEE5), *grlR* and *grlA* (LEE regulator) was 2.8 ± 0.5, 1.3 ± 0.4, 5.5 ± 0.4, 4.8 ± 0.4, 6.4 ± 0.4, 4.7 ± 0.4, and 7.6 ± 0.4, respectively. Western blot analysis revealed that the expression of Tir and EspA was enhanced in the *rpoS* mutants of EDL933 (Figure 4.6). Similar results were obtained in cultures grown in DMEM media, another LEE induction condition (Figure 4.6). Consistent with previous results, neither Tir nor EspA could be detected in LB without sodium bicarbonate (data not shown).

Negative regulation by RpoS

As mentioned above, we found 536 genes expressed higher in *rpoS* mutants in stationary phase (Table 4.3). These genes are involved in many cellular functions, including metabolism (e.g., *thiI* and *guaBA*), nutrient transport (e.g., *ampG*, *cmr* and *uraA*), and DNA modification (e.g., *endA* and *nth*). The expression of almost all genes in the purine biosynthesis pathway was enhanced in the *rpoS* mutant (Figure 4.7). The *rsxABCDGE* operon that is required for the reduction of SoxR was also expressed higher in the *rpoS* mutants (Table 4.3). Interestingly, the flagellar genes and the TCA cycle genes, whose expression is negatively regulated by RpoS in *E. coli* K12 strains (Patten *et al.*, 2004), were not differentially expressed in the *rpoS* mutant of EDL933. The flagellar sigma factor FliA, was expressed similarly in wild type EDL933 and *rpoS* mutants (Figure 4.8).

Verification of microarray results

To validate the microarray results, we determined the expression level and RpoS dependence of candidate genes by qPCR (Figure 4.9). The RpoS-dependence levels of all

12 genes tested were in good correlation between results of microarray and qPCR. Because the *rpoS* sequence is absent in the *rpoS* null mutant tested in this study, the signal difference for *rpoS* between wild type EDL933 and *rpoS* mutant strains serves as an internal control for the sensitivity of microarray data. We found the expression difference of the two *rpoS* probe sets was about 5,000 fold between wild type and *rpoS* mutants. As expected, we also found many known RpoS-regulated genes (e.g., *osmY*, *katE* and *astC*) were identified as RpoS-controlled genes in this study.

4.6 Discussion

In this study, we have characterized the RpoS regulon of the important pathogenic *E. coli* O157:H7 strain EDL933. Comparison with previous data obtained using laboratory K12 strains reveals substantial differences between the composition of RpoS regulon in K12 and O157:H7 EDL933. As might be expected, the RpoS-regulon identified in EDL933 is much larger than that of K12, which is partly attributable to the larger number of genes present in the pathogenic strain. Another factor may be different levels of the expression of RpoS itself. Indeed, we found that the level of RpoS was higher in EDL933 than in MG1655 in early stationary phase, consistent with previous results that RpoS levels vary among *E. coli* isolates (King *et al.*, 2004). Though there is a core set of genes regulated by RpoS in both K12 and EDL933 strains, the RpoS-dependence of a large number of genes (~80% of RpoS-dependent genes in EDL933) is strain-specific, including a group of RpoS-dependent genes on O-islands and several virulence determinant genes. RpoS has a larger effect on exponential phase gene expression in K12 strain than in EDL933 (Dong *et al.*, 2008b; Rahman *et al.*, 2006).

These results suggest that RpoS regulation may be strongly dependent on strain background. Consistent with this, there are many known phenotypic differences between K12 and EDL933. For example, MG1655 and EDL933 differ in utilization of nutrients and location of colonization during *in vivo* growth in mouse intestine (Fabich *et al.*, 2008; Jones *et al.*, 2008; Miranda *et al.*, 2004).

The expression of a large number of genes was higher in the *rpoS* mutants, indicating negative control of RpoS on gene expression. As a sigma factor, negative control exerted by RpoS is likely an indirect effect, probably resulting from sigma factor competition (Farewell *et al.*, 1998a). Because the number of sigma factors exceeds that of core RNA polymerase, different sigma factors compete for binding to the core enzyme (Ishihama, 2000). Deletion of RpoS, a major sigma factor in stationary phase, may thus result in increased amount of core enzyme associated with other sigma factors and their-directed gene expression. In *E. coli* K12 strain, there is also a large number of genes negatively regulated by RpoS (Patten *et al.*, 2004). For example, expression of genes for chemotaxis and flagella is negatively regulated by RpoS in K12 (Dong & Schellhorn, 2009a; Patten *et al.*, 2004). However, this was not the case in EDL933 (this study), suggesting the negative regulation of RpoS was also strain-specific. In other pathogens, the effect of RpoS on flagella expression is variable (Table 4.6) (Adams *et al.*, 2001; Cogan *et al.*, 2004; Dong & Schellhorn, 2009a; Hammer *et al.*, 2002; Hulsmann *et al.*, 2003; Ito *et al.*, 2008; Kutsukake, 1997; Makinoshima *et al.*, 2003; Nielsen *et al.*, 2006; Patten *et al.*, 2004; Schuster *et al.*, 2004). In *P. aeruginosa*, expression of the flagellar gene *fliF* as well as genes for chemotaxis is positively regulated by RpoS (Schuster *et al.*,

2004). In *Vibrio cholerae,* RpoS positively controls the expression of chemotaxis and flagellar genes during pathogenesis (Nielsen *et al.*, 2006). In *Legionella pneumophila* and *S. typhimurium*, RpoS is important for expression of flagella (Cogan *et al.*, 2004; Hammer *et al.*, 2002). However, flagella gene expression is independent of RpoS in *S. typhimurium* strain LT2 (Kutsukake, 1997), which has a mutant allele of RpoS (Swords *et al.*, 1997).

The intestinal growth environment inhabited by EHEC *E. coli* is complex. Utilization of glycogen (Jones *et al.*, 2008), maltose (Jones *et al.*, 2008), L-fucose (Autieri *et al.*, 2007), galactose (Fabich *et al.*, 2008), arabinose (Fabich *et al.*, 2008), and ribose (Fabich *et al.*, 2008) is important for colonization by *E. coli.* We found that an *rpoS* mutation attenuates the expression of genes involved in metabolism of these sugars (Figure 4.5), suggesting a role of RpoS in regulation of bacterial colonization. This is consistent with our previous findings in an animal model that wild type *C. rodentium* colonizes mouse colon better than *rpoS* mutants (Dong *et al.*, 2009b). The contribution of RpoS-regulated metabolism to *in vivo* colonization needs to be further evaluated through construction of mutations in relevant pathways to identify specific causal factors.

The expression of most genes on the LEE island is under control of Ler, a LEE-encoded regulator (Kaper *et al.*, 2004; Mellies *et al.*, 1999), and thus LEE genes is expected to be expressed similarly. However, previous results have shown that this is not the case (Bergholz *et al.*, 2007; Kendall *et al.*, 2007). Consistent with this, our results show that RpoS had an opposing effect on LEE gene expression, suggesting that LEE genes are under differential control for expression. The difference in expression of LEE

genes may be due to the lack of induction signals for LEE expression in LB. Under induction conditions, all LEE genes tested were expressed higher in the *rpoS* mutants (this study).

A recent microarray study reviewed differences in the heat shock response of *E. coli* O157:H7 EDL933 and K12 strains, and attributed discrepancies to experimental conditions and/or genomic compositions (Carruthers & Minion, 2009). About 30 EDL933 specific genes are differentially expressed during heat shock (Carruthers & Minion, 2009). Only four of the top 25 heat shock response genes were RpoS-dependent (this study), suggesting that other regulators (e.g., the heat shock sigma factor RpoH) are required for the full heat shock response. Again, differences in methodology (e.g., array platforms and experimental conditions) make it difficult to directly compare results.

Gene expression profiling has greatly improved our knowledge of the role of RpoS in regulation of genes and many cellular functions. However, we are still far from fully understanding the physiological role of RpoS. For example, a large portion of RpoS-regulated genes are those with unknown or putative functions. Factors responsible for strain-specific effects also remain elusive. Furthermore, the regulation of RpoS itself is not fully understood. Recent studies have identified two anti-adaptor proteins, IraM (previously known as YcgW) (Bougdour *et al.*, 2008) and IraD (YjiD) (Merrikh *et al.*, 2009), which stabilize RpoS through inhibition of RssB-ClpXP directed proteolysis. RpoS activity has also been found to be transiently inhibited by FliZ in post exponential phase (Pesavento *et al.*, 2008). It is likely that there are other unidentified factors involved in the regulatory network of RpoS.

In conclusion, our results reveal the first snapshot overview of RpoS-regulated transcriptome expression in non-K12 strains. This, together with previous results regarding RpoS control in laboratory strains, provides a useful database for understanding how global regulators (e.g., RpoS) can gain addition functions in pathogenic *E. coli* strains.

Table 4.1 RpoS-dependent genes in exponential phase (MER ≥ 2, P < 0.05).

Gene	RpoS-dependence (MER)	Function	Major regulator
*motAB**	5/6	Flagellar motor complex	RpoF CpxR
yciF	6	Putative structural protein	H-NS
yhjH	8	Protein involved in flagellar function	RpoF FlhDC
Z1344	2	Putative endonuclease	
Z2774	3	Unknown	
Z3023	2	Putative secreted protein	
Z3024	4	Unknown	
Z3026	2	Putative secreted protein	
Z3672	4	Unknown	
Z4850	2	Putative O-methyltransferase	

* Indicates that some genes in the known operon are not listed because these genes did not satisfy the criteria to be RpoS-dependent.

Table 4.2 Top 100 most RpoS-dependent genes in stationary phase.

Gene	RpoS-dependence (MER)	Function	Major regulator
*abgABT**	24/41/26	Aminoacyl aminohydrolase family proteins/transporter	AbgR
aceBA	164/422	Glyoxylate cycle	IclR FruR IHF CRP ArcA
acs-yjcH-actP	541/357/163	Acetyl-CoA synthetase/ Unknown/Acetate permease	Fis IHF CRP
aidB	79	Isovaleryl CoA dehydrogenase	RpoS Ada Lrp
puuCB	576/214	Putrescine degradation II	
astCADBE	3492/1270/2402 /512/388	Arginine degradation	RpoS RpoN ArgR NtrC
blc	568	Outer membrane lipoprotein	RpoS
*csiD-ygaF-gabD**	357/67/44	Carbon starvation-induced gene/L-2-hydroxyglutarate oxidase/ succinate semialdehyde dehydrogenase	RpoS CRP HNS CsiR Lrp
csiE	792	Stationary phase inducible protein	RpoS CRP HNS
cstA	46	Peptide transporter	CRP
ddpXA	39/31	D-ala-D-ala dipeptidase/transporter	RpoN NtrC
*dppABDF**	74/64/148/122	Dipeptide ABC transporter	FNR IHF PhoB
ecnB	67	Entericidin B	RpoS
espI	78	Virulence protein	
fadBA	26/125	Fatty acid β-oxidation I	Fis ArcA FadR
fadE	74	Fatty acid β-oxidation I	FadR ArcA
fadH	64	2,4-dienoyl-CoA reductase	
*fadI**	77	Fatty acid β-oxidation I	FadR ArcA
fucAO	32/123	Fucose catabolic process	FucR CRP
gadAXW	66/46/2	Glutamate dependent acid resistance	RpoS Fis FNR GadEXW CRP H-NS TorR
galS	140	GalS transcriptional dual regulator	GalS GalR CRP
garD	41	Galactarate dehydratase	CdaR
*garPLR**	40/56/21	Degradation of D-glucarate and D-galactarate	H-NS FNR CadR
hcaR	46	Transcriptional activator of hca cluster	HcaR ArcA
katE	416	Catalase HPII	RpoS Fis

Gene	RpoS-dependence (MER)	Function	Major regulator
*lsrABF**	46/118/124	Putative ABC transporter	RpoS CRP LsrR
lsrR	46	LsrR transcriptional repressor	CRP LsrR
malKLM	40/5/6	Maltose transport	RpoS MalT CRP
*msyB**	40	Acidic protein	RpoS
osmY	27	Osmotically inducible protein	RpoS IHF CRP Fis
otsBA	211/220	Trehalose biosynthesis I	RpoS
phnB	56	Unknown	
*potFGH**	52/18/4	Putrescine ABC transporter	RpoN NtrC
poxB	787	Pyruvate oxidase	
prpR	416	DNA-binding transcriptional activator	PrpR RpoN CRP
psiF	73	Phosphate starvation-induced protein	
puuA	393	Putrescine degradation II	
*sufABCDS**	124/88/71/43/25	Fe-S cluster assembly	OxyR IHF IscR Fur RpoS
talA	67	Transaldolase A	RpoS
tam	86	Trans-aconitate methyltransferase	RpoS
tdcBCD	41/5/5	Threonine degradation I	
tktB	168	Transketolase II	RpoS
tnaLAB	443/189/750	Tryptophan catabolism	RpoS CRP TorR
treF	45	Cytoplasmic trehalase	
ugpBAECQ	161/129/46/184/4	Glycerol-3-P ABC transporter	PhoB CRP
xylFGHR	265/7/10/5	Xylose ABC transporter	RpoS Fis CRP XylR
yahO	241	Unknown	RpoS
ybaST	19/70	Glutaminase/ABC transporter	GadX RpoS
ybgS	82	Unknown	RpoS
ybhPO	251/7	Predicted DNase/cardiolipin synthase	RpoS
ycaC	653	Predicted hydrolase	BaeR Fnr RpoS
ycaP	66	Unknown	
ycgB	478	Unknown	RpoS
yciGFE	205/405/38	Unknown	RpoS HNS
ydbC	100	Predicted oxidoreductase	
*ydcST**	125/22	Putative ABC transporter	RpoS
yeaGH	771/458	Protein kinase/Unknown	RpoS RpoN NtrC
yeaT	106	Unknown	

Gene	RpoS-dependence (MER)	Function	Major regulator
yeaX	48	Predicted oxidoreductase	
yebV	72	Unknown	
yedI	60	Unknown	
yedK	43	Unknown	
yedK	43	Unknown	
yegP	185	Unknown	RpoS
yegS	112	Lipid kinase	
*yehZYX**	787/95/60	ABC transporter	RpoS RpoH
yeiCN	64/31	Unknown	
yfcG	187	Glutathione S-transferase	
ygaM	155	Stress-induced protein	RpoS
ygdI	90	Unknown	
ygeV	55	Putative transcriptional regulator	
yghA	326	Unknown	
yhbO	231	Stress response protein	RpoS
yhcO	214	Unknown	RpoS
yhfG-fic	133/111	Unknown /Stationary phase protein	RpoS
yhjD	41	Unknown	
yhjY	55	Putative lipase	
yiaG	449	Predicted transcriptional regulator	RpoS
yjfN	43	Unknown	
yjgB	55	Putative oxidoreductase	
yjjM	70	Predicted transcriptional regulator	
ykgC	127	Predicted oxidoreductase	
yncB	57	Predicted oxidoreductase	
yniA	63	Unknown	
yodD	290	Unknown	
yphA	135	Inner membrane protein	
ytfQRT-yjfF	879/76/36/34	Putative ABC transporter	
Z0608	55	Putative outer membrane protein	
Z1504	93	Unknown	
Z1629	117	Unknown	
Z1923	64	Prophage CP-933X protein	
Z1924	137	Prophage CP-933X protein	
Z2296	57	Unknown	

Gene	RpoS-dependence (MER)	Function	Major regulator
Z2297	254	Unknown	
Z2298	55	Unknown	
Z3624	64	D-fructokinase	
Z3625	139	Sucrose hydrolase	
Z4874	60	Unknown	
Z5000	48	Putative regulatory protein	
Z5352	125	Unknown	

* Indicates that some genes in the known operon are not listed because these genes did not satisfy the criteria to be RpoS-dependent.

Table 4.3 Top 50 RpoS-negatively regulated genes in stationary phase.

MER: mean expression ratio (*rpoS*/WT).

Gene	MER	Function	Major regulator
ampG	-13	Muropeptide Major facilitator superfamily (MFS) transporter	
ansP	-12	L-asparagine permease	
*ccmBC**	-8/-24	Protoheme IX ABC transporter	
cmr	-9	MFS multidrug transporter	
codBA	-26/-5	Cytosine transporter/deaminase	Nac PurR
dusC	-13	tRNA dihydrouridine synthase	
emrAB	-4/-11	EmrAB-TolC multidrug efflux	MprA
endA	-9	DNA-specific endonuclease I	
guaBA	-16/-6	Purine nucleotides *de novo* biosynthesis I	Fis CRP PurR DnaA
lpxT	-14	Und-PP pyrophosphatase	
mscK	-9	Mechanosensitive (MS) channel	
napFD	-13/-4	Ferredoxin-type protein/chaperone for NapA	NarL NarP FNR FlhDC ModE
ndh	-12	NADH dehydrogenase II	Fis FNR ArcA PdhR IHF
pdhR	-10	Pyruvate dehydrogenase regulator	CRP FNR PdhR
proVWX	-10/-6/-2	Proline ABC transporter	H-NS
purEK	-22/-18	Purine nucleotides de novo biosynthesis I	PurR
purT	-27	Purine nucleotides *de novo* biosynthesis I	
pyrD	-21	Dihydroorotate oxidase	PurR Fis
pyrL	-39	Pyr operon leader peptide	
rarD	-9	Putative permease	
rhlE	-18	ATP-dependent RNA helicase	
rsxABCDGE-nth	-10/-4/-7/-13/-26/-7/-16	SoxR reducing system/ endonuclease III	
speC	-10	Putrescine biosynthesis III	CRP
thiI	-12	Thiamine biosynthesis	
tyrP	-15	Tyrosine transporter	TyrR
uhpABC	-5/-9/-18	Uptake of hexose phosphates	
uraA	-13	Uracil transport	
xseA	-10	Exonuclease VII	CRP
yaaH	-11	Inner membrane protein	

Gene	MER	Function	Major regulator
yccFS	-36/-27	Inner membrane protein	
ychM	-27	Unknown function	
ydeA	-35	MFS transporter	
ydeP	-12	Acid resistance protein	EvgA
yegD	-14	Actin family protein	
ygiR	-12	Unknown function	
yhfC	-40	MFS transporter	ArcA
yhhQ	-15	Unknown function	
yhjV	-14	Putative transporter protein	
yieG	-17	Putative transporter protein	
yliG	-14	Unknown function	
ynjE	-22	Putative sulfur transferase	
yoaG	-28	Unknown function	
yobD	-28	Unknown function	
Z2059	-11	Prophage CP-933O protein	
Z2274	-20	Unknown function	
Z2389	-9	Prophage CP-933R protein	
Z2605	-20	Putative arginine/ornithine antiporter	
Z2751	-15	Unknown function	
Z3622	-9	Putative resolvase	
Z4223	-13	Unknown function	

* Indicates that some genes in the known operon are not listed because these genes did not satisfy the criteria to be RpoS-dependent.
- Indicates negative regulation.

Table 4.4 RpoS-dependent EDL933-specific O-island genes (MER ≥ 2, P < 0.05).

These are not present in *E. coli* K12 MG1655. MER: mean expression ratio (WT/*rpoS*).

Gene	Expression (log2)		MER	Position	Function
	WT	*rpoS*			
Z0321	12.4 ± 0.0	10.0 ± 0.3	6	O-Island 8	Putative regulator (prophage CP-933H)
Z0443	10.0 ± 0.1	6.7 ± 0.1	10	O-Island 19	Unknown
Z0463	7.2 ± 0.8	2.2 ± 0.0	32	O-Island 20	Putative response regulator
Z0608	10.8 ± 0.4	5.0 ± 1.0	55	O-Island 28	Putative outer membrane export protein
Z0609	6.5 ± 0.6	2.2 ± 0.0	20	O-Island 28	Unknown
Z0701	5.6 ± 0.3	3.7 ± 0.3	4	O-Island 30	Unknown
Z0702	10.4 ± 0.2	9.2 ± 0.1	2	O-Island 30	Unknown (Rhs Element Associated)
Z0957	12.0 ± 0.1	10.6 ± 0.2	3	O-Island 36	Unknown (prophage CP-933K)
Z0958	11.8 ± 0.4	10.0 ± 0.1	3	O-Island 36	Unknown (prophage CP-933K)
Z0984	5.7 ± 0.2	4.2 ± 0.2	3	O-Island 36	Unknown (prophage CP-933K)
Z1129	9.1 ± 0.2	7.9 ± 0.3	2	O-Island 43	Putative enzyme
Z1185	11.5 ± 0.2	10.3 ± 0.2	2	O-Island 43	Unknown
Z1190	12.2 ± 0.7	7.9 ± 0.2	20	O-Island 43	Putative enzyme
Z1193	10.2 ± 0.8	6.3 ± 0.8	15	O-Island 43	Unknown
Z1385	11.8 ± 0.1	10.5 ± 0.3	2	O-Island 44	Unknown (cryptic prophage CP-933M)
Z1386	7.1 ± 0.3	5.8 ± 0.2	2	O-Island 44	Unknown (cryptic prophage CP-933M)
Z1528	6.5 ± 0.3	3.3 ± 0.7	9	O-Island 47	Unknown
Z1629	12.2 ± 0.8	5.3 ± 0.3	117	O-Island 48	Putative enzyme
Z1764	9.0 ± 0.2	7.3 ± 0.2	3	O-Island 50	Putative enzyme (prophage CP-933N)
Z1922	9.9 ± 0.8	4.8 ± 0.2	35	O-Island 52	Unknown (prophage CP-933X)
Z1923	8.9 ± 1.0	2.9 ± 0.1	64	O-Island 52	Unknown (prophage CP-933X)
Z1924	11.1 ± 0.9	4.0 ± 0.2	137	O-Island 52	Unknown (prophage CP-933X)
Z2048	4.1 ± 0.2	2.3 ± 0.1	3	O-Island 57	Unknown (prophage CP-933O)
Z2057	5.9 ± 0.2	4.3 ± 0.4	3	O-Island 57	Putative enzyme (prophage CP-933O)
Z2124	6.0 ± 0.2	5.0 ± 0.1	2	O-Island 57	Unknown (prophage CP-933O)

Gene	Expression (log2)		MER	Position	Function
	WT	*rpoS*			
Z2149	13.4 ± 0.4	10.1 ± 0.3	10	O-Island 57	Unknown (Phage or Prophage Related)
Z2150	10.4 ± 0.6	5.3 ± 0.4	33	O-Island 57	Unknown (Phage or Prophage Related)
Z2151	11.6 ± 0.4	8.6 ± 0.1	8	O-Island 57	Unknown (Phage or Prophage Related)
Z2164	6.8 ± 0.1	4.3 ± 0.6	6	O-Island 59	Putative regulator
Z2254	6.9 ± 0.2	4.7 ± 0.6	5	O-Island 64	Unknown (Rhs Element Associated)
Z2994	8.9 ± 0.2	6.8 ± 0.1	4	O-Island 76	Unknown (prophage CP-933T)
Z3391	9.9 ± 0.5	7.1 ± 0.4	7	O-Island 95	Putative enzyme
Z3392	8.4 ± 0.4	5.0 ± 0.2	11	O-Island 95	Putative enzyme
Z3393	7.4 ± 0.3	2.2 ± 0.0	36	O-Island 95	Putative enzyme
Z3394	6.0 ± 0.1	2.3 ± 0.0	13	O-Island 95	Putative transporter
Z3623	9.4 ± 0.3	4.8 ± 0.1	24	O-Island 102	Sucrose permease
Z3624	8.5 ± 0.2	2.5 ± 0.0	64	O-Island 102	D-fructokinase
Z3625	9.4 ± 0.1	2.2 ± 0.0	139	O-Island 102	Sucrose hydrolase
Z3947	8.3 ± 0.4	4.0 ± 0.5	19	O-Island 108	Unknown (Phage or Prophage Related)
Z4488	7.8 ± 0.2	5.6 ± 0.4	4	O-Island 126	Putative enzyme
Z4803	6.4 ± 0.9	2.4 ± 0.1	17	O-Island 134	Putative enzyme
Z5114	7.4 ± 0.3	4.9 ± 0.4	6	O-Island 148	LEE-encoded virulence protein CesF
Z5139	14.0 ± 0.4	12.0 ± 0.5	4	O-Island 148	LEE-encoded virulence protein
Z5140	14.2 ± 0.3	12.6 ± 0.3	3	O-Island 148	LEE-encoded regulator Ler
Z5199	9.7 ± 0.3	6.6 ± 0.5	8	O-Island 152	Unknown
Z5200	9.0 ± 0.7	3.3 ± 0.2	53	O-Island 152	Unknown
Z5619	7.3 ± 0.3	6.0 ± 0.3	3	O-Island 166	Putative regulator
Z5684	7.3 ± 0.1	3.4 ± 0.5	15	O-Island 167	Putative regulator
Z5887	8.3 ± 0.1	6.2 ± 0.3	4	O-Island 172	Unknown
Z6024	9.3 ± 0.3	3.0 ± 0.1	78	O-Island 71	EspI, essential for virulence

Table 4.5 RpoS negatively regulated genes on the O-islands (P < 0.05).

MER: mean expression ratio (*rpoS*/WT).

Gene	Expression (log2)		MER	Position	Function
	WT	*rpoS*			
Z0264	7.8 ± 0.1	9.0 ± 0.0	-2	O-Island 7	Unknown
Z0372	11.4 ± 0.3	12.6 ± 0.2	-2	O-Island 11	Unknown
Z0397	5.1 ± 0.3	6.2 ± 0.1	-2	O-Island 14	Unknown
Z0955	9.7 ± 0.3	11.5 ± 0.0	-4	O-Island 36	Unknown (prophage CP-933K)
Z1146	11.7 ± 0.3	12.7 ± 0.3	-2	O-Island 43	Putative urease accessory protein E
Z1144	11.3 ± 0.2	12.4 ± 0.2	-2	O-Island 43	Putative urease structural subunit B
Z1142	10.9 ± 0.3	12.1 ± 0.2	-2	O-Island 43	Putative urease accessory protein D
Z1164	12.1 ± 0.1	13.4 ± 0.0	-2	O-Island 43	Unknown
Z1143	10.9 ± 0.3	12.3 ± 0.2	-3	O-Island 43	Putative urease structural subunit A
Z1160	3.7 ± 0.1	5.5 ± 0.4	-4	O-Island 43	Unknown
Z1163	7.5 ± 0.5	9.4 ± 0.4	-4	O-Island 43	Unknown
Z1346	11.9 ± 0.1	13.0 ± 0.2	-2	O-Island 44	Unknown (cryptic prophage CP-933M)
Z1348	10.8 ± 0.1	11.9 ± 0.2	-2	O-Island 44	Unknown (cryptic prophage CP-933M)
Z1324	4.4 ± 0.1	5.8 ± 0.3	-3	O-Island 44	Putative exoDNaseVIII
Z1347	10.0 ± 0.0	11.5 ± 0.2	-3	O-Island 44	Unknown (cryptic prophage CP-933M)
Z1326	3.4 ± 0.3	5.5 ± 0.3	-4	O-Island 44	Putative inhibitor of cell division
Z1325	4.1 ± 0.4	6.3 ± 0.3	-5	O-Island 44	Unknown (cryptic prophage CP-933M)
Z1456	12.8 ± 0.2	13.8 ± 0.3	-2	O-Island 45	Unknown (bacteriophage BP-933W)
Z1503	8.0 ± 0.5	10.2 ± 0.5	-4	O-Island 45	Unknown (bacteriophage BP-933W)
Z1794	5.6 ± 0.3	6.8 ± 0.3	-2	O-Island 50	Putative holin protein
Z1878	13.0 ± 0.2	14.7 ± 0.1	-3	O-Island 52	Putative Bor protein
Z2146	5.8 ± 0.2	7.0 ± 0.1	-2	O-Island 57	Putative OMP Lom precursor
Z2100	2.4 ± 0.1	3.7 ± 0.2	-2	O-Island 57	Unknown (prophage CP-933O)
Z2045	9.9 ± 0.1	11.4 ± 0.1	-3	O-Island 57	Regulator of DicB
Z2105	8.8 ± 0.2	10.3 ± 0.1	-3	O-Island 57	Unknown (prophage CP-933O)

Gene	Expression (log2)		MER	Position	Function
	WT	*rpoS*			
Z2101	3.8 ± 0.0	5.3 ± 0.3	-3	O-Island 57	Putative endonuclease
Z2103	10.5 ± 0.1	12.0 ± 0.1	-3	O-Island 57	Unknown (prophage CP-933O)
Z2144	5.9 ± 0.2	7.6 ± 0.2	-3	O-Island 57	Putative tail component of CP-933O
Z2059	5.3 ± 0.3	8.7 ± 0.3	-11	O-Island 57	Unknown (prophage CP-933O)
Z2510	5.0 ± 0.4	7.0 ± 0.2	-4	O-Island 70	Putative transcriptional repressor
Z3201	12.0 ± 0.3	13.2 ± 0.2	-2	O-Island 84	O antigen flippase Wzx
Z3361	7.3 ± 0.2	8.3 ± 0.1	-2	O-Island 93	Putative regulatory protein
Z3360	11.8 ± 0.1	13.0 ± 0.2	-2	O-Island 93	Unknown (prophage CP-933V)
Z3322	5.0 ± 0.2	6.3 ± 0.2	-2	O-Island 93	Putative major tail subunit
Z3622	6.9 ± 0.2	10.1 ± 0.7	-9	O-Island 102	Putative resolvase
Z4048	8.4 ± 0.2	10.4 ± 0.1	-4	O-Island 110	Putative regulator
Z4789	3.1 ± 0.2	4.4 ± 0.1	-2	O-Island 133	Unknown
Z4851	7.4 ± 0.0	8.6 ± 0.2	-2	O-Island 138	Unknown
Z4855	9.4 ± 0.2	10.5 ± 0.1	-2	O-Island 138	Unknown
Z4852	8.9 ± 0.2	10.1 ± 0.1	-2	O-Island 138	Putative acyltransferase
Z4857	3.5 ± 0.3	4.9 ± 0.3	-3	O-Island 138	Unknown
Z4854	8.7 ± 0.3	10.2 ± 0.1	-3	O-Island 138	Putative acyl carrier protein
Z4861	3.2 ± 0.5	5.7 ± 0.4	-6	O-Island 138	Unknown
Z4860	6.3 ± 0.3	8.8 ± 0.2	-6	O-Island 138	Unknown
Z5051	10.2 ± 0.3	11.4 ± 0.1	-2	O-Island 145	Putative LPS biosynthesis enzyme
Z5049	11.7 ± 0.3	13.5 ± 0.3	-3	O-Island 145	Putative LPS biosynthesis enzyme
Z5089	3.8 ± 0.2	4.9 ± 0.1	-2	O-Island 148	Putative transposase
Z5110	7.6 ± 0.2	8.9 ± 0.1	-2	O-Island 148	LEE-encoded virulence protein Eae
Z5225	3.6 ± 0.2	4.7 ± 0.2	-2	O-Island 154	Putative major fimbrial subunit

- Indicates negative regulation.

Table 4.6 Effect of RpoS on expression of flagella and chemotaxis genes.

Species	Flagella or Motility	Chemotaxis	Reference
E. coli K12	Down	Down	(Ito *et al.*, 2008; Makinoshima *et al.*, 2003; Patten *et al.*, 2004)
E. coli O157:H7	-[a]	-	This study
Legionella pneumophila	Up	ND[b]	(Hammer *et al.*, 2002)
Pseudomonas aeruginosa	Up	Up	(Schuster *et al.*, 2004)
Salmonella enteritidis	Up	ND	(Cogan *et al.*, 2004)
S. typhimurium LT2	-	ND	(Kutsukake, 1997)
S. typhimurium SL1344	Up	ND	(Adams *et al.*, 2001)
Vibrio cholerae	Up	Up	(Nielsen *et al.*, 2006)
Vibrio vulnificus	UP	ND	(Hulsmann *et al.*, 2003)

[a] Indicates no effect.
[b] Not determined.

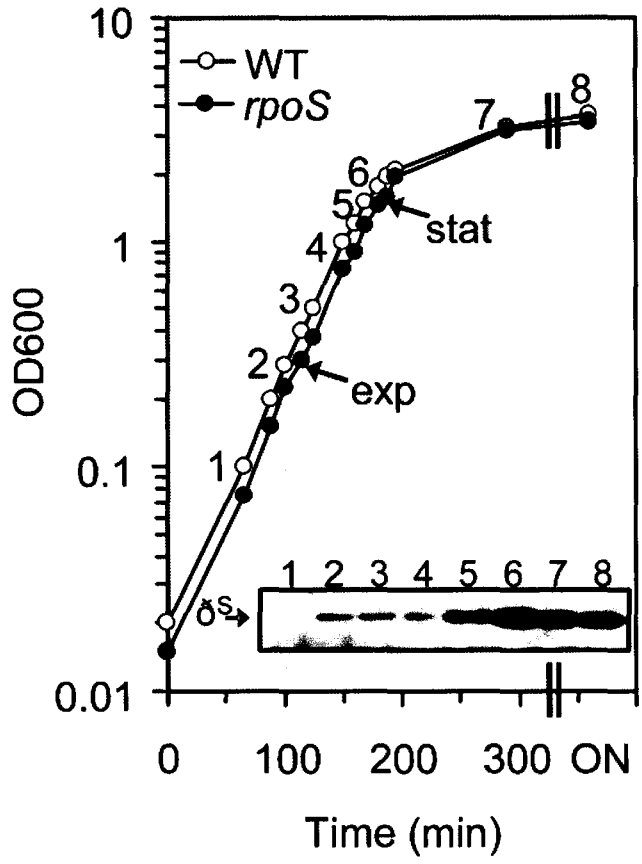

Figure 4.1 Growth of EDL933 in LB media.

Cultures were inoculated from overnight cultures to a starting OD_{600} = 0.0001 and incubated aerobically at 37 °C at 200 rpm. RNA samples were isolated at OD_{600} = 0.3 and 1.5 as indicated. RpoS (δ^S) protein levels were tested by Western blot analyses using monoclonal anti-RpoS antiserum as described in Materials and Methods. This experiment was performed in triplicate using independent isolates. Averaged values were used for construction of the growth curve.

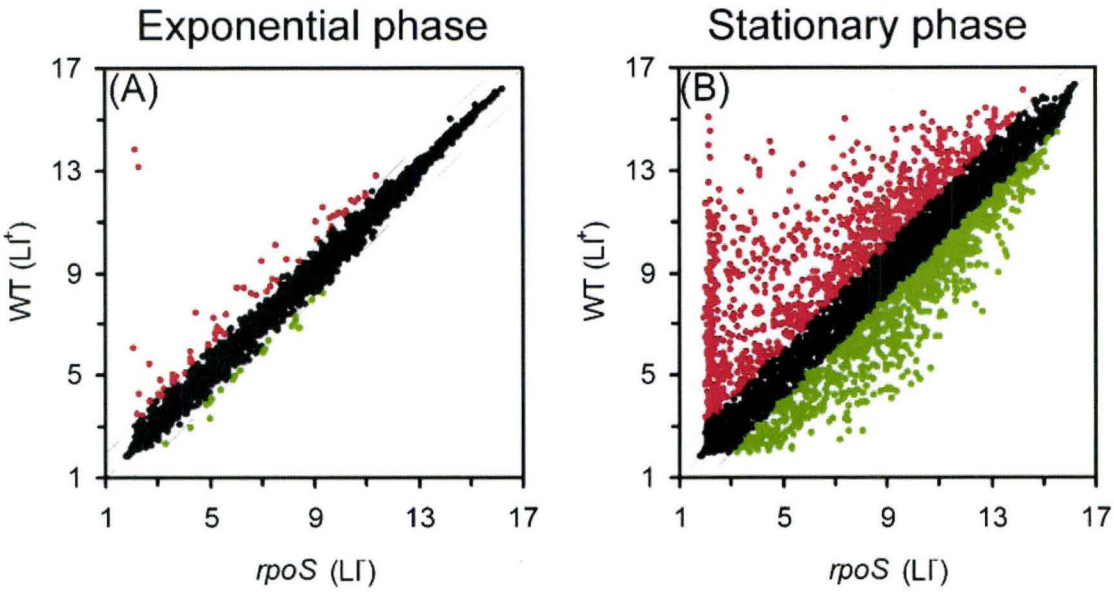

Figure 4.2 Transcriptome profiles of WT EDL933 and *rpoS* mutants.

Scatterplot was used to examine the effects of RpoS on gene expression in exponential (A) and stationary (B) phase. Probe sets (including genes and intergenic regions) are outlined by two parallel lines into three different groups: probe sets expressed at least twofold higher in the WT (red), those expressed more than twofold higher in *rpoS* mutants (green), and those not differentially expressed (black). LI: $\log_2$-transformed expression intensity.

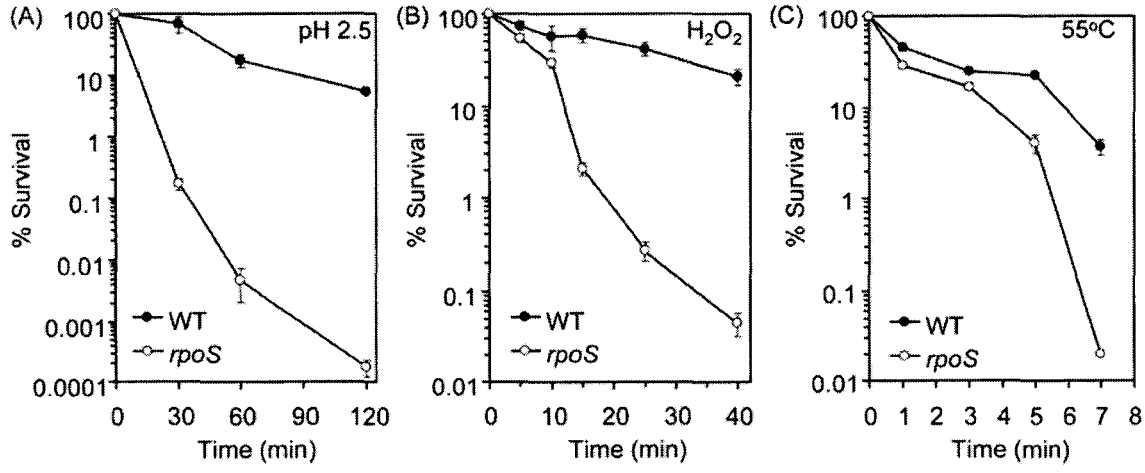

Figure 4.3 Effect of *rpoS* mutation on survival under stress.

Stationary phase cultures were washed and diluted in 0.9% NaCl before exposure to low pH (2.5) (A), H_2O_2 (15mM) (B), and heat (55°C) (C). WT, wild type EDL933; *rpoS*, *rpoS* mutant.

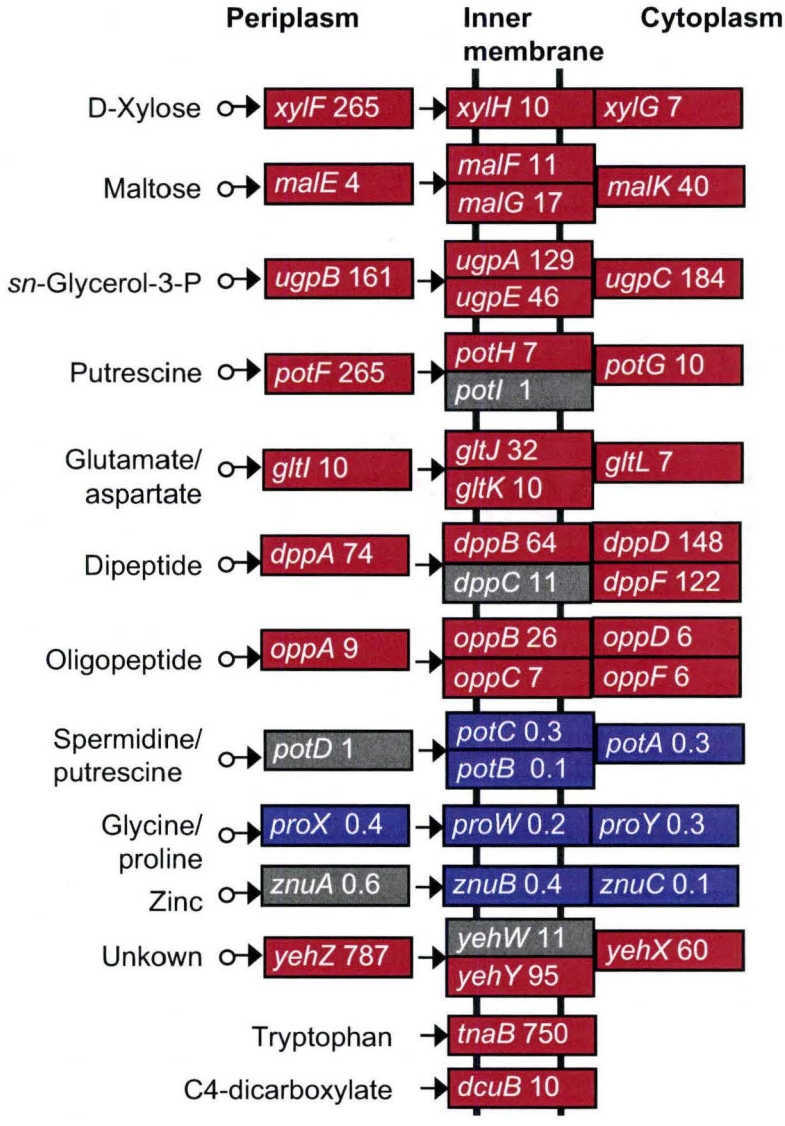

Figure 4.4 Effect of RpoS on expression of transporter genes.

The mean expression ratio (MER/RpoS-dependence level) is given after each gene. Genes highlighted in red were expressed higher in wild type, those in blue were expressed higher in the *rpoS* mutant, and those in grey were not found to be significantly different ($P > 0.05$).

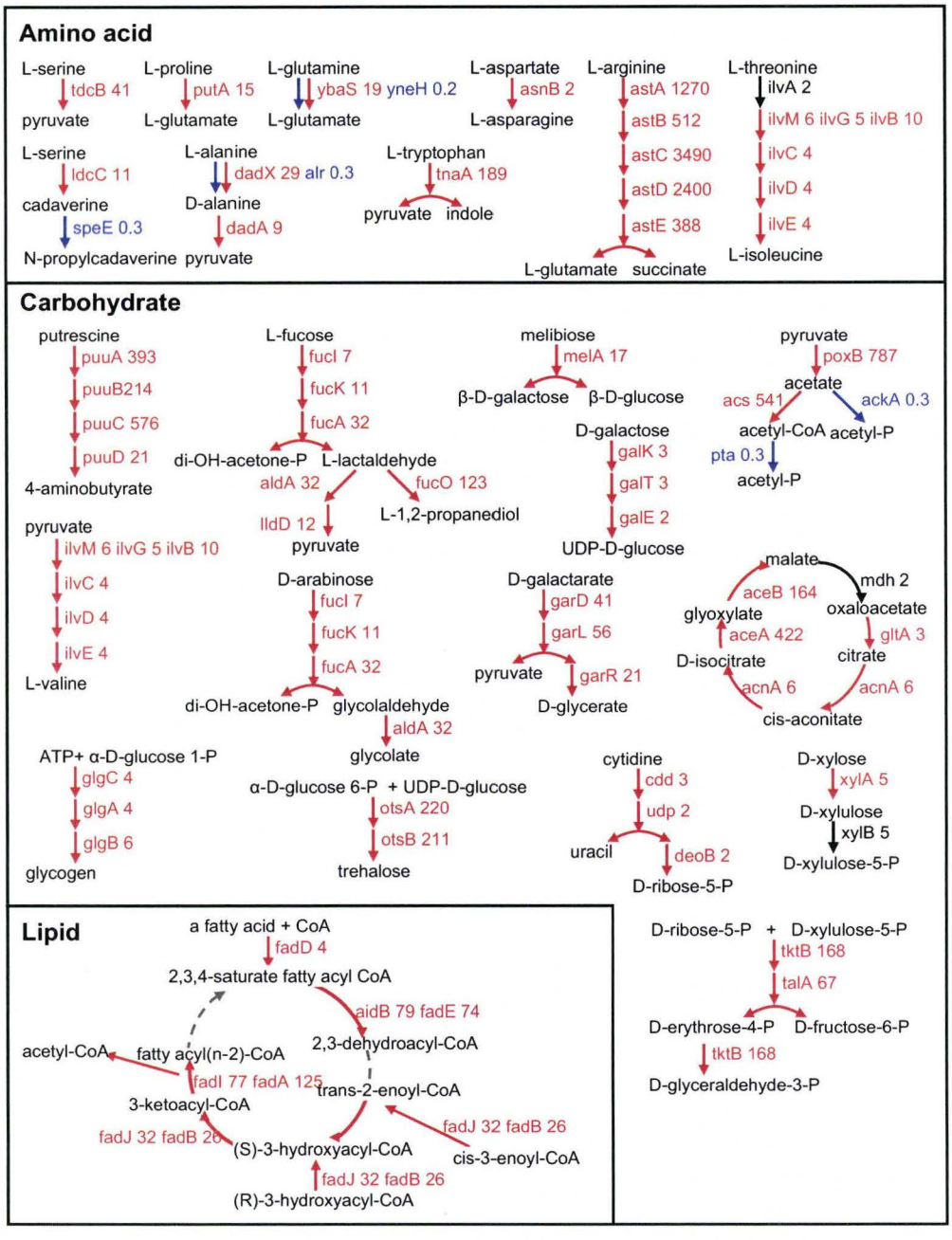

Figure 4.5 Metabolic pathways that are regulated by RpoS in stationary phase.

Genes expressed higher in wild type are colored red and those expressed higher in *rpoS* mutants are blue. Genes whose differential expression was not significant ($P > 0.05$) are in black. The mean expression ratio (MER: WT/*rpoS*) is indicated after each gene.

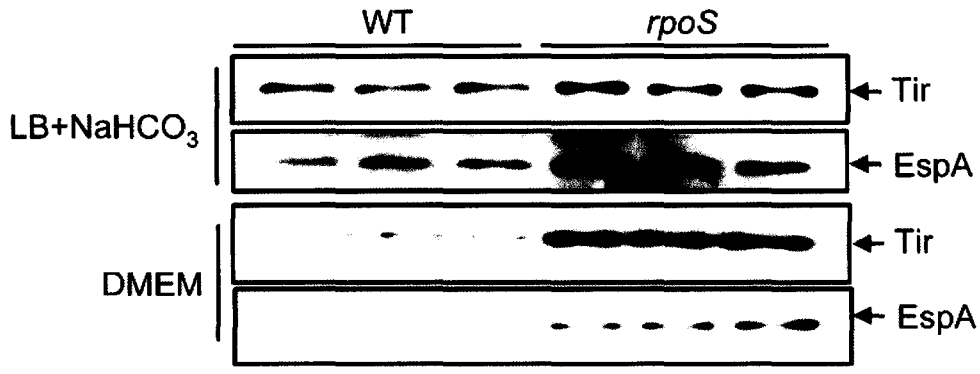

Figure 4.6 Western blot analysis of Tir and EspA expression in wild type and *rpoS* mutants.

Cultures were grown aerobically at 37°C in LB media supplemented with 44mM $NaHCO_3$ to OD_{600} = 1.5 or in DMEM media in 5% CO_2 (two known LEE-induction conditions). Cell pellets were resuspended in SDS loading buffer and boiled for 5 min. Resultant cell extracts were resolved on a 10% SDS-PAGE gel. Proteins were transferred to a PVDF membrane by electrophoresis, followed by incubation of the membrane with anti-Tir or anti-EspA specific antibody. Signals were detected using ECL solution and Hyperfilm-ECL film (Amersham).

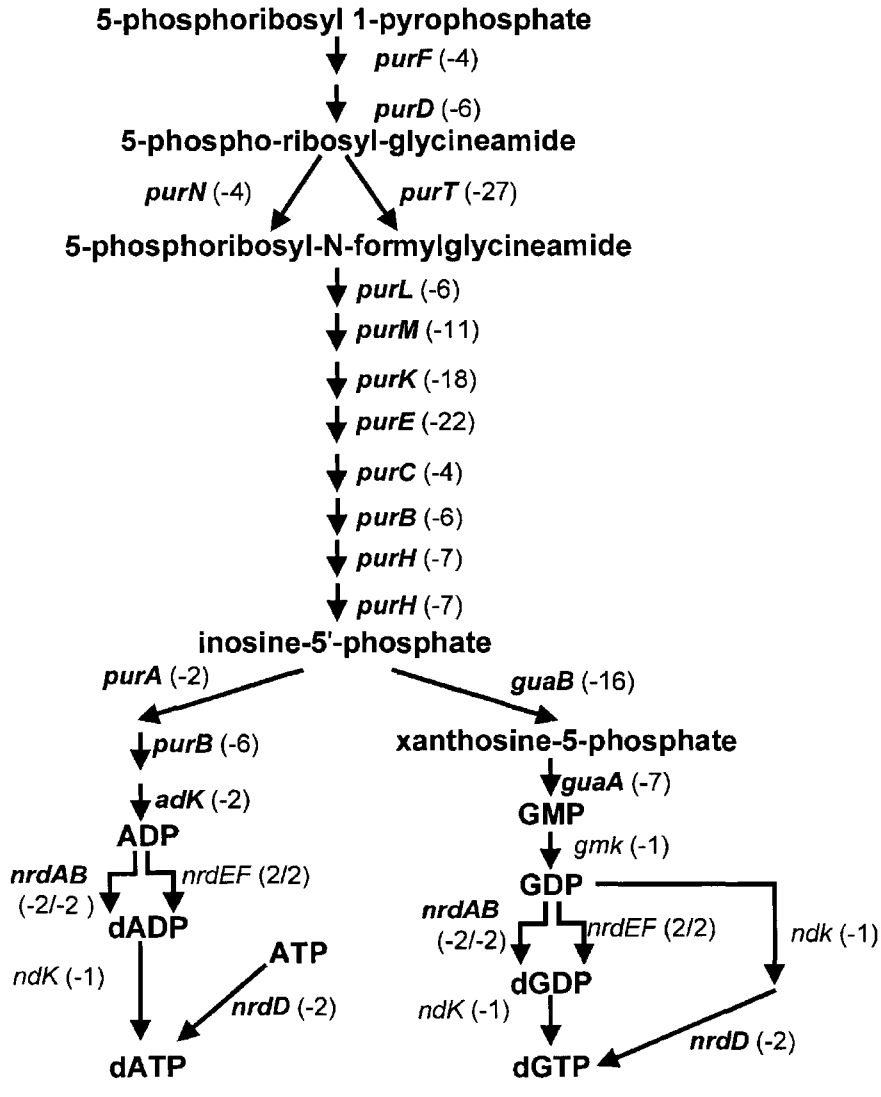

Figure 4.7 RpoS-regulation of genes required for de novo biosynthesis of purine nucleotides pathway I in stationary phase.

RpoS-dependence (MER) is indicated in parentheses. A negative value (-) denotes RpoS-negative regulation. The pathway map is adapted from the EcoCyc database. Genes that were significantly differentially expressed ($P < 0.05$) are highlighted in bold.

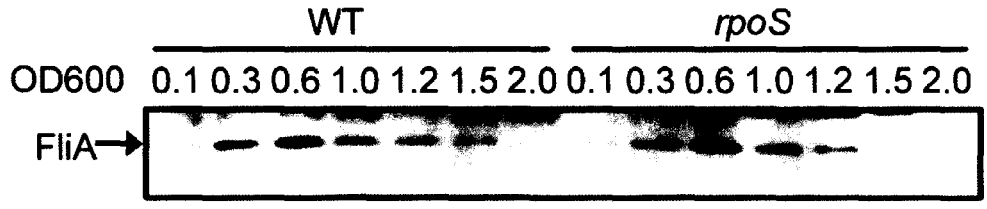

Figure 4.8 Expression of FliA in WT and *rpoS* mutants of EDL933 in LB.

Western blot analyses of the expression of the flagella sigma factor FliA were performed using monoclonal antibody to FliA as described in Material and Methods. To confirm equal protein loading, another protein gel run in parallel was stained by Coomassie blue R250.

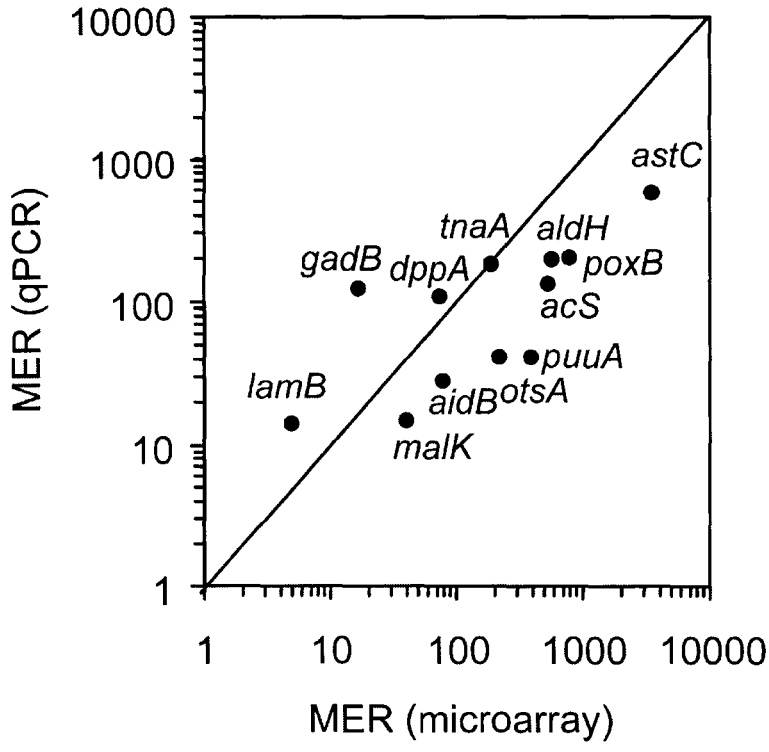

Figure 4.9 Confirmation of microarray data using qPCR.

RpoS dependence is represented by the mean expression ratio (WT/*rpoS*).

CHAPTER V

Role of RpoS in the virulence of *Citrobacter rodentium*

from

Dong, T., Coombes, B.K., and Schellhorn, H.E. 2009. Role of RpoS in the virulence of *Citrobacter rodentium*. Infection and Immunity 77:501-507

5.1 Preface

The contents of this chapter were contributed primarily by the author of this thesis. The text was written, in its entirety, by Tao Dong. This chapter is adapted from a published article in Infection and Immunity to fit the format requirements of the thesis.

5.2 Abstract

Citrobacter rodentium (CR) is a mouse enteropathogen closely related to *Escherichia coli*, that causes severe colonic hyperplasia and bloody diarrhea. CR infection requires the expression of genes on the LEE pathogenicity island and simulates the infection of enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) in human intestine, thus providing an effective model for studying enteropathogenesis. In this study we investigated the role of RpoS, the stationary phase sigma factor, in virulence in CR. Sequence analysis showed that the *rpoS* gene was highly conserved between CR and *E. coli,* sharing 92% identity. RpoS was critical for survival in heat shock and H_2O_2 exposure and positively regulated the expression of catalase KatE (HPII). The development of RDAR (red dry and rough) morphotype, an important virulence trait in *E. coli*, was also mediated by RpoS in CR. Unlike *E. coli*, CR grew well in the mouse colon and the wild type strain colonized significantly better than did the *rpoS* mutants. However, mutation in *rpoS* conferred a competitive growth advantage over the wild type both *in vitro* in LB media and *in vivo* in the mouse colon. Survival analysis shows that the *rpoS* mutant was attenuated in virulence. The expression of genes on the LEE pathogenicity island, which are essential for colonization and virulence, was reduced in the *rpoS* mutant. In conclusion, RpoS is important for stress response and is required for full virulence in CR.

5.3 Introduction

Intestinal disease caused by bacterial infection, such as by *Escherichia coli* O157:H7, is a major threat to public health (Rangel *et al.*, 2005). It is imperative to fully understand how these pathogens transmit, propagate, and cause disease in the host. During transmission, pathogens likely have to survive many stresses including environmental stresses (e.g., nutrient-limitation) and host-internal stresses (e.g., acidic exposure in the stomach and host defense), implicating the importance of stress response systems. One of the most important regulators in stress response is RpoS, an alternative sigma factor of RNA polymerase that exists primarily in gamma-proteobacteria including *E. coli* and *Salmonella* (Dong *et al.*, 2008a; Hengge-Aronis, 2002a).

RpoS is important for cell survival in stress conditions, such as oxidative stress and acid exposure, in many pathogens including *Salmonella* sp. (Fang *et al.*, 1992), *Vibrio cholerae* (Yildiz & Schoolnik, 1998), *Pseudomonas aeruginosa* (Suh *et al.*, 1999), and *Yersinia enterocolitica* (Iriarte *et al.*, 1995). However, RpoS plays distinct roles in pathogenesis of these organisms. RpoS is essential for virulence in *Salmonella* (Fang *et al.*, 1992) and important for the invasion of brain microvascular endothelial cells in *E. coli* K1 strains (Wang & Kim, 2000), but is not required for virulence in *P. aeruginosa* (Suh *et al.*, 1999) and *Y. enterocolitica* (Iriarte *et al.*, 1995). The effect of RpoS on virulence may also differ within a given species. For example, RpoS has been found to be either important (Merrell *et al.*, 2000) or not required (Klose & Mekalanos, 1998; Yildiz & Schoolnik, 1998) for colonization of the mouse intestine by *V. cholerae*. This discrepancy is likely caused by differences in strain backgrounds or animal models.

Although *rpoS* mutants of *E. coli* outcompete wild type cells during competitive colonization in the mouse large intestine (Krogfelt *et al.*, 2000), the role of RpoS in enteropathogenesis of *E. coli* has not been clearly resolved yet, due to the lack of an effective animal model. The human pathogens, enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) strains, do not cause severe disease in mice (Mundy *et al.*, 2005; Mundy *et al.*, 2006; Wiles *et al.*, 2006).

Citrobacter rodentium (CR) is a natural mouse pathogen that causes colonic hyperplasia and diarrhea (Mundy *et al.*, 2005). Similar to EPEC and EHEC strains in human, CR utilizes attaching and effacing (A/E) lesions, induced by genes on the LEE (locus of enterocyte effacement) pathogenicity island, to colonize the large intestine of the host (Mundy *et al.*, 2005). The LEE island carries 41 open reading frames (ORF) that are organized into five polycistronic operons, encoding a type III secretion system and virulence factors essential for virulence (Deng *et al.*, 2004). Expression of LEE genes is controlled by three LEE-encoded global regulators: Ler (the major regulator), Orf11 (GrlA, the Ler activator) and Orf10 (GrlR, the Ler repressor) (Deng *et al.*, 2004).

Compared with the extensive information available regarding RpoS in *E. coli*, little is known in CR. In this study, we examined the role of RpoS in virulence in CR, which may help to understand the physiological function of stress response genes in human intestinal disease caused by enteric bacteria.

5.4 Materials and Methods

Media and growth conditions

Cultures were grown in Luria-Bertani (LB) broth or on LB plates with 1.5% agar. In cell motility assay, 0.3% agar was used on LB plates. Samples isolated from mouse colon were plated on Brilliant Green Agar (BGA) (Oxoid, Nepean, ON), an indicator medium that differentiates CR from *E. coli* and other bacteria. Cell growth was monitored spectrophotometrically at 600nm. When necessary, antibiotics were added at the following concentrations: ampicillin (200 μg/ml) and chloramphenicol (30 μg/ml). To visualize the RDAR (red, dry and rough) morphotype, an indicator for production of extracellular components such as curli and cellulose (Romling *et al.*, 1998; Romling, 2005), cells were streaked out on RDAR plates (non-salt LB media with 1.5% agar, 40 μg/ml of Congo Red dye, and 20 μg/ml of Coomassie Blue R-250) and incubated at 25°C for 48h (Bokranz *et al.*, 2005).

Construction of an *rpoS::cat* mutant in CR

A precise *rpoS* deletion mutant of CR was constructed using the Red recombination method (Datsenko & Wanner, 2000). The *rpoS* sequence was retrieved from the genome sequencing project of CR (http://www.sanger.ac.uk/Projects/C_rodentium/). The *rpoS* gene was replaced by homologous recombination with the chloramphenicol-resistance gene *cat*, which was amplified using the pKD3 plasmid (template) and primers FP1 (CCTCACAGAGACTGGTCTTTTCTGATGGAACGGTGCGTGTAGGCTGGAG CTGCTTC) and RP1 (GCTTGTTTTGTCAAGGGATCACGGGTAGGAGCCACCTT ATATGAATATCCTCCTTAG).

Native polyacrylamide gel electrophoresis (PAGE) for catalase activity

Cultures were grown in LB broth aerobically at 37°C to OD_{600} 0.3 (exponential phase) and OD_{600} 1.5 (stationary phase), harvested by centrifugation at 4, 000 x g for 15 min at 4 °C and washed three times in potassium phosphate buffer (50 mM, pH 7.0). Cell extracts were prepared by sonication for 5 min at 4°C using a Heat Systems sonicator (Misonix, Inc.). Cell debris was removed by centrifugation for 15 min at 12,000 x g at 4°C. Protein concentration was determined by the Bradford assay using bovine serum albumin as a standard (Bradford, 1976). Ten μg of each protein sample was loaded on a 10% native polyacrylamide gel and resolved at 160V for 50 min. The gel was then stained for catalase activity using horseradish peroxidase and diaminobenzidine (Clare *et al.*, 1984; Schellhorn & Stones, 1992). Parallel gels were stained with Coomassie Blue R-250 to verify equal protein loading.

Resistance to H_2O_2 and heat exposure

Cell resistance to H_2O_2 and heat was tested as described (Lange & Hengge-Aronis, 1991b). Stationary phase LB cultures were washed with 0.9% NaCl and resuspended to cell density of 10^8 cells/ml and 5,000 cells/ml for H_2O_2 and heat exposure, respectively. Viable cells were enumerated by serial plating on LB media. Survival was determined as the ratio of the number of viable cells after treatment to the starting number of cells.

In vitro competition

Equal volumes of overnight LB cultures (25 ml) of wild type and *rpoS* mutants (chloramphenicol resistant) were mixed together, incubated at 37 °C at 200 rpm, and sampled daily for 6 days. Colony forming units (CFU) were measured by serial plating

on LB media (LB) and LB with chloramphenicol (LBC). The competitive index was calculated as $(\text{mutant/wild type})_{output}/(\text{mutant/wild type})_{input}$.

In vivo colonization and competition

All experiments using mice were performed in accordance with Canadian Council on Animal Care (CCAC) guidelines. Female 6- to 8-week-old C3H/Hej mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice (groups of 5) were infected by oral gavage with $1x10^8$ CFU of CR WT, *rpoS* mutants, and a mixed inoculum (1:1) of WT and *rpoS* mutants (CI), respectively. Mice were sacrificed at day 6 post infection. The colon of each animal was homogenized in 1 ml ice-cold PBS buffer, and CFU was determined by serial plating on selective Brilliant Green Agar (BGA). Colonies were replica-plated on BGA chloramphenicol plates for enumeration of *rpoS* mutants.

Survival analysis

To test whether RpoS is required for virulence, C3H/Hej mice were inoculated with 1×10^8 CFU wild type cells or *rpoS* mutants by oral gavage (Deng *et al.*, 2004) and were euthanized when they exhibited 20% loss of body weight.

Quantification of expression of the LEE genes by qPCR

Expression of the LEE genes was quantified by qPCR using an Mx3000P QPCR System (Stratagene, La Jolla, CA). Cultures for RNA isolation were prepared as described (Deng *et al.*, 2003). Briefly, wild type and *rpoS* mutants were inoculated into LB media in triplicate, incubated aerobically at 37 °C overnight, subcultured at 1:50 dilution into DMEM media, and grown in 5% CO_2 at 37 °C to OD_{600nm} of 0.7. RNA samples were extracted using acidic hot phenol, purified using NucleoSpin RNA II

(Clontech, Palo Alto, CA), and reverse transcribed to cDNA using the M-MuLV reverse transcriptase (NEB, Beverly, MA). A serial dilution of genomic DNA of CR was used as a standard for quantification. The *rrsA* gene, encoding the 16S RNA, was tested as an endogenous control to normalize RNA quantity (Dong *et al.*, 2008b; Shalel-Levanon *et al.*, 2005).

LEE protein secretion assay

Protein secretion of *E. coli* and CR was examined as described (Deng *et al.*, 2003). Cultures were grown in triplicate in DMEM media in 5% CO_2 at 37°C to OD_{600nm} of 0.70, and centrifuged twice at 12,000g for 10 min to completely remove cells from the supernatants. The supernatants were precipitated by 10% TCA (trichloroacetic acid), and the resultant protein pellets were dissolved in Laemmli SDS-loading buffer (Laemmli, 1970). Proteins were then resolved by 10% SDS-PAGE and stained by Coomassie Blue.

Phylogenetic analysis and sequence alignment.

The *rpoS* sequences were retrieved from the Genome project of CR at the Sanger Institute and GenBank. Sequences were aligned using ClustalW (Thompson *et al.*, 1997). The phylogenetic tree was then generated by the neighbor-joining method (Saitou & Nei, 1987) with bootstrap analysis (1000 iterations).

5.5 Results

RpoS is conserved between CR and *E. coli*

As an important stress response regulator, RpoS has been found in about 30 bacterial genera, primarily in the class of gamma-proteobacteria (Dong *et al.*, 2008a; Hengge-Aronis, 2000). Of particular interest is that RpoS exists in many human pathogens. The *rpoS* gene is highly variable (Ferenci, 2003; Robbe-Saule *et al.*, 2003). Among different strains of *E. coli*, *rpoS* polymorphisms are common (Atlung *et al.*, 2002; Chen *et al.*, 2004; Ferenci, 2003; Herbelin *et al.*, 2000). Thus, we first examined the evolutionary relationship of *rpoS* between CR and several representative pathogens. As expected, *rpoS* is highly similar to that in *E. coli* and distinct from *P. aeruginosa* and *V. cholerae* (Figure 5.1). The *rpoS* genes share 92% consensus sequence between *E. coli* O157:H7 EDL933 and CR. All but three variant sites are synonymous mutations and the RpoS protein sequences only differ in three amino acids (codon: E19D, G21R, D243R). To avoid the possibility that the observed difference between CR and *E. coli* is due to genome sequencing errors that have been previously reported in *E. coli* strains (Coldewey *et al.*, 2007; Hayashi *et al.*, 2006), we sequenced the *rpoS* region of CR independently and the results were consistent with the CR genome sequence.

RpoS is important for resistance to H_2O_2 and heat exposure

Because of conservation of the *rpoS* gene between *E. coli* and CR, we expected RpoS in CR to play a similar role in stress response. To test this, we examined the effect of the *rpoS* mutation on cell survival against oxidative stress and heat exposure (Figure 5.2). Upon exposure to H_2O_2, the viability of *rpoS* mutants was substantially reduced

compared with wild type (Figure 5.2). Under heat stress, though the viability of both wild type and *rpoS* mutants decreased, the CFU of wild type was about 100 fold higher than that of *rpoS* mutants after 7 min exposure. These results show that RpoS is required for both oxidative resistance and heat-tolerance in CR.

Expression of catalase HPII (KatE) is highly RpoS-dependent

In *E. coli*, there are two catalases, HPI encoded by *katG* and HPII by *katE*, which protect cells from oxidative stress. Expression of *katE* is highly RpoS-dependent (Schellhorn & Hassan, 1988). Since RpoS is critical for oxidative response in CR, we set out to examine the effect of RpoS on catalase production. Genes homologous to *E. coli katG* and *katE* were found in CR genome using the BLAST algorithm (data not shown). Native-PAGE catalase analyses show that catalase HPII was induced in stationary phase (Figure 5.3). HPII (KatE) was the major catalase expressed under the condition investigated and was highly RpoS-dependent.

Effect of RpoS on RDAR morphotype, an important trait for virulence

Production of extracellular components, such as curli fimbriae and cellulose, is important for cell attachment in pathogenesis of *E. coli* and *Salmonella*, and is positively regulated by RpoS (Romling *et al.*, 1998; Romling, 2005; Uhlich *et al.*, 2006). The expression of curli and cellulose can be visualized by growing cells at room temperature on RDAR plates, producing a specific red dry and rough (RDAR) colony phenotype termed RDAR morphotype. This morphotype positively correlates with virulence (Uhlich *et al.*, 2002). To test whether RDAR is produced and/or is controlled by RpoS in CR, wild type and isogenic *rpoS* mutants were plated on RDAR plates and incubated at room

temperature for 48 h. The *E. coli* O157:H7 strain EDL933 and its isogenic *rpoS* mutants were also included for comparison. RDAR development was impaired in the *rpoS* mutants in *E. coli*, consistent with previous findings (Romling *et al.*, 1998) (Figure 5.4). Wild type CR exhibited a more pronounced RDAR morphotype than did the *rpoS* mutants, indicating that RDAR is under positive regulation by RpoS.

Mutation in *rpoS* confers a growth advantage *in vitro* and *in vivo*

In *E. coli*, *rpoS* mutants have a growth advantage in stationary phase, a phenotype named GASP (growth advantage in stationary phase) (Zambrano *et al.*, 1993; Zambrano & Kolter, 1996). To test whether *rpoS* mutants of CR also exhibit GASP, we monitored cell population changes in a 1:1 mixture of LB overnight cultures of wild type and *rpoS* mutants *in vitro*. The *rpoS* mutants dominated the co-culture in about 24 h (Figure 5.5). After 3 days incubation, more than 90% of living cells in the mixture were *rpoS* mutants. During this time, the total CFU dropped dramatically from about 10^8 cells/ml (day 0) to about 10^4 cells/ml (day 6).

We then tested whether *rpoS* mutations confer a growth advantage *in vivo* in mouse colon, a more complex environment than *in vitro*. Mice were infected with a 1:1 mixed inoculum of wild type and *rpoS* mutants. Results indicate that *rpoS* mutants outcompeted wild type CR at day 6 (Figure 5.6), with an average competitive index (CI) value of 5.9 (geometric mean), indicating that *rpoS* mutants have a growth advantage *in vivo* as well. Unlike *in vitro* results, the CFU of the mixture remained at a similar level (10^8 -10^9 cells per colon) to the initial inoculum. To test whether RpoS is important for colonization in the colon, mice were infected with either wild type or *rpoS* mutant strains

and colon samples were taken at day 6. The CFU per colon infected with wild type cells was significantly higher than *rpoS* mutants ($P < 0.05$) (Figure 5.6). These results indicate that RpoS of CR may be beneficial for colonization but is likely a disadvantage for competition in the colon (see Discussion). Colonic hyperplasia, a typical symptom of CR infection, was observed in mice infected with either wild type or *rpoS* mutants, suggesting that the cause of CR-induced mortality was the same for both groups.

Because of the important role of RpoS in stress response, it is also possible that the difference in colonization is because wild type cells survived better than *rpoS* mutants during passage through the gastrointestinal tract. However, this is not likely since the number of bacteria in mouse stools (CFU/g) after 5 h infection was the same between wild type and *rpoS* mutants (data not shown).

RpoS controls the expression of the LEE genes

The type III secretion system and virulence factors such as Tir and Eae on the LEE island are important for colonization through the formation of A/E lesions (Deng *et al.*, 2003; Deng *et al.*, 2004). We therefore sought to examine the role of RpoS in LEE expression. In *E. coli*, there are conflicting reports regarding the effect of RpoS on the expression of the LEE genes, which may be due to differences in strains and conditions tested. It has been shown that RpoS positively controls the expression of the LEE3 operon and of Tir (Sperandio *et al.*, 1999). However, expression of the LEE genes has also been reported to be negatively regulated by RpoS (Iyoda & Watanabe, 2005; Tomoyasu *et al.*, 2005). Interestingly, the transcription of *ler* (a global regulator of LEE

genes) is enhanced in *rpoS* mutants, while the activation of Ler (a global regulator of LEE genes) by DsrA requires RpoS in *E. coli* (Laaberki *et al.*, 2006).

Using a LEE-induction condition (5% CO_2, 37 °C, DMEM media) (Kenny *et al.*, 1997), we examined the transcription of representative genes from each of the five LEE operons including three LEE regulators, Ler, GrlA and GrlR, by qPCR. The transcription of all genes tested was higher in the wild type than in the *rpoS* mutants (Figure 5.7), though the RpoS effect seems to be moderate (less than twofold difference). The similar expression ratio of these LEE genes may be the result of the global regulatory effect from Ler, GrlA and GrlR (Deng *et al.*, 2004; Elliott *et al.*, 2000; Mellies *et al.*, 2007).

Many LEE genes encode either the type III secretion apparatus or secreted virulence effectors, and the secreted protein profile of wild type CR has been characterized (Deng *et al.*, 2004). We tested the effect of RpoS on LEE protein secretion. In *E. coli* O157:H7, the secretion of LEE factors is negatively controlled by RpoS (Iyoda & Watanabe, 2005). In this study, we included *E. coli* O157:H7 EDL933 and its derivative *rpoS* knockout mutant in our assay as a control (Figure 5.8). Consistent with previous reports, the secreted protein profile of *rpoS* mutants was enhanced in *E. coli*. However, in CR, there were more secreted proteins in wild type cultures than in the *rpoS* mutants, though the difference was moderate, consistent with the transcription results. Wild type and *rpoS* mutants of CR grew similarly in DMEM media with a generation time of ~76 min. Therefore, it is unlikely that the difference in LEE expression results from growth difference.

RpoS is required for full virulence

Many genes on the LEE island are essential for virulence (Deng *et al.*, 2004). Some non-LEE encoded virulence effectors also require the LEE Type III secretion system for delivery to the host (Wickham *et al.*, 2007). In addition, RDAR morphotype is associated with increased virulence in *E. coli* (Uhlich *et al.*, 2002). Because of the positive effect of RpoS on LEE expression and RDAR development, RpoS may play an important role in virulence in CR. To test this hypothesis, we performed a survival analysis using C3H/HeJ mice, a strain highly susceptible to CR infection. Though *rpoS* mutants were still lethal to C3H/HeJ mice, mice infected with *rpoS* mutants survived significantly longer than mice infected with wild type ($P = 0.003$, Wilcoxon test), indicating that virulence of CR is attenuated by *rpoS* mutation (Figure 5.9).

5.6 Discussion

Infection of mice by CR provides a robust model to examine enteropathogenesis and the interaction between the pathogen and its natural host under physiological conditions (Deng *et al.*, 2004; Mundy *et al.*, 2005; Wiles *et al.*, 2006). Because CR utilizes the same A/E lesions as in EPEC and EHEC strains, two serious classes of human pathogens, to initiate infection (Mundy *et al.*, 2005), results obtained from CR may be readily relevant to pathogenesis in humans. Here we used CR to examine the role of RpoS in virulence under physiological conditions. Our results show that RpoS was conserved in sequence as well as in function between *E. coli* and CR. RpoS positively regulated the expression of LEE genes and the development of RDAR. Though *rpoS* mutants caused lethality in mice, there was a significant delay in both onset of disease

symptoms and lethality of infected mice. Thus, RpoS is important for full virulence of CR. This phenotype is similar to that caused by mutations in several LEE virulence genes (e.g., *rorf1*, *espF*, and *cesF*), which result in 2-3 days delay in survival of infected mice (Deng *et al.*, 2004).

RpoS controls a large regulon consisting of 10% of the genome in *E. coli* (Lacour & Landini, 2004; Patten *et al.*, 2004; Weber *et al.*, 2005). We expect a similar size of the RpoS regulon in CR, since CR and *E. coli* are closely related (Mundy *et al.*, 2005). Because *rpoS* mutations cause pleiotropic physiological effects in *E. coli*, we cannot be sure that a single RpoS-controlled gene accounts for the observed virulence. It is likely that the attenuated virulence of *rpoS* mutants *in vivo* may be due to a decrease in not only virulence factor expression but also other RpoS-controlled functions. In this study, we found that the LEE genes were expressed higher in wild type CR *in vitro*. Since the LEE genes encode for essential functions for virulence, decreased expression of the LEE genes in *rpoS* mutants may, at least in part, contribute to the longer survival of mice infected with *rpoS* mutants than that of mice infected with wild type CR. However, other non-LEE encoded RpoS-regulated genes that have not been identified or tested yet may also contribute to virulence. Further studies are required to examine gene expression at the genomic scale to fully understand the role of RpoS during infection.

Flagella are essential for *Salmonella* pathogenesis *in vivo* (Stecher *et al.*, 2004), while in *E. coli*, mutants deficient in flagella synthesis colonize the mouse intestine much better than wild type (Leatham *et al.*, 2005). Expression of flagella is higher in *rpoS* mutants in *E. coli* (Dong & Schellhorn, 2009a; Patten *et al.*, 2004). We considered it

possible that flagellar genes may be differentially expressed in CR wild type and *rpoS* mutants as well, and this might have also contributed to the observed difference in colonization. However, this does not appear to be the case. Both wild type and *rpoS* mutants of CR were nonmotile on soft agar plates (data not shown). The loss of motility of CR may contribute to its better colonization ability relative to *E. coli* in mouse colon.

It seems paradoxical that, though RpoS was important for colonization of CR, *rpoS* mutants possessed a substantial growth advantage over wild type and predominated in co-colonization experiments (this study). Our results, however, are consistent with a previous study in which the effect of RpoS on colonization was examined in a non-pathogenic model using the *E. coli* BJ4 strain in mice (Krogfelt *et al.*, 2000). The fact that *rpoS* mutants outcompete wild type *in vivo* is similar to the previously-reported *in vitro* growth phenotype GASP of *E. coli* (Zambrano *et al.*, 1993; Zambrano & Kolter, 1996). During *in vitro* growth in stationary phase, although RpoS is critical for long-term survival (Lange & Hengge-Aronis, 1991b), *rpoS* mutants are dominant in mixed cultures with wild type cells (Zambrano *et al.*, 1993). The growth advantage of *rpoS* mutants may be explained by the sigma factor competition model (Farewell *et al.*, 1998a; Ferenci, 2003; Nystrom, 2004a). Sigma factors compete for a limited number of RNA core polymerase, and mutations in *rpoS* may increase the number of RpoD-associated polymerase, the housekeeping sigma factor, thus increasing the transcription of many housekeeping genes that are important for nutrient scavenging in a nutrient-limiting environment. However, growth in the mouse colon is quite different from growth *in vitro*. We found that the population of bacteria in the colon did not drop drastically post

infection (as *in vitro* experiments), but rather remained constant, suggesting that nutrients in the colon are not limiting but are sufficient to support a stable population. In contrast, both wild type and *rpoS* mutants rapidly lost viability to 0.1% (for wild type) or 0.001% (for *rpoS* mutants) during *in vitro* growth. How can *rpoS* mutants out-compete wild type cells? There are at least two possible explanations. First, since RpoS is known to negatively regulate the expression of a large set of genes including those involved in the TCA cycle (Patten *et al.*, 2004), *rpoS* mutants may produce a factor, such as a secondary metabolite, that inhibits wild type growth. Alternatively, it is possible that *rpoS* mutants can better utilize key limiting nutrients than wild type cells (King *et al.*, 2004), perhaps through increased nutrient transport (King *et al.*, 2004; Maharjan *et al.*, 2007).

In conclusion, this study shows that RpoS is important for full virulence in CR in its natural host. Because of the high similarity of RpoS in sequence and function between CR and *E. coli*, the use of CR-mouse model is well-suited to examine the role of RpoS in enteropathogenesis for future studies. The effect of RpoS on LEE gene expression was found to be different between CR and *E. coli* O157:H7, suggesting the involvement of CR-specific factors. Since whole genome profiling has been successfully used to investigate carbon source utilization during *E. coli* colonization (Chang *et al.*, 2004), it would be interesting to use microarrays to examine the expression of RpoS-regulated genes during infection in CR to infer when or what kind of stresses that bacteria may encounter during growth and pathogenesis process, as well as to fully understand how RpoS functions *in vivo*.

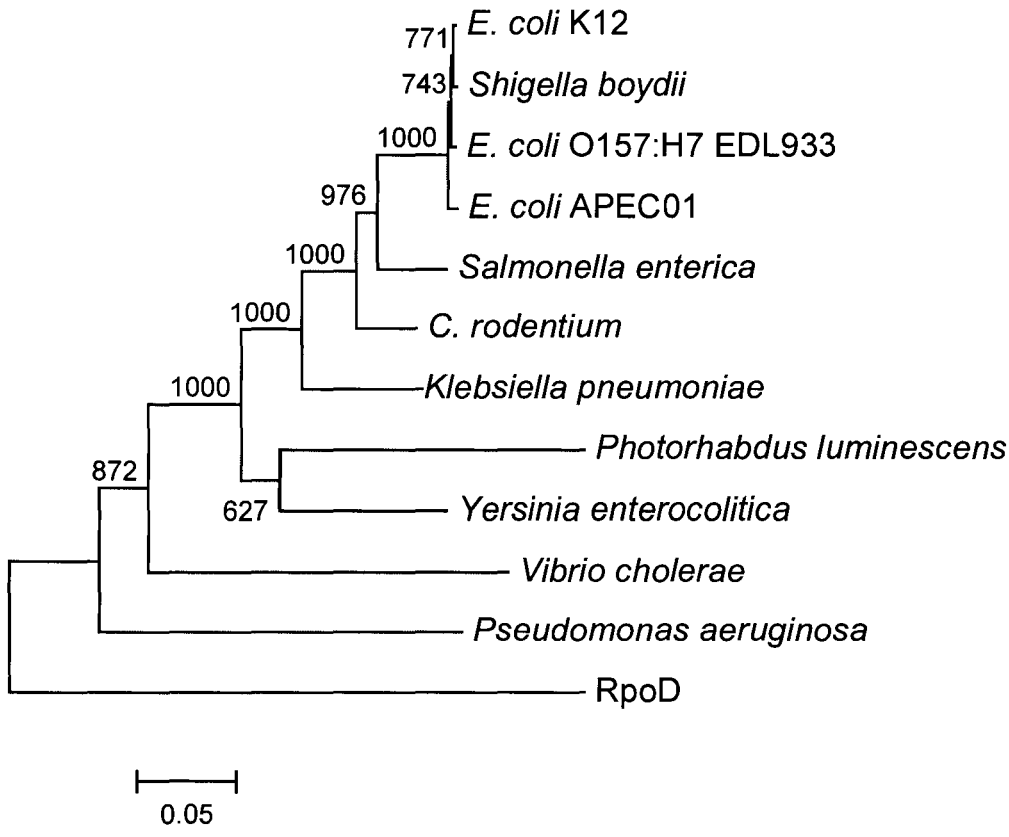

Figure 5.1 Phylogeny of the *rpoS* gene from representative pathogens.

The tree was constructed by the neighbor-joining method with bootstrap analysis (1000 iterations). The scale bar shows 5% sequence divergence. The *rpoD* gene of *E. coli* was included as an outgroup.

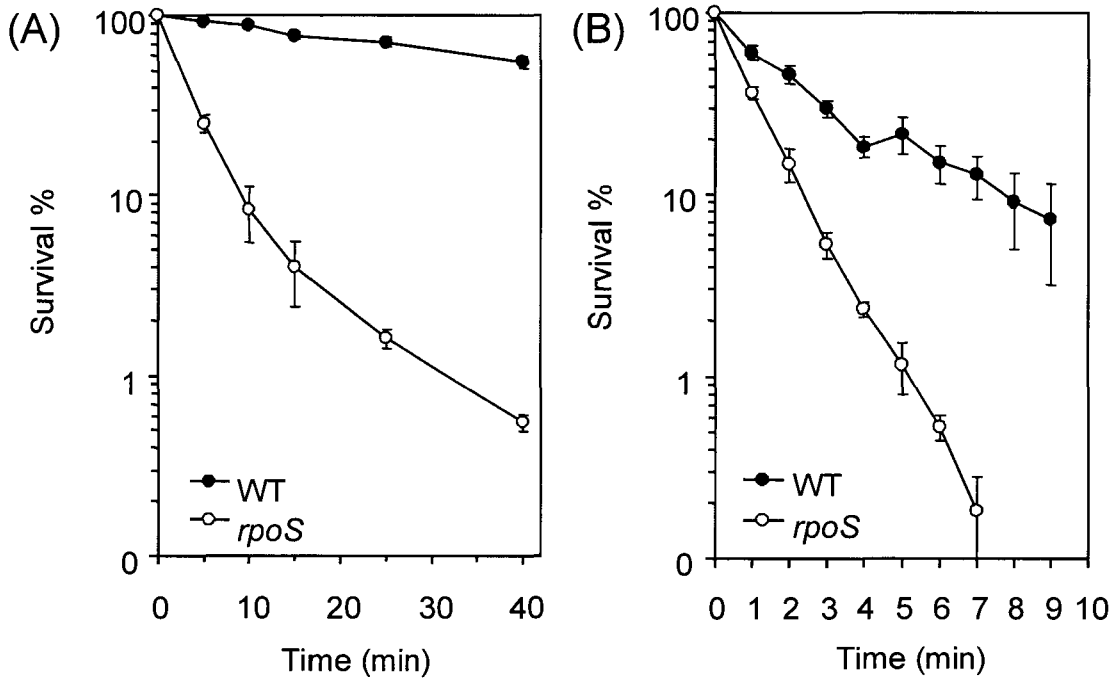

Figure 5.2 Effect of RpoS on H_2O_2 resistance and thermotolerance.

Stationary phase cultures were washed with 0.9% NaCl and resuspended to 10^8 cells/ml and 5,000 cells/ml for H_2O_2 resistance and thermotolerance, respectively. Cells were exposed to 15mM H_2O_2 (A) or 55°C (B), and enumerated by plating on LB media. Survival was determined as the percentage of the number of viable cells compared with the starting number of cells before treatment.

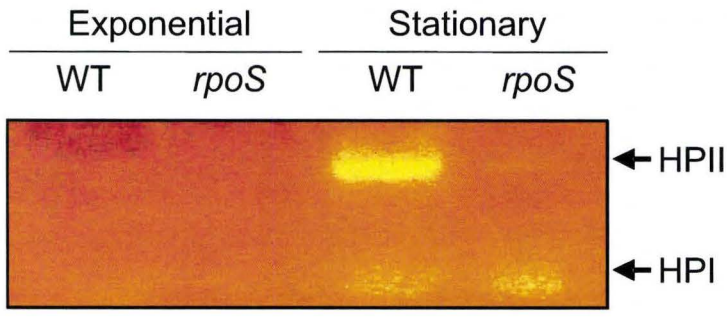

Figure 5.3 Expression of catalase is highly RpoS-dependent.

Cultures were collected in exponential phase (OD_{600} 0.3) and in stationary phase (OD_{600} 1.5), and washed three times in ice-cold phosphate buffer prior to sonication. Protein was quantified by the Bradford assay (1976). Ten μg of each protein sample was loaded on the 10% native-PAGE gel. Catalase activity staining was performed as described in Materials and Methods.

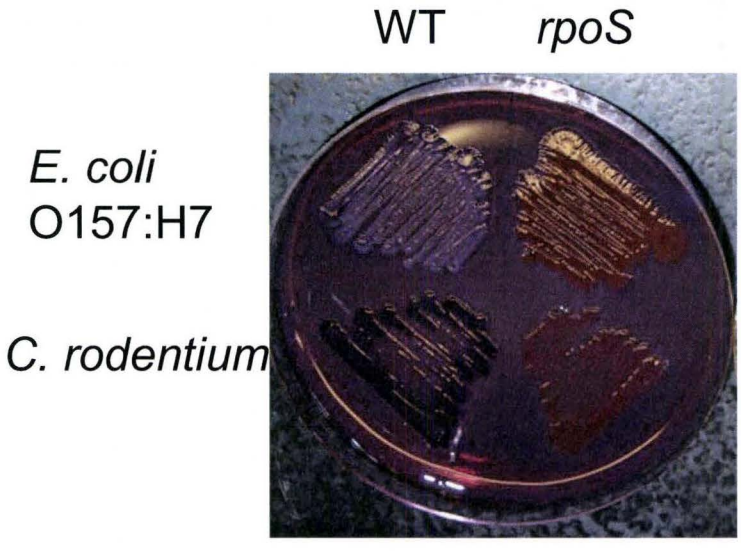

Figure 5.4 Effect of RpoS on RDAR morphotype development.

Cells were grown at 37°C in non-salt LB media overnight and streaked out on RDAR plates. RDAR plates were incubated at room temperature for 48 h. Production of cellulose and curli was visualized by staining with Coomassie Blue and Congo-red in the RDAR plates.

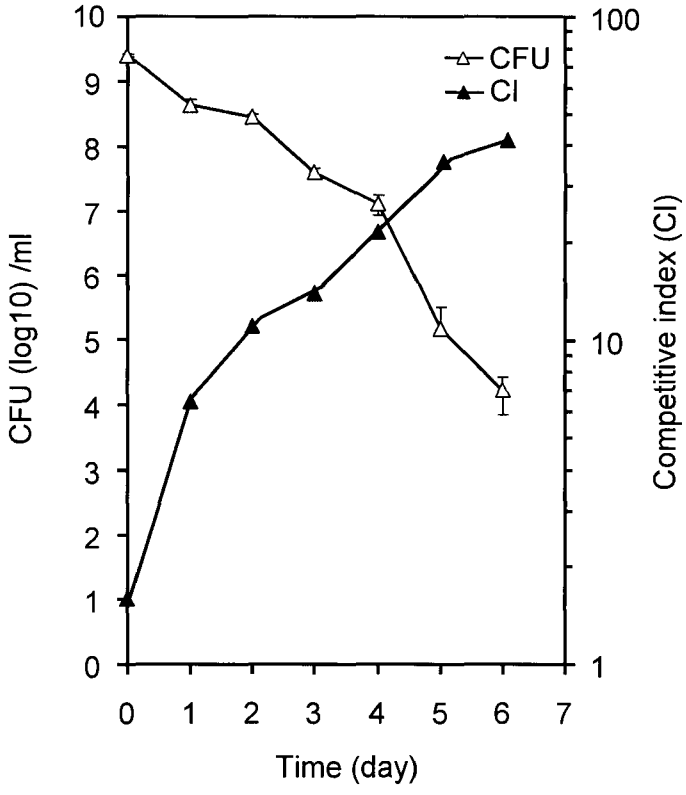

Figure 5.5 Competition between wild type and *rpoS* mutants in vitro.

Equal volumes of overnight cultures of wild type and *rpoS* mutants were mixed together and incubated aerobically at 37°C. The CFU was enumerated by serial plating on LB media (LB chloramphenicol for *rpoS* mutants). Competitive index was calculated as $[rpoS/\text{wild type}]_{output}/\ [rpoS/\text{wild type}]_{input}$.

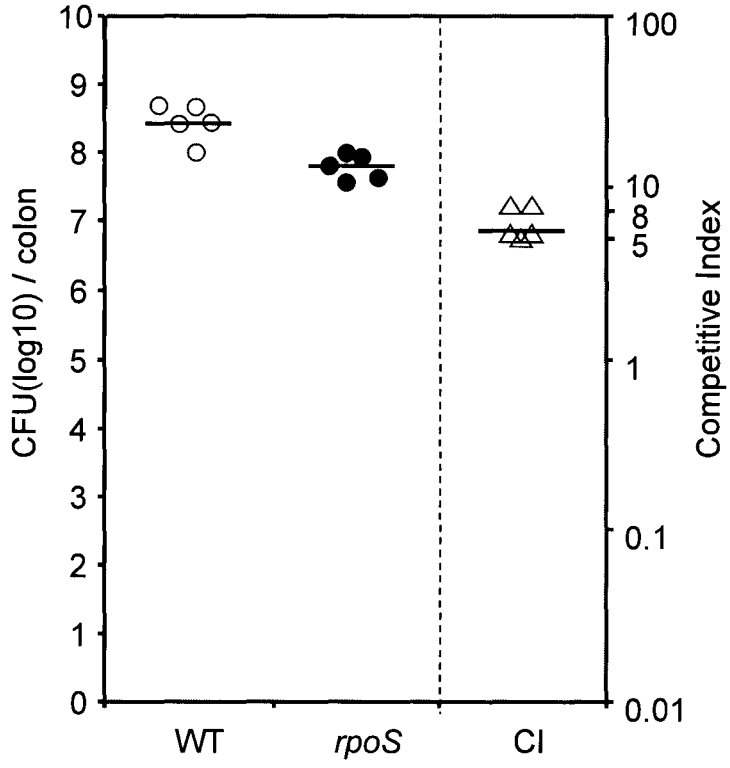

Figure 5.6 Colonization and competitive index of wild type (WT) and *rpoS* mutants of CR.

C3H/Hej mice were infected by oral gavage with 10^8 CFU of WT, *rpoS* mutants, and 1:1 mixture of WT and *rpoS* mutants (CI), respectively. Mice were sacrificed at day 6 post infection. The colon of each animal was homogenized in 1.0 ml PBS buffer, and the CFU was determined by serial plating on Brilliant Green Agar (BGA). Each point represents data from one animal.

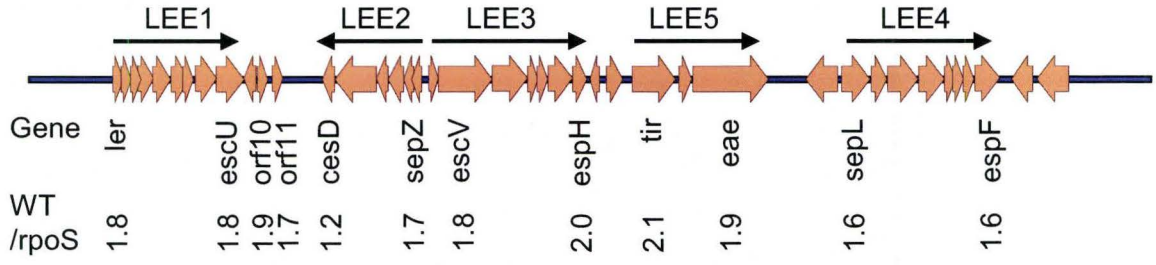

Figure 5.7 Expression of the LEE genes in wild type and the *rpoS* mutants in CR.

RNA samples were extracted from cultures grown in triplicate in DMEM media to OD_{600nm} 0.7 in 5% CO_2 at 37 °C. Differential gene expression was determined by qPCR, and is represented by the ratio of WT/rpoS.

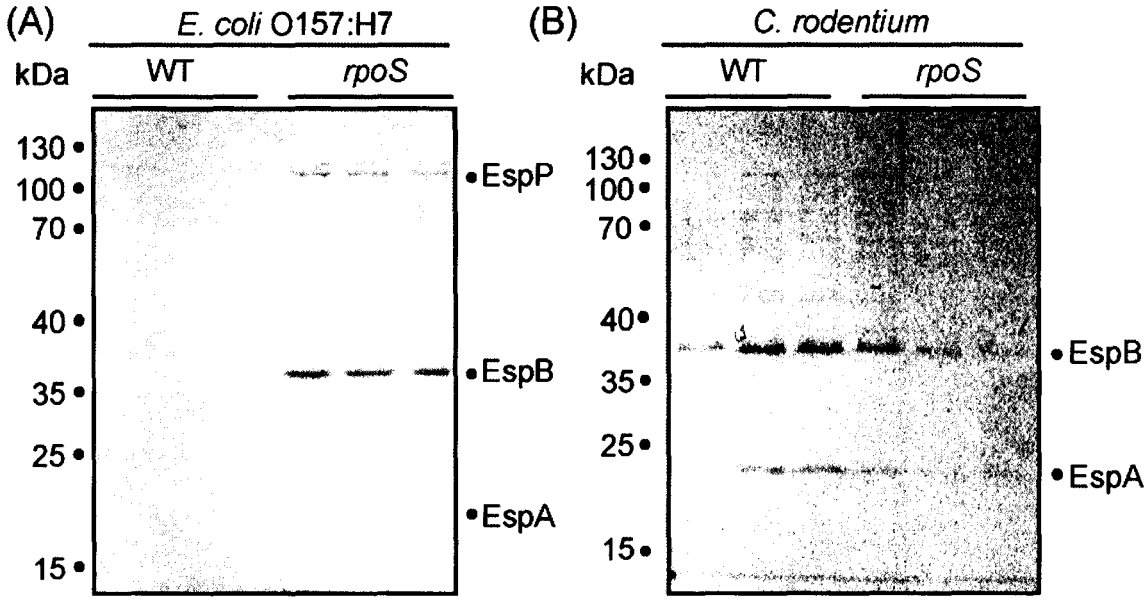

Figure 5.8 LEE protein secretion of wild type E. coli O157:H7 EDL933 (A) and CR (B) and derivative *rpoS* mutants, respectively.

Known secreted proteins are indicated.

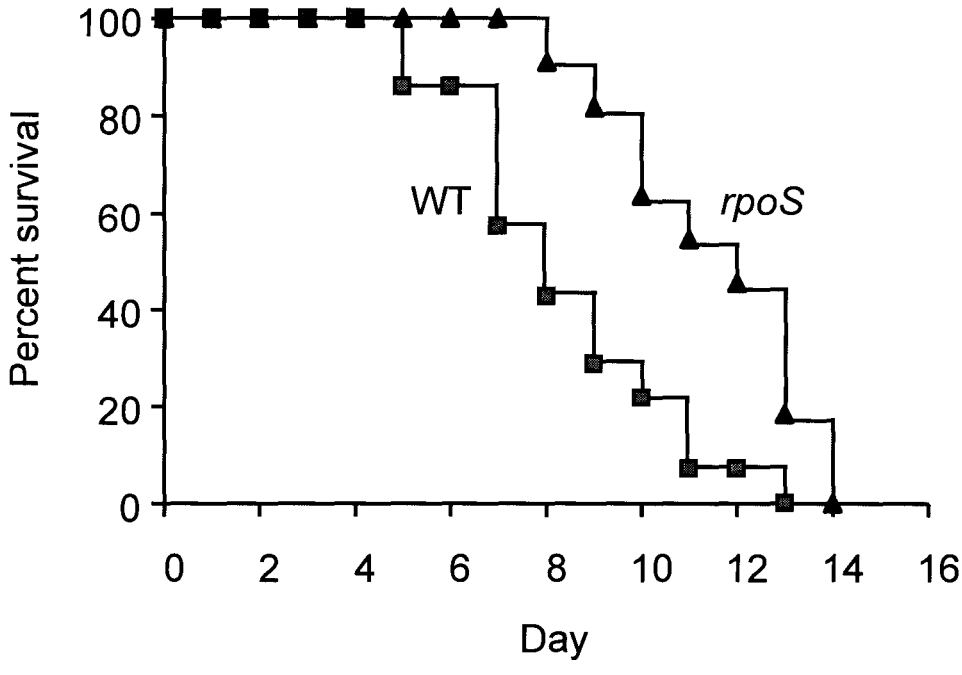

Figure 5.9 Survival analysis.

C3H/Hej mice were infected with 10^8 CFU wild type or *rpoS* mutants and euthanized at the loss of 20% body weight. Mice infected with *rpoS* mutants survived significantly longer than mice with wild type (P = 0.003, Wilcoxon test). Results were obtained from two independent experiments.

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CHAPTER VI

Polymorphism and selection of *rpoS*

in pathogenic *Escherichia coli*

from

Dong, T., Chiang, S.M., Joyce, C., Yu, R., and Schellhorn, H.E. 2009. Polymorphism and selection of *rpoS* in pathogenic *Escherichia coli*. BMC Microbiology 9:118

6.1 Preface

The contents of this chapter were contributed primarily by the author of this thesis. Tao Dong performed all the experiments and analyses, and wrote the manuscript in its entirety. This chapter is adapted from a published article in BMC Microbiology to fit the format requirements of the thesis.

6.2 Abstract

Though RpoS is important for survival of pathogenic *Escherichia coli* in natural environments, polymorphism in the *rpoS* gene is common. However, the causes of this polymorphism and consequential physiological effects on gene expression in pathogenic strains are not fully understood. In this study, we found that growth on non-preferred carbon sources can efficiently select for loss of RpoS in seven of ten representative verocytotoxin-producing *E. coli* (VTEC) strains. Mutants (Suc^{++}) forming large colonies on succinate were isolated at a frequency of 10^{-8} mutants per cell plated. Strain O157:H7 EDL933 yielded mainly mutants (about 90%) that were impaired in catalase expression, suggesting the loss of RpoS function. As expected, inactivating mutations in *rpoS* sequence were identified in these mutants. Expression of two pathogenicity-related phenotypes, cell adherence and RDAR (red dry and rough) morphotype, were also attenuated, indicating positive control by RpoS. For the other Suc^{++} mutants (10%) that were catalase positive, no mutation in *rpoS* was detected. In conclusions, The selection for loss of RpoS on poor carbon sources is also operant in most pathogenic strains, and thus is likely responsible for the occurrence of *rpoS* polymorphisms among *E. coli* isolates.

6.3 Introduction

Adaptation is important for survival of bacteria in various natural environments, but the underlying mechanisms are not fully understood. Bacteria are often present in large communities (e.g., biofilm (Stoodley *et al.*, 2002)) in nature, and adaptation can

occur at population levels. An important adaptive strategy is the generation of variants to maximize bacteria fitness at the population level in response to fluctuating environments (Davidson & Surette, 2008; Wolf *et al.*, 2005). These variants may result from spontaneous mutations selected within a population or from non-genetic changes. For example, to evade host immune system, some pathogens can alter surface antigen structure (Lederberg & Iino, 1956), termed phase variation (Hallet, 2001; Lederberg & Iino, 1956), through revertible high frequency mutation of genes encoding surface proteins (Davidson & Surette, 2008; Hallet, 2001). Bacteria also exhibit cell-to-cell variation in gene expression, termed individuality (Davidson & Surette, 2008), even in an isogenic population. For example, under suboptimal induction conditions, the *lac* operon in *Escherichia coli* exhibits two distinct expression states, either fully induced or non-induced, but not an intermediate (Tolker-Nielsen *et al.*, 1998). Gene expression noise due to stochastic events also results in phenotypic variation within isogenic *E. coli* populations (Davidson & Surette, 2008; Ozbudak *et al.*, 2002). Both genetic selection and individuality are likely important for bacterial adaptation in natural environments (Davidson & Surette, 2008).

An important adaptation regulator is the alternative sigma factor RpoS widely found in *E. coli* and many other proteobacteria (Dong *et al.*, 2008a; Hengge-Aronis, 2000). RpoS controls a large regulon (Dong *et al.*, 2008b; Dong & Schellhorn, 2009a; Lacour & Landini, 2004; Patten *et al.*, 2004; Weber *et al.*, 2005) and plays a critical role in survival against stresses, such as prolonged starvation (Lange & Hengge-Aronis, 1991b), low pH (Small *et al.*, 1994), thermal stress (Hengge-Aronis *et al.*, 1991), near-

UV exposure (Sammartano *et al.*, 1986) and oxidative stress (Sammartano *et al.*, 1986). Despite the importance of RpoS, many attenuating mutations in the *rpoS* gene have been identified in both laboratory and natural *E. coli* strains. For example, some K12 strains possess an amber mutation (TAG) at codon 33 (Atlung *et al.*, 2002), while others have Glu (GAG), Tyr (TAT), or Gln (GAG) at the same position (Atlung *et al.*, 2002; Subbarayan & Sarkar, 2004). GAG is commonly found in natural non-K12 *E. coli* isolates (Atlung *et al.*, 2002; Subbarayan & Sarkar, 2004). Mutations in *rpoS* have also been identified in Shiga-like toxin-producing *E. coli* strains (Waterman & Small, 1996).

Polymorphism of *rpoS* appears to be paradoxical to the central role that RpoS plays in survival. Mutants of *rpoS* can be selected under nutrient limitation and exhibit enhanced metabolic potential (King *et al.*, 2004), suggesting a regulatory trade-off for fitness between stress resistance and nutrient scavenging (King *et al.*, 2004). Growth on weak acids, including succinate (Chen *et al.*, 2004) and acetate (Spira *et al.*, 2008), strongly selects for mutations in *rpoS* in laboratory *E. coli* strains (Chen *et al.*, 2004). Considering that the weak acid (e.g., acetate) concentration is relatively high in human colon (80 mM) where *E. coli* colonize (Cummings *et al.*, 1987; Rosenthal *et al.*, 2006), *E. coli* may face a similar selective pressure within the host environment. Selection for loss and gain of RpoS function may be an important adaptive mechanism, like phase variation, to ensure that *E. coli* can survive in complex natural environments.

However, whether this selection is responsible for the observed *rpoS* polymorphism in natural *E. coli* isolates remains unclear, primarily because most studies have been done with laboratory *E. coli* K12 strains. The genomes of *E. coli* isolates differ

substantially and constitute a pangenome consisting of 13,000 genes, of which 2,200 genes are conserved among all isolates (Rasko *et al.*, 2008). Since RpoS mostly controls expression of genes encoding non-essential functions (Dong *et al.*, 2008a; Hengge-Aronis, 2000; Patten *et al.*, 2004; Weber *et al.*, 2005), RpoS likely plays a considerable role in the expression of non-conserved genes in the pangenome. Given that *E. coli* K12 strains only possess about 1/3 of all genes found in the pangenome of *E. coli* (Rasko *et al.*, 2008), it is possible that *rpoS* selection is limited to laboratory strains. Interestingly, selection for *rpoS* could not be observed in a natural *E. coli* isolate ECOR10 under nutrient limitation (see Fig 5 in (King *et al.*, 2004)).

In this study, we wished to address three outstanding questions. First, can *rpoS* mutants be selected in clinical strains isolated from natural environments? Of particular interest is whether this selection occurs in pathogenic strains, which may have important medical relevance because of the potential role of RpoS in bacterial pathogenesis. Second, are there other factors involved in the selection for enhanced metabolic abilities in natural strains? Finally, is there any evidence that this selection occurs in natural environments? To address these questions, we employed a succinate selection strategy as a tool (Chen *et al.*, 2004) and examined the selection using a group of ten representative verocytotoxin-producing *E. coli* (VTEC) strains from all five identified seropathotypes as our model strains. VTEC strains, including the O157:H7 serotype, are responsible for most *E. coli* foodborne outbreaks and can cause severe diseases, including diarrhea, hemorrhagic colitis and the hemolytic uremic syndrome (Karmali, 1989). Our results show that the selection for loss of RpoS is operant in most pathogenic *E. coli* strains. Virulence traits

including RDAR morphotype and cell adherence were attenuated as a result of *rpoS* mutations. In addition, although *rpoS* mutants constituted most of the metabolic enhanced mutants, there was a small fraction of mutants that had intact RpoS function, indicating that other factors can also increase metabolic potential under conditions examined. Interestingly, three of ten tested VTEC strains grew well on succinate, and no growth-enhanced mutants could be selected. One of these three strains possessed a null *rpoS* mutation. This indicates that an adaptation to poor carbon source may have occurred in natural *E. coli* populations.

6.4 Materials and Methods

Bacterial strains, media, and growth conditions

Pathogenic strains examined in this study are listed in Table 6.1. Strains were routinely grown in Luria-Bertani (LB) broth aerobically at 37°C with shaking at 200 rpm. Cell growth was monitored spectrophotometrically at 600nm. M9 minimal media was supplemented with glucose (0.4% wt/vol), succinate (1%), fumarate (1%) or malate (1%) as a sole carbon source (Miller, 1992). Media was supplemented with ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml) as indicated. All chemicals and media were supplied by Invitrogen, Fisher Scientific, or Sigma-Aldrich. The generation time was determined using exponential phase cultures ($g = t/ (3.3 (\log N - \log N_0))$; g = generation time; t = time of exponential growth; N_0 = initial cell number; N= final cell number) (Madigan *et al.*, 2003).

HepG2 cell growth

HepG2 cells were grown at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS).

Selection of Suc^{++} mutants

Cultures were inoculated into LB broth from single colonies. After overnight incubation, cells were washed 3 times with M9 minimal salts to eliminate media carryover, plated on succinate minimal media (approximately 10^9 cells) and incubated at 37°C for 48h. Several large colonies (Suc^{++}) from each plate were picked and purified by serial streaking on succinate plates. The selection for Suc^{++} mutants was performed in triplicate using independent colonies to ensure isolated mutants were not clones descended from single variants. Three independent mutants, selected from independently-grown cultures of each strain, were sequenced using *rpoS* flanking primers as described below.

Amplification of the *rpoS* region and sequencing

The *rpoS* region of wild type strains and putative *rpoS* mutants that were catalase-deficient was amplified using primers FP1 (CAACAAGAAGTGAAGGCGGG) and RP1 (TGGCCTTTCTGACAGAT GCTTAC) by whole colony PCR. A single colony from each strain was resuspended into 30 µl ddH_2O, heated at 95°C for 5 min, and 4 µl was used in a standard 20 µl PCR reaction. PCR products were purified by QIAquick Purification Kit (Qiagen, Inc.) and sequenced by MOBIX lab (McMaster University).

Construction of EDL933 *rpoS* deletion mutant

A precise *rpoS* deletion mutant of EDL933 was constructed using the Red recombination system (Datsenko & Wanner, 2000), and served as a negative control for the following experiments. The *rpoS* gene was replaced by homologous recombination with the chloramphenicol resistant gene *cat,* which was amplified using pKD3 plasmid (the template) and primers FP2 (CCTCGCTTGAGACTGGCCTTTCTGAAGAGCTAC ATGGAGCTGCTTC) and RP2 (ATGTTCCGTCAAGGGACACGGGTAGGGCCACT AAAATATCCTCCTTAG). The *cat* gene was further removed from the chromosome by recombination with the FLP recombinase. The resultant mutant lost the entire *rpoS* ORF. The mutation was confirmed by PCR using primers flanking the deleted region.

Catalase assay

Native polyacrylamide gel electrophoresis (PAGE) was performed to examine the catalase activity in selected Suc^{++} mutants. Overnight cultures were harvested by centrifugation at 4,000 X g for 15 min at 4°C, and washed three times in potassium phosphate buffer (50 mM, pH 7.0). Cells were resuspended to OD_{600nm} = 15 in potassium phosphate buffer (50 mM, pH 7.0) and disrupted by sonication using a Heat Systems sonicator (Misonix, Inc., Farmingdale, New York). Cell debris was removed by centrifugation for 15 min at 12,000 X g at 4°C. Protein concentration was determined by the Bradford assay using bovine serum albumin as a standard (Bradford, 1976). Ten μg of each protein sample were loaded on a 10% native polyacrylamide gel and resolved at 160V for 50 min. The gel was then stained with horseradish peroxidase and diaminobenzidine as described by Clare *et al.* (1984). Parallel gels were stained with

Coomassie Blue R-250 to verify equal protein loading. Plate catalase assays were used to qualitatively test the Suc^{++} mutants for loss of catalase activity by dropping 10 μl of 30% H_2O_2 on the plates, an indicator for *rpoS* status because catalase production is highly-RpoS dependent (Schellhorn & Hassan, 1988).

Western blot analysis

Protein samples were prepared as described for catalase staining. Samples (10 μg) were boiled for 5 min, loaded on a 10% SDS-PAGE gel, and fractioned at 160V for 50 min. Protein samples were then transferred from the gel onto a PVDF membrane by electrophoresis at 90V for 1h. The PVDF membrane was incubated with anti-RpoS (a gift from R. Hengge, Freie Universität Berlin) or anti-AppA sera (a gift from C.W. Forsberg, University of Guelph) and secondary antibody of goat anti-rabbit immunoglobulin (Bio-Rad). Signals were detected using enhanced chemiluminescence (Amersham Bioscience).

Growth under aerobic and anaerobic conditions

Culture samples were collected after overnight incubation in LB media, and washed 3 times in M9 salts. To obtain isolated mutant colonies, serial dilutions were plated on M9 minimal media with either glucose (0.4%) or succinate (1%) as the sole carbon source, and incubated for 72h at 37°C under aerobic or anaerobic conditions as indicated. Anaerobic conditions were maintained in Brewer anaerobic jars (Becton Dickinson) using the BBL GasPak anaerobic system as described previously (Chang *et al.*, 1999). Potassium nitrate (40 mM) was supplemented to all the media to provide an electron receptor for respiration under anaerobic conditions (Chang *et al.*, 1999). The diameter of individual colonies was determined at 40X magnification.

Test of pathogenicity-related traits

(a) RDAR morphotype. To visualize RDAR (red, dry and rough) cell morphotype (Bokranz *et al.*, 2005), a single colony of each strain was resuspended in non-salt LB media (1% tryptone and 0.5% yeast extract) in a 96-well microtiter plate, transferred to Congo Red (CR) plates (non-salt LB media with 1.5% agar, 40 μg/ml of Congo Red dye, and 20 μg/ml of Coomassie Blue R-250) by replica plating, and grown at 25°C for 48h (Bokranz *et al.*, 2005).

(b) Adherence assay. Quantitative adherence assays were performed as described by Torres and Kaper (Torres & Kaper, 2003). Wild type *E. coli* EDL933 and derivative *rpoS* and Suc^{++} mutants were tested for adherence to human liver epithelial HepG2 cells. Confluent HepG2 cultures grown in DMEM were incubated with 10^8 CFU *E. coli* overnight grown cells for 6h at 37°C in 5% CO_2. Adhered *E. coli* cells were washed with PBS buffer, released by 0.1% Triton X-100 and enumerated by serial plating on LB media. The adherence is reported as the percentage of cells that remain adherent following the washing process. The statistical significance of differences between treatment groups was determined using an unpaired Student's *t*-test (Bliss, 1970).

Phenotype Microarray analysis

To assess the effect of RpoS on metabolism, we compared wild type MG1655 *E. coli* strain and a derivative null-*rpoS* mutant (Patten *et al.*, 2004) using a commercial high-throughput phenotype screening service, Phenotype Microarray (PM) analysis (Biolog, Hayward, CA), that permits evaluation of about 2,000 cellular phenotypes including utilization of carbon, nitrogen, phosphate and sensitivity to various stresses

(Bochner *et al.*, 2001; Bochner, 2003). PM analysis assesses substrate-dependent changes in cell respiration using tetrazolium as an electron acceptor and has been widely used to test growth phenotypes (Ihssen & Egli, 2005; Loh *et al.*, 2006; Zhou *et al.*, 2003).

Sequence alignment

The *rpoS* sequences of VTEC *E. coli* strains and isolated mutants were aligned by ClustalW (Thompson *et al.*, 1997) and graphically depicted using Vector NTI 10 (Invitrogen, Carlsbad, CA).

6.4 Results

Polymorphisms of *rpoS* in wild type VTEC strains

The ten representative VTEC strains examined in this study (Table 6.1) belong to five seropathotypes that have been categorized on the basis of virulence and outbreak frequency (Karmali *et al.*, 2003). To test whether selection for loss of RpoS function can occur in these isolates, we first examined the *rpoS* sequences of these strains. Many nucleotide base substitutions were found in *rpoS* (Table 6.2). However, these substitutions did not result in changes in protein sequence, except for a single transversion (G to T) in strain N99-4390 which formed a premature stop codon, resulting in a loss of 86 amino acids at the C-terminal end of RpoS. As expression of catalase HPII encoded by *katE* is highly RpoS-dependent (Mulvey *et al.*, 1988; Schellhorn & Hassan, 1988), catalase production in all strains could be used to assess RpoS activity using plate catalase assays. Only N99-4390 exhibited a low catalase activity, consistent with the expected effect of the identified mutation in this strain. All tested VTEC strains were

found to have a GAG at codon 33, in contrast to CAG in the laboratory K12 strain MG1655 (Table 6.2).

Selection of Suc^{++} mutants

Our primary goal was to determine if loss of RpoS in VTEC strains can be selected by growing cells on non-preferred carbon sources. Mutants forming large colonies (Suc^{++}) were readily isolated from seven of ten tested strains at a frequency of 10^{-8} per cell plated on succinate media, consistent with the frequencies obtained for laboratory strains (Chen *et al.*, 2004). Interestingly, strains CL3, R82F2 and N99-4390 grew uniformly well on succinate plates, much better than the other wild type strains, thus no Suc^{++} mutants were obtained. Similar results were obtained by growing cells on fumarate, another TCA cycle intermediate (data not shown), indicating that this selection is not limited to succinate alone.

A group of 12 independent representative Suc^{++} mutants were selected from each strain to test their RpoS status using catalase plate assays (Chen *et al.*, 2004). Most of the Suc^{++} mutants (depending on parental strain background) were impaired in catalase production (Table 6.1). In *E. coli*, there are two catalases, HPI (KatG) and HPII (KatE), but only catalase HPII (KatE) is highly RpoS-dependent (Chen *et al.*, 2004). To confirm the plate assay results and to differentiate between the expression of KatE and KatG, we tested the catalase activity in the isolated catalase-negative Suc^{++} mutants from three representative VTEC strains EDL933, CL106, and EC3-377 using native-PAGE gels. As expected, all Suc^{++} mutants exhibited substantially reduced HPII catalase activity (Figure 6.1A). The higher expression of HPI in Suc^{++} mutants (Figure 6.1A) is not entirely

unexpected. Low levels of HPII may lead to higher accumulation of intracellular hydrogen peroxide which can activate OxyR, the main regulator of HPI (Christman *et al.*, 1985).

The enhanced growth of Suc^{++} mutants was assessed in liquid media by comparing the growth of wild type EDL933 and the derived mutants. There was no difference between growth of mutants and wild type cultures on glucose. However, growth of wild type strains on succinate was much lower compared with that of mutant strains, with a 10-fold longer generation time (Table 6.3). In addition, the Suc^{++} mutants grew similarly to an *rpoS*-null deletion mutant on succinate and glucose (Table 6.3).

Characterization of *rpoS* mutations in Suc^{++} mutants

To determine if the loss of RpoS function in Suc^{++} mutants resulted from acquired mutations in *rpoS*, the *rpoS* region of VTEC Suc^{++} mutants exhibiting catalase deficiency was amplified and sequenced in both directions. Inactivating mutations, predicted to result in premature termination of RpoS, were identified in the *rpoS* gene in all the Suc^{++} catalase deficient mutants. These acquired mutations included transitions, transversions, deletions and duplications. To ensure that enhanced growth on succinate was attributable to acquisition of *rpoS* mutations (rather than to secondary mutations), selected Suc^{++} mutants carrying *rpoS* null mutations were complemented with a plasmid-borne functional *rpoS* (Kitagawa *et al.*, 2005). As expected, the growth of transformed cells on succinate was much slower than that of the Suc^{++} parental strains, confirming that acquired mutations in *rpoS* are responsible for the enhanced growth of Suc^{++} mutants (data not shown). To examine the effect of mutation on RpoS levels, Western analysis

using polyclonal antisera to RpoS was performed. In the selected representative Suc^{++} mutants, RpoS protein was absent (Figure 6.1B). In addition, the expression of AppA, a RpoS-dependent protein which has both acid phosphatase and phytase activities (Atlung *et al.*, 1997; Golovan *et al.*, 2000), was substantially decreased in Suc^{++} mutants to about 25% of the expression level in isogenic wild type strains (Figure 6.1B).

Growth of VTEC strains and derivative Suc^{++} mutants under aerobic and anaerobic conditions

Effective utilization of succinate as a carbon source depends on the availability of an external electron receptor such as oxygen. However, in the human intestine, low oxygen tension permits *E. coli* to grow by fermentation or respiration using an alternative electron acceptor. As nitrate is readily available in the human intestine (14 μmol/kg (Saul *et al.*, 1981)) and can be readily utilized by intestinal bacterial flora including *E. coli* (Forte *et al.*, 1999; Witter *et al.*, 1981) we examined succinate selection using this alternate electron receptor. Interestingly, host nitrate synthesis can be stimulated in response to infections caused by gastroenteric pathogens (Forte *et al.*, 1999). To test if selection for loss of RpoS can occur under low oxygen conditions, cultures were grown in anaerobic jars (see Methods). We first compared the anaerobic growth of wild type and aerobically-selected Suc^{++} mutants on glucose and succinate plates. Wild type EDL933 grew as well as an isogenic *rpoS* knockout mutant and derivative Suc^{++} mutants on glucose, while the *rpoS* and Suc^{++} mutants grew much better than wild type on succinate under both aerobic and anaerobic conditions (Figure 6.2). The growth of Suc^{++} mutants was similar to that of the control *rpoS* null mutant under all conditions tested.

All VTEC strains were then tested for selection on succinate under anaerobic conditions. As under aerobic conditions, Suc^{++} mutants could be selected from all tested strains, except for CL3, R82F2 and N99-4390. Most (87%) of the Suc^{++} had reduced catalase activity. We sequenced the *rpoS* region of 15 Suc^{++} mutants isolated from EDL933 and found mutations in *rpoS*, resulting in impaired RpoS function, in 13 mutants while the *rpoS* gene in the other two Suc^{++} mutants remained unchanged (data not shown).

Expression of virulence-related traits, RDAR and cell adherence

Mutations in *rpoS* may affect virulence factor expression in pathogenic strains (Fang *et al.*, 1992; Norel *et al.*, 1992). To test this, we examined two virulence-related traits, the RDAR morphotype and cell adherence. Extracellular components, such as curli fimbriae and cellulose, are correlated with biofilm formation and virulence in *Salmonella sp.* and *E. coli* strains (Bian *et al.*, 2000; Romling, 2005; Uhlich *et al.*, 2002). The expression of curli and cellulose can be visualized by staining with Congo Red dye to produce a red, dry and rough morphotype (RDAR) (Bokranz *et al.*, 2005; Romling, 2005). Biosynthesis of both curli and cellulose is positively regulated by RpoS through a transcriptional regulator CsgD in *E. coli* K12 (Romling *et al.*, 1998; Weber *et al.*, 2006). However, to our knowledge, the role of RpoS in expression of RDAR has not been previously tested in pathogenic *E. coli* isolates. Wild type EDL933 exhibited a more pronounced RDAR morphotype than an isogenic *rpoS* null deletion mutant and Suc^{++} mutants (Figure 6.3A), suggesting that RpoS is important for RDAR development. Similar results were also obtained for other VTEC strains (data not shown). Cell adherence assays were performed using human liver epithelial cell HepG2. The

adherence of wild type EDL933 to HepG2 cells in tissue culture was two-fold higher than that of *rpoS* and Suc^{++} mutants ($P<0.05$) (Figure 6.3B), indicating that Suc^{++} mutants are impaired in cell adherence due to loss of RpoS function. This is consistent with previous results that over-expression of RpoS stimulates cell adherence (Bhagwat *et al.*, 2005).

Suc^{++} mutants with an intact RpoS function (*rpoS*$^+$)

During the screening for the Suc^{++} phenotype, we found that a small proportion of Suc^{++} mutants from strains EDL933 (8%), CL106 (16%), and EC6-484 (33%) were catalase-positive, a presumptive indication that RpoS was functional. To confirm this, we sequenced the *rpoS* region of five such Suc^{++} mutants (three aerobically isolated and the other two anaerobically isolated) of strain EDL933. As expected, there was no mutation in the *rpoS* gene in these mutant strains. However, these grew much better than wild type when grown on succinate (generation time: 240 ± 31 min) and fumarate (generation time: 306 ± 33 min) (Table 6.3). These data suggest that non-*rpoS* mutations are a minor component in the poor carbon selection process.

Effect of the *rpoS* mutation on metabolism by Phenotype Microarray analysis

RpoS is known to negatively control many genes involved in metabolism (Dong *et al.*, 2008b; Patten *et al.*, 2004; Rahman *et al.*, 2006), and therefore, mutations in *rpoS* are likely to exert pleiotropic effects on metabolism. To test this, we compared wild type MG1655 and its derivative *rpoS* deletion mutants (Patten *et al.*, 2004) using Phenotype Microarray analysis (Biolog, Hayward, CA). The *rpoS* mutants exhibited better respiration on 8 carbon sources and 92 nitrogen sources but less respiration on four carbon sources and one nitrogen source (Table 6.4). The substantial impact of *rpoS*

mutations on nutrient utilization suggest that the beneficial effect of loss of RpoS in one selection condition may be extended to other conditions as well.

Enhanced growth of Suc^{++} (*rpoS*$^{+}$ and *rpoS*$^{-}$) mutants is not limited to the TCA cycle intermediates

To extend the phenotype screening results to pathogenic *E. coli*, we tested the growth of EDL933 and derivative *rpoS* and Suc^{++} (*rpoS*$^{+}$ and *rpoS*$^{-}$) mutants on selected carbon sources (20 mM each) that best supported differential respiration of *rpoS* mutants relative to wild type (Figure 6.4). Glucose and succinate were also tested as controls for comparison. As expected, compared with wild type, the *rpoS* and Suc^{++} mutants grew similarly on glucose but much better on succinate. Among the Biolog compounds tested, the *rpoS* and Suc^{++} mutants, including the Suc^{++} (*rpoS*$^{+}$) mutants, grew better than wild type on D-glucuronic acid or glutamine as the sole carbon source. However, none of these strains could grow on threonine or proline as the sole carbon source, which is likely due to differences in strain background and experimental conditions. The enhanced growth of mutants on D-glucuronic acid and glutamine confirmed that mutations selected on succinate have pleiotropic effects on utilization of other nutrient sources.

6.5 Discussion

Understanding how pathogens adapt and mutate in response to growth environments is critical in deciphering many of the unknowns regarding pathogenesis, such as the emergence of new pathogens, the increased resistance to antibiotics, and the long-term persistence in host environment. In this study, we report that a metabolic selection mechanism for loss of RpoS, a central stress and adaptation regulator, in

representative verocytotoxin-producing *E. coli* strains, may be responsible for the occurrence of *rpoS* mutations among pathogenic *E. coli* isolates. In surveying the *rpoS* gene among *E. coli* isolates, we found many mutations in *rpoS*, some of which result in loss of RpoS function. Among the VTEC strains tested, most grow poorly on succinate (like laboratory K12 strains) but some strains grow well. Those that grow poorly all have intact *rpoS*. In contrast, strains that grow well on succinate can be distinguished into two groups, one with intact *rpoS* and the other with truncated *rpoS*. The difference in utilization of succinate and *rpoS* status of these natural isolates is likely the result of certain selection that has occurred in natural environments. By testing growth-enhanced mutants (Suc^{++}) selected from strains with intact *rpoS* on succinate, we identified two groups of mutants, one with impaired RpoS while the other with functional RpoS, a finding that is in agreement with the two parallel groups found in natural VTEC isolates. This correlation provides support that metabolic selection is a natural process relevant to pathogenic strains.

Most of the selected Suc^{++} mutants had lost RpoS function, confirmed by both DNA sequencing and Western analyses. The positive selection pressure for *rpoS* mutations may result from the known negative effect of RpoS on a large group of genes including those in the TCA cycle (Dong *et al.*, 2008b; Jung *et al.*, 2006; Patten *et al.*, 2004; Rahman *et al.*, 2006). In *E. coli*, the number of sigma factors greatly exceeds the number of RNA core polymerase, and thus there is a strong competition among sigma factors for binding to the core polymerase (Ishihama, 2000). Genes involved in the TCA cycle are primarily transcribed by RpoD, the vegetative sigma factor (Ishihama, 2000).

The absence of RpoS, caused by *rpoS* mutation or low levels of expression, may thus result in an increase in RpoD-associated RNA polymerase, thereby leading to enhanced expression of the TCA cycle genes (Farewell *et al.*, 1998a; Ferenci, 2003; Patten *et al.*, 2004).

Mutations in *rpoS* result in substantial phenotypic modification. A previous study using similar Biolog screening technology has shown that the mutation of *rpoS* stimulates metabolism of about 20 carbon compounds in some *E. coli* strains but only has a minor effect in MG1655 (King *et al.*, 2004). By comparing respiration rates instead of final OD employed in the previous study, we extended previous results and found that the respiration of the *rpoS* deletion mutant (Patten *et al.*, 2004) increased in over 100 new compounds compared with wild type MG1655. Thus, we suggest that RpoS, known as a master stress regulator, can be also envisioned as a central metabolism repressor, whose inactivation results in enhanced nutrient utilization abilities. RpoS, therefore, is a critical control in cellular fitness, which can be defined as better survival or growth depending on environmental conditions. During stress conditions, activation of RpoS promotes survival by protecting cells from multiple stresses. During growth on poor carbon sources, however, mutating RpoS results in better growth by conferring cells enhanced metabolic abilities. In either case, cell fitness is effectively achieved through modulation of a single factor, RpoS.

What are the potential effects for loss of RpoS in pathogenic *E. coli*? On one hand, mutations in *rpoS* in Suc^{++} mutants may attenuate RpoS-mediated stress resistance and virulence functions. Suc^{++} mutants were deficient in RDAR morphotype development, an

indicator for expression of extracellular components that are important for bacterial pathogenesis (Uhlich *et al.*, 2002). We also found that adherence to epithelial cells was impaired in *rpoS* and Suc^{++} mutants, indicating a decrease in pathogenesis. On the other hand, because *rpoS* mutants can better utilize non-preferred carbon sources (Chen *et al.*, 2004), *rpoS* mutations may help *E. coli* compete with other bacteria in the human intestine, a highly-competitive environment harboring at least 1,000 different species (Sears, 2005). It has been reported that *rpoS* mutants outcompete wild type strains in colonizing mouse intestine (Krogfelt *et al.*, 2000). Although mutations in *rpoS* may increase the sensitivity of *E. coli* cells to exogenous stresses (due to the loss of protective functions such as catalase), enhanced metabolism of less-preferred carbon sources may offset this deficiency and lead to, on the whole, selection for *rpoS* mutations even in a competitive environment (Ferenci, 2003). This has led to the proposal by Ferenci and co-workers that the loss of RpoS may be viewed as an increase in metabolic fitness at the expense of a loss of protective functions (King *et al.*, 2006). A slightly different scenario may be operant in VTEC strains where loss of pathogenic functions, such as curli fimbriae, may occur during selection for enhanced metabolic fitness (this study), even in the host environment where *rpoS* mutants can be isolated (Waterman & Small, 1996). It is also important to note that mutants of *rpoS* were isolated at a low frequency close to spontaneous mutation frequency (10^{-8}), suggesting that naturally occurred *rpoS* mutants would constitute, at least initially, only a small fraction of *E. coli* population unless there is a prolonged strong selective condition (i.e., poor carbon source).

Although loss of RpoS appears to be the usual consequence of selection for metabolic fitness, clearly other mutation(s) can also occur and result in an enhanced growth phenotype (e.g., five of 30 EDL933-derived Suc^{++} mutants characterized did not acquire mutations in *rpoS*). The occurrence of non-*rpoS* mutations may be strain-specific, since such mutations could not be selected from K12 strains (Chen *et al.*, 2004) or from some of the tested VTEC strains in this study. The non-*rpoS* mutations may represent another adaptation strategy of *E. coli* in natural environments, in which metabolic fitness is achieved without the cost of RpoS-controlled stress resistance system (Figure 6.5). Of the ten tested wild type VTEC strains, three grew well on succinate, among which two strains (CL3 and R82F2) are $RpoS^{+}$ and one (N99-4390) is $RpoS^{-}$. It is possible that both *rpoS* and non-*rpoS* mutations for enhanced growth could have occurred in nature among *E. coli* isolates. Given the importance of RpoS in cell survival, growth-enhanced mutations that retain RpoS functions may be better preserved among *E. coli* natural populations. Using representative natural commensal *E. coli* isolates from the ECOR collection (Ochman & Selander, 1984), we recently found that seven of ten wild type ECOR strains can utilize succinate well; six of them were $RpoS^{+}$ and one was $RpoS^{-}$ (Dong and Schellhorn, unpublished data).

In summary, non-preferred carbon sources can select for *rpoS* mutations in pathogenic VTEC *E. coli* strains. The resultant Suc^{++} mutants also exhibited growth advantages on succinate minimal media under anaerobic conditions with nitrate as a respiratory electron receptor. Suc^{++} mutants harboring *rpoS* mutations were impaired in the development of RDAR morphotype and the ability of adherence to epithelial cells.

Heterogeneity of *rpoS* as a result of the selection may thus contribute to differences in pathogenesis among pathogenic *E. coli* strains.

Table 6.1 Suc^{++} mutants selected from VTEC strains with attenuated or intact RpoS functions.

Twelve Suc^{++} mutants from each strain were tested for catalase activity using a plate catalase assay. Mutants impaired in catalase were considered as putative *rpoS* mutants. Detailed VTEC strain information is described elsewhere (Karmali *et al.*, 2003). N/A: no Suc^{++} mutant selected.

Sero-pathotype	Serotype	Strain	Source	Host	Number of mutants		Ratio of
					Suc^{++}	*rpoS*	*rpoS*/Suc^{++}
A	O157:H7	EDL933	J. Kaper	Human	12	11	0.92
B	O121:H19	CL106	LFZ	Human	12	10	0.83
	O111:NM	R82F2	LFZ	Human	N/A		N/A
C	O5:NM	N00-4067	BCCDC, NLEP	Human	12	12	1.00
	O113:H21	CL3	LFZ	Human	N/A		N/A
	O121:NM	N99-4390	BCCDC, NLEP	Human	N/A		N/A
D	O103:H25	N00-4859	BCCDC, NLEP	Human	12	12	1.00
	O172:NM	EC6-484	LFZ	Bovine	12	8	0.67
E	O84:NM	EC2-044	LFZ	Bovine	12	12	1.00
	O98:H25	EC3-377	LFZ	Bovine	12	12	1.00

Table 6.2 Polymorphic codons in *rpoS* among VTEC strains.

The *rpoS* gene in *E. coli* K-12 MG1655 strain was used as the reference for comparison. The G-C transition at codon 33 in MG1655 results in a conversion of glutamate to glutamine, while the G-T transversion in N99-4390 at codon 243 forms a stop codon resulting in a truncated RpoS protein. The other polymorphic sites are synonymous mutations.

Codon	33	54	119	129	154	181	191	243	273	317
	Glu	Val	Leu	Arg	Ile	Thr	His	Glu	Val	Leu
Consensus	GAG	GTG	CTT	CGC	ATT	ACC	CAT	GAG	GTG	CTG
MG1655	**C**..	...	...	...	...	...	...	...	...	...
EDL933	...	...	...	..T	...	..A	...	...	..A	...
CL106	...	...	...	...	...	...	...	...	...	...
R82F2	...	..A	...	...	...	...	...	...	...	...
N00-4067	...	...	...	...	...	...	...	...	...	..A
CL3	...	...	...	...	...	...	...	...	...	...
N99-4390	...	...	..G	...	..C	...	..C	**T**..		
N00-4859	...	...	...	...	...	...	...	...	...	...
EC6-484	...	...	...	...	...	...	...	...	...	..A
EC2-044	...	...	...	...	...	...	...	...	...	...
EC3-377	...	...	...	...	...	...	...	...	...	...

Table 6.3 Growth of EDL933 and isogenic mutants in M9 minimal media with glucose, succinate, fumarate or malate as the sole carbon source.

M9 minimal media with glucose (0.4%), succinate (1%), fumarate (1%), or malate (1%) were prepared as described in Methods. Cells were grown in LB to an OD_{600} of 0.6, washed with 1X M9 salts at 4°C, and inoculated into fresh minimal media at a starting OD600nm of 0.05. Cultures were incubated at 37°C and sampled every hour. This experiment was performed in triplicate.

Substrate	Generation time (min)		
	WT	*rpoS*	Suc^{++}
Glucose	94 ± 8	102 ± 28	106 ± 8
Succinate	1,443 ± 250	93 ± 10	116 ± 14
Fumarate	2,780 ± 422	135 ± 12	139 ± 6
Malate	2,107 ± 731	1,443 ± 31	1,147 ± 16

Table 6.4 Phenotypic Microarray (PM) analyses of growth changes resulted from *rpoS* mutations.

PM-value shows the growth difference between WT and *rpoS* mutants on these nutrients as carbon or nitrogen sources. Positive values show phenotypes gained in *rpoS* mutants while negative values show phenotypes lost because of *rpoS* mutations. In total, *rpoS* mutants grew better on 92 nitrogen sources tested, and the top 10 are listed.

Carbon source	PM-value	Nitrogen source	PM-value
β-Methyl-D-Glucuronic Acid	102	Gly-Phe-Phe	157
L-Galactonic Acid-g-Lactone	98	Guanosine	137
L-Threonine	92	Nitrite	133
L-Alaninamide	70	D-Valine	125
L-Glutamine	67	Phe-b-Ala	124
L-Proline	66	L-Tyrosine	124
D-Trehalose	64	Tyr-Phe	120
D-Saccharic Acid	50	Phe-Phe	119
Propionic Acid	-51	Tyr-Ile	118
Glycyl-L-Proline	-69	L-Glutamic Acid	113
α-Keto-Butyric Acid	-86	Ser-Gln	-67
α-Hydroxy-Butyric Acid	-110		

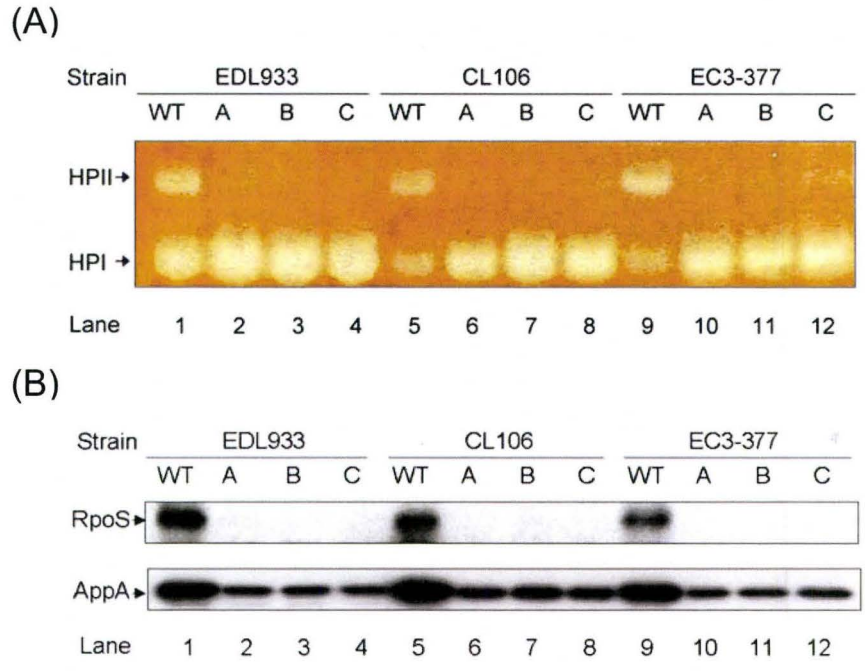

Figure 6.1 Catalase activity and RpoS expression in representative Suc^{++} mutants of VTEC strains EDL933, CL106 and EC3-377.

(A) Samples were separated by native PAGE and stained for catalase activity. Catalase HPI (KatG) and HPII (KatE) are indicated. (B) Expression of RpoS and RpoS-regulated AppA by Western analysis. Mutations in *rpoS* were identified in these tested Suc^{++} mutants by sequencing. To confirm equal protein loading, identical gels were run in parallel and stained by Coomassie Blue R-250 (Dong *et al.*, 2009b; Dong & Schellhorn, 2009a).

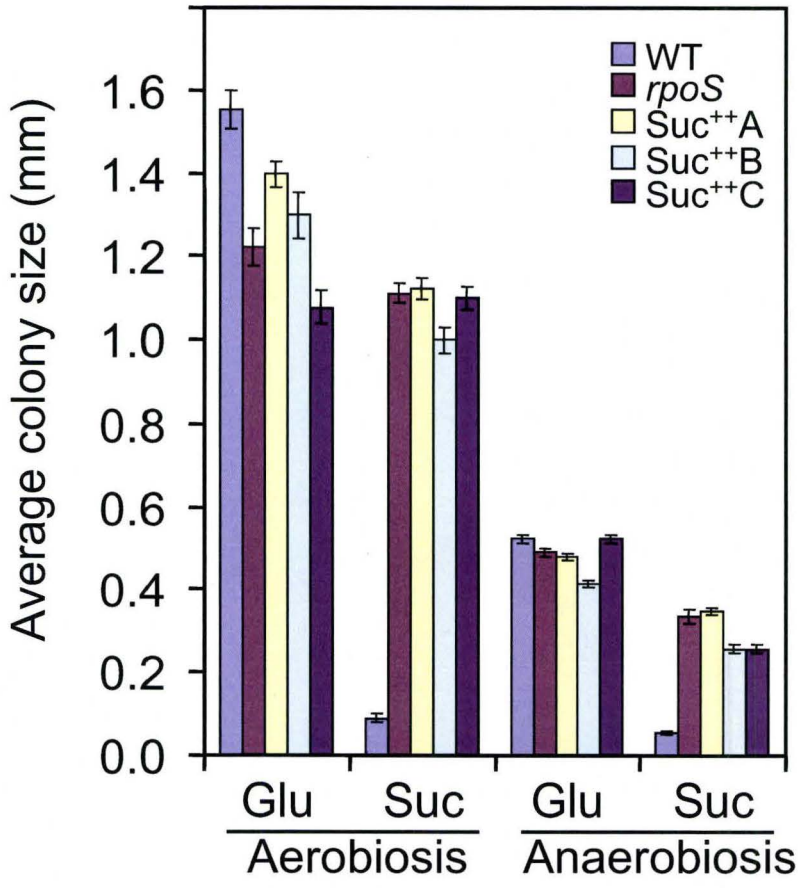

Figure 6.2 Growth of EDL933 and derivative Suc^{++} mutants on M9 glucose (Glu) and succinate (Suc) media.

Colony size (diameter) was determined under a light microscope at 40x magnification.

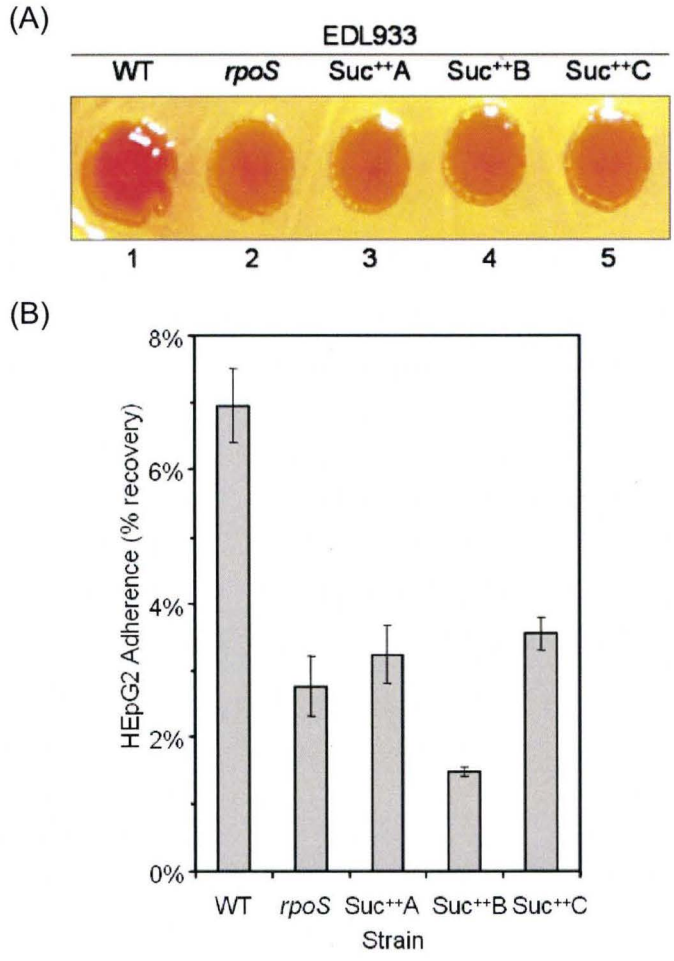

Figure 6.3 Virulence-related traits, RDAR and cell adherence.

(A) Development of RDAR morphotype is impaired in Suc^{++} mutants. Cells were replica-plated on CR (Congo Red) plates and incubated at 25°C for 48h. (B) Cell adherence to epithelial cells. The adherence was expressed as the percentage of cells surviving the washing process. *rpoS* designates the constructed *rpoS* null-deletion mutant.

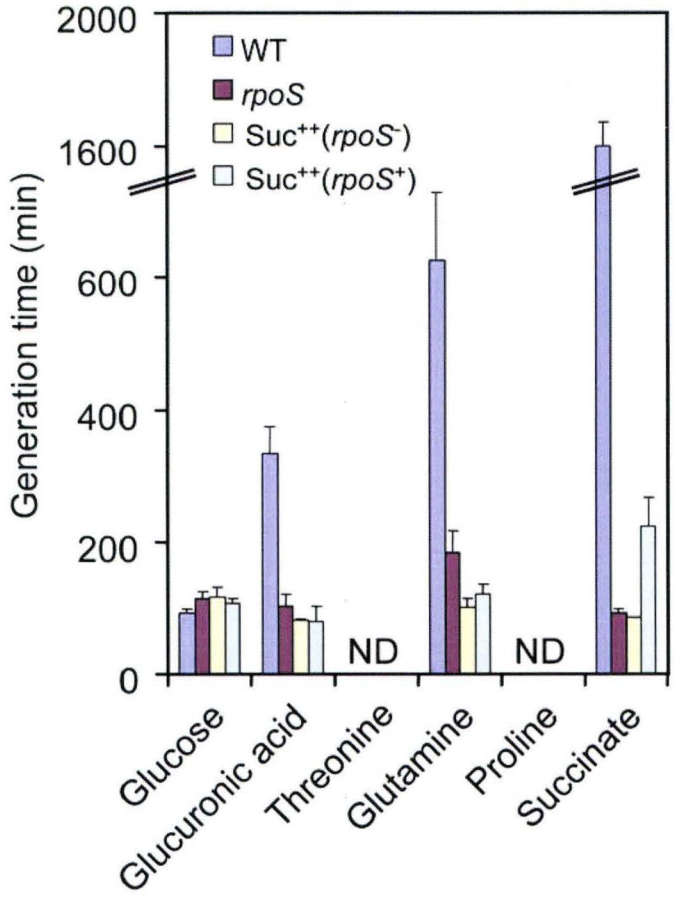

Figure 6.4 Growth of EDL933 and derivative mutants on different carbon sources.

"ND": not detected. Cells were grown in LB media to OD_{600} 0.6, washed and inoculated to fresh media to a starting OD_{600} of 0.05. Cultures were then grown at 37°C with vigorous shaking (200 rpm) and sampled every hour for 10 hours to monitor growth. D-glucuronic acid, threonine, glutamine or proline were added to M9 minimal media as the sole carbon source to a final concentration of 20 mM.

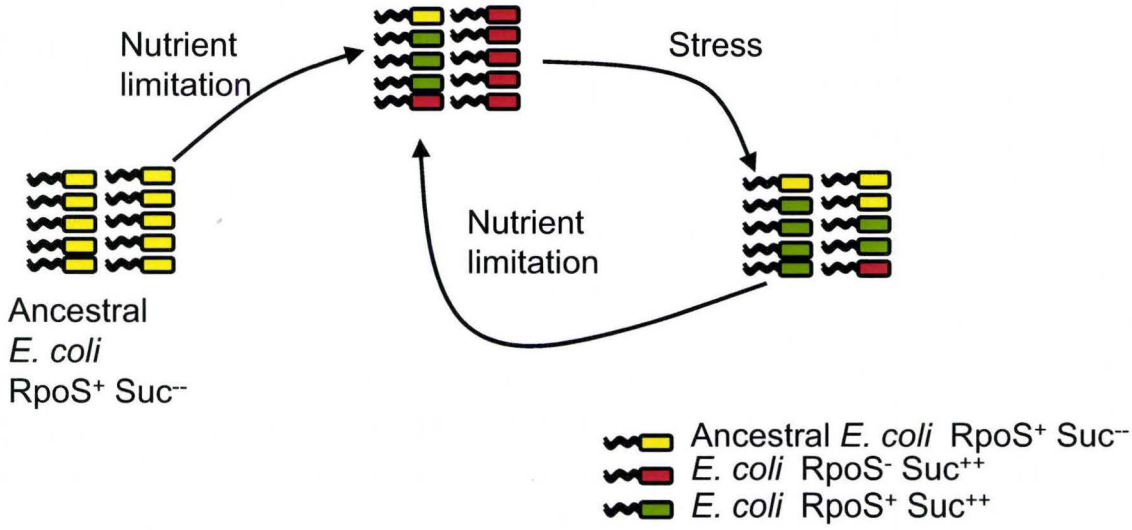

Figure 6.5 Dynamic view of RpoS status and metabolic fitness in natural *E. coli* populations.

It is postulated that the ancestral *E. coli* strain possesses a functional *rpoS* allele ($RpoS^+$) but cannot grow well on poor nutrient sources (Suc^{--}). Upon exposure to nutrient limitation, mutants (Suc^{++}) exhibiting enhanced metabolic activity can be selected and become dominant among the population. These mutants consist of two groups, $RpoS^+$ and $RpoS^-$. Under stress conditions, however, the proportion of $RpoS^-$ mutants decreases because of the loss of protection by RpoS-controlled functions, and the abundance of strains with functional RpoS increases. Because cells likely are constantly facing selection between nutrient limitation and stress in nature, the population of *E. coli* isolates is in a dynamic status in terms of RpoS function and metabolic fitness.

CHAPTER VII

Role of RpoS in virulence of pathogens

from

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7.1 Preface

The contents of this chapter were contributed primarily by the author of this thesis. The text was written, in its entirety, by Tao Dong. This chapter is adapted from a manuscript accepted for publication in Infection and Immunity to fit the thesis format requirements.

7.2 Abstract

Understanding mechanisms of bacterial pathogenesis is critical for infectious disease control and treatment. Infection is a sophisticated process that requires the participation of global regulators to coordinate expression of not only genes encoding for virulence factors but also those involved in other physiological processes, such as stress response and metabolic flux, to adapt to host environments. RpoS is a key response regulator to stress conditions in *E. coli* and many other proteobacteria. In contrast to its conserved well-understood role in stress response, effects of RpoS on pathogenesis are highly variable and dependent on species. RpoS contributes to virulence through either enhancing survival against host defense systems or directly regulating expression of virulence factors in some pathogens, while RpoS is dispensable, or even inhibitory, to virulence in others. In this review, we focus on the distinct and niche-dependent role of RpoS in virulence by surveying recent findings in many pathogens.

7.3 Introduction

RpoS is an alternative sigma factor of RNA polymerase primarily found in beta- and gamma-proteobacteria (Dong *et al.*, 2008a; Hengge-Aronis, 2000). RNA core polymerase requires a sigma factor for promoter recognition and transcription initiation. In addition to housekeeping sigma factors that control transcription of essential genes, bacteria also possess alternative sigma factors that recognize the promoters of a specific set of genes. There are seven known sigma factors in the gram-negative model bacterium *Escherichia coli* (Ishihama, 2000) and 18 in the gram-positive bacterium *Bacillus subtilis*

(Gruber & Gross, 2003). The contribution of alternative sigma factors to virulence can be direct through regulated expression of virulence genes or indirect by enhancing survival against host defense and other stress conditions (Kazmierczak *et al.*, 2005).

Pathogenic bacteria experience many stresses during transmission and infection. For example, the enterohemorrhagic *E. coli* O157:H7 strain may face nutrient limitation and heat exposure in natural environments, and acid stress and host defense after entry into human hosts. The ability to quickly adapt to changing environments is therefore critical for bacterial pathogens to successfully transmit and infect hosts. One of the most important adaptation factors in *E. coli* is RpoS (Dong *et al.*, 2008a; Hengge-Aronis, 2000). The RpoS regulon, comprising 10% of *E. coli* genes (Dong *et al.*, 2008b; Dong & Schellhorn, 2009a; Lacour & Landini, 2004; Patten *et al.*, 2004; Weber *et al.*, 2005), plays a critical role in survival to several stresses, including acid (Small *et al.*, 1994), heat (Hengge-Aronis *et al.*, 1991), oxidative stress (Sammartano *et al.*, 1986), starvation (Lange & Hengge-Aronis, 1991b), and near-UV exposure (Sammartano *et al.*, 1986). In *E. coli*, the levels of RpoS are low in exponential phase (Dong *et al.*, 2008b; Lange & Hengge-Aronis, 1994), due to reduced transcription (Lange & Hengge-Aronis, 1994), attenuated translation (Lange & Hengge-Aronis, 1994) and, most importantly, rapid proteolysis mediated by RssB, a chaperone protein that binds to RpoS and directs the RssB-RpoS complex to the ClpXP protease (Lange & Hengge-Aronis, 1994; Muffler *et al.*, 1996a; Pratt & Silhavy, 1996; Zhou & Gottesman, 1998). The degradation of RpoS is suppressed in stationary phase (Becker *et al.*, 1999; Zhou & Gottesman, 1998), resulting in increased RpoS levels (Lange & Hengge-Aronis, 1994). Expression of RpoS is

sensitive to environmental changes and is under control of many regulatory factors, such as acetate, ppGpp and cAMP (reviewed in (Dong *et al.*, 2008a; Hengge-Aronis, 2000)).

The RpoS-bearing bacteria have a broad host range, including human pathogens (e.g., *E. coli* and *Vibrio cholerae*), animal pathogens (e.g., *Citrobacter rodentium* and *Salmonella typhimurium*), insect pathogens (e.g., *Serratia entomophila* and *Xenorhabdus nematophilus*), and plant pathogens (e.g., *Burkholderia plantarii, Erwinia carotovora, Ralstonia solanacearum*). RpoS is required for resistance to many stresses in these bacteria (Table 7.1). However, the effect of RpoS on virulence is variable, differing even in closely-related species. RpoS is required for virulence in some pathogens, including *Salmonella enterica*, *V. cholerae*, *B. plantarii*, and *S. entomophila*, but is less important in other species (Table 7.1). Despite the considerable accumulated information on RpoS control of virulence functions in specific bacteria, there is, as yet, no comprehensive review on this topic. Therefore, this review summarizes the involvement and contribution of RpoS in virulence of RpoS-bearing pathogens. We place special focus on studies that have tested *rpoS* in host (animal or plant) or cell culture models.

7.4 Enteric pathogens

E. coli commensal strains are a common component of human intestinal flora, but there are many *E. coli* pathogens, including enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC), which can cause severe gastrointestinal disease. Though the regulation of RpoS in gene expression is best-studied in *E. coli*, RpoS involvement during enteropathogenesis is unresolved, probably due to the lack of effective animal models (Mundy *et al.*, 2005). Infection of *E. coli* in mice does not cause

intestinal disease as it does in humans (Mundy *et al.*, 2005). However, several virulence traits are known to be controlled by RpoS. For example, the production of curli, important for colonization, is dependent on RpoS (Olsen *et al.*, 1993). RpoS also controls the expression of the *ehxCABD* operon, encoding enterohemolysin, in *E. coli* O157:H7 (Li *et al.*, 2008). A common characteristic of EPEC and EHEC infection is the formation of attaching/effacing (A/E) lesions, which requires expression of genes on a pathogenicity island, the locus of enterocyte effacement (LEE) (Elliott *et al.*, 1998). The LEE island harbors five polycistronic operons, which encode a type III secretion system (T3SS) and secreted proteins essential for virulence (Deng *et al.*, 2004). The effect of RpoS on the expression of LEE genes has been studied by several independent groups and variable results have been reported. Expression of *lacZ* fusions to promoters of LEE3 and EspA is higher in wild type K12 strain than in isogenic *rpoS* mutants (Beltrametti *et al.*, 1999; Sperandio *et al.*, 1999). However, other studies report that RpoS is a negative regulator of LEE genes (Iyoda & Watanabe, 2005; Laaberki *et al.*, 2006; Tomoyasu *et al.*, 2005). It is known that expression of LEE genes varies among *E. coli* species (Dowd & Ishizaki, 2006; Roe *et al.*, 2003), though the basis for this is not yet fully understood. One likely contributing factor is the sequence variation in the *pch* prophage adjacent genomic regions that affects expression of LEE genes in *E. coli* O157:H7 subpopulations (Dowd & Ishizaki, 2006). Expression of LEE genes is also dependent on environmental conditions (Abe *et al.*, 2002; Kenny *et al.*, 1997). We recently found that RpoS positively regulates expression of Ler, a LEE encoded regulator, in stationary phase in LB media (a non-inducing condition for LEE expression), but negatively regulates expression of Ler

and other LEE genes under LEE induction conditions (Dong & Schellhorn, 2009b). Interestingly, mutations in Hfq, a small RNA chaperone protein that is required for effective RpoS translation (Muffler *et al.*, 1996b), also result in elevated expression of LEE genes through post-transcriptional control (Hansen & Kaper, 2009). However, this effect is RpoS-independent (Hansen & Kaper, 2009).

Because of its importance in the bacterial stress responses, RpoS may be required for *E. coli* to survive passage through the gastrointestinal tract. When *rpoS* mutants and wild type of *E. coli* O157:H7 are fed to mice and calves, the recovery of wild type in feces is much higher that that of *rpoS* mutants, probably due to the RpoS-regulated acid resistance response (Price *et al.*, 2000). RpoS also plays a role in intestinal colonization of *E. coli* strain BJ4 in streptomycin-treated mice (Krogfelt *et al.*, 2000). Colonization in mice by *rpoS* mutants is as high as that of wild type in separate infection (Krogfelt *et al.*, 2000). However, *rpoS* mutants can outcompete wild type in mouse colon during co-infection, suggesting that *rpoS* mutants may be able to better utilize a specific limiting nutrient in colon (Krogfelt *et al.*, 2000).

In non-enteric *E. coli* pathogens, RpoS also controls expression of virulence traits. In *E. coli* K1 strains that can cause neonatal meningitis, RpoS is important for invasion of brain microvascular endothelial cells, though the mechanism has yet to be identified (Wang & Kim, 2000). RpoS also positively controls motility and biofilm formation in uropathogenic *E. coli* (UPEC) strain UTI89 (Kulesus *et al.*, 2008). However, mutations in *rpoS* have little effect on biofilm formation in UPEC strain 536 (Beloin *et al.*, 2006) or on colonization of urethra, bladder, and kidney in UPEC CFT073(Culham *et al.*, 2001).

Citrobacter rodentium is a natural murine enteropathogen closely related to *E. coli*, and, similar to EPEC and EHEC strains, utilizes the LEE-encoded type III secretion system for delivery of virulence factors (Mundy *et al.*, 2005). Infection of mice using *C. rodentium* provides a promising alternative model to study enteropathogenesis in natural hosts (Mundy *et al.*, 2005; Wiles *et al.*, 2006). The *rpoS* mutant of *C. rodentium* is more sensitive to heat and oxidative stress than the wild type, indicating a conserved RpoS function (Dong *et al.*, 2009b). Colonization and virulence of *C. rodentium* is attenuated in *rpoS* mutants during infection in mice (Dong *et al.*, 2009b). However, *rpoS* mutants outcompete wild type during co-infection in mouse colon (Dong *et al.*, 2009b). In contrast to the negative regulation of LEE genes by RpoS in *E. coli*, RpoS has a moderate yet positive effect on expression of LEE genes in *C. rodentium* (Dong *et al.*, 2009b).

Salmonella serovars can cause severe systemic infection (typhoid fever) or non-systemic gastroenteritis depending on the serotypes (Ohl & Miller, 2001). The systemic infection of *S. typhimurium* in mice resembles the severe infection of *S. typhi* in humans causing typhoid fever (Ohl & Miller, 2001). RpoS plays a critical role in *Salmonella* virulence (Fang *et al.*, 1992). Specifically, RpoS is important for persistence in lymphoid organs, such as the spleen (Coynault *et al.*, 1996; Kowarz *et al.*, 1994) and liver (Coynault *et al.*, 1996), and for initial stages of infection in murine Peyer's patches (Coynault *et al.*, 1996; Nickerson & Curtiss, III, 1997). RpoS acts primarily through positive regulation of expression of the plasmid-borne *spvR* and *spvABCD* genes that are required for intracellular growth and systemic infection in mice and humans (Fang *et al.*, 1992; Norel *et al.*, 1992). RpoS positively regulates the expression of SpvR, a LysR-

family regulator, which accounts for the RpoS-dependence of *spvABCD* (Abe *et al.*, 1994; Chen *et al.*, 1995; Heiskanen *et al.*, 1994; Kowarz *et al.*, 1994). Interestingly, *rpoS* mutants are also less virulent than plasmid-cured wild type in mouse infections, suggesting that RpoS regulates chromosomal virulence determinants as well (Fang *et al.*, 1992). Identified chromosomal virulence factors in *Salmonella* include YedI, SodCII, and genes for curli synthesis. The *yedI* gene is RpoS-dependent and is important for persistence during infection in mice by *S. typhimurium* (Erickson & Detweiler, 2006). The *yedI* mutants are impaired in competition with wild type during oral infection and sensitive to polymyxin B, a cationic antimicrobial peptide (Erickson & Detweiler, 2006). Genes encoding for curli production, *csgD* and *csgAB*, are positively regulated by RpoS in *Salmonella* (Romling *et al.*, 1998). There are two *sodC* alleles, *sodC*I and *sodC*II, encoding superoxide dismutase in *Salmonella*. Most *Salmonella* serotypes possess *sodC*II (Fang *et al.*, 1999). SodCII is controlled by RpoS and is important for virulence likely by protecting bacteria against superoxide-dependent host defense (Fang *et al.*, 1999; Sansone *et al.*, 2002a; Sly *et al.*, 2002). However, other studies show that only SodCI but not SodCII contributes to virulence (Krishnakumar *et al.*, 2004; Sansone *et al.*, 2002b). The small RNA chaperone protein Hfq, an important RpoS regulator, also plays an essential role in virulence in *Salmonella* through post-transcriptional regulation of many virulence genes (Sittka *et al.*, 2007). This virulence effect, however, is largely independent of RpoS (Sittka *et al.*, 2007).

In addition to regulating virulence functions, RpoS is essential for survival against stresses, such as oxidative stress, starvation, DNA damage, and low pH, which

Salmonella likely encounters during intracellular growth in host macrophages (Fang *et al.*, 1992). In *S. typhimurium*, RpoS and RpoS-regulated genes, including *katE* and *spv*, are induced after invasion of epithelial cells and macrophages (Chen *et al.*, 1996). RpoS is also important for survival of *S. typhi* in mouse peritoneal macrophages through protection from nitric-oxide produced by macrophages (Alam *et al.*, 2006). Though not required for survival of *S. typhi* in the human promonocytic macrophage THP-1, RpoS is required for the effective induction of macrophage apoptosis by *S. typhi* during intracellular infection (Khan *et al.*, 1998).

Interestingly, *Salmonella* infect and grow intracellularly in cultured epithelial and macrophage cells but not fibroblasts and other non-phagocytic cells (Matsui *et al.*, 2000). Using a random mutagenesis strategy, Cano and colleagues (2001) have found that mutations in genes *phoP/Q*, *rpoS*, *spvR*, and *spvB* can allow for growth in fibroblasts (NRK-49F, rat kidney cell) (Cano *et al.*, 2001). This growth repression in fibroblasts by these genes is likely restricted to specific cell lines, since mutations in *phoP/Q* result in enhanced growth in the 3T3 mouse fibroblast cell line, but not in Hela cells (Cano *et al.*, 2001). The viable but not growing intracellular state in fibroblasts could conceivably aid in bacterial persistence within infected non-phagocytic cells (Cano *et al.*, 2001).

Because of the virulence deficiency of *rpoS* mutants, these mutants are potential candidate vaccine agents (Coynault *et al.*, 1996; Curtiss, III *et al.*, 2009). However, the potential of using *rpoS* mutants as vaccine is serotype-dependent (Coynault & Norel, 1999). Protection from infection of wild type *S. dublin* can be achieved with pre-

inoculation of *rpoS* mutants of *S. dublin* but not with a heterologous preparation made from *S. typhimurium* (Coynault & Norel, 1999).

During outbreaks, *Salmonella* spreads through contaminated food sources including vegetables. In an alfalfa sprout model, *S. enterica* serovar Newport wild type strain colonizes the plant tissue much better than *rpoS* mutants by 24 h, though the number of cells reaches to a similar level after 48h (Barak *et al.*, 2005). Interestingly, studies in the plant pathogens *Erwinia carotovora* (Andersson *et al.*, 1999) and *Pseudomonas putida* (Miller *et al.*, 2001) have shown that RpoS is important for colonization on tobacco, bean, and cucumber.

Vibrio cholerae is another major foodborne human pathogen. During infection, *V. cholerae* adheres to the epithelial cells in the small intestine and secretes enterotoxins to disrupt ion transport of attached cells, resulting in severe diarrhea (Faruque *et al.*, 1998). RpoS mutants are impaired in survival under starvation, osmotic shock, and oxidative stress in *V. cholerae* (Yildiz & Schoolnik, 1998). The HA/protease that processes cholera enterotoxins is positively controlled by RpoS (Yildiz & Schoolnik, 1998). Though HA/protease is not required for colonization and virulence in infant rabbits, it may allow *V. cholerae* to detach from epithelial cells to be released into the environment (Finkelstein *et al.*, 1992).

RpoS is required for efficient colonization of *V. cholerae* in suckling CD1 mice (Merrell *et al.*, 2000). However, another study reports that, after co-infection with wild type *V. cholerae* in infant mice, the proportion of *rpoS* mutants remains stable by 20h, indicating that RpoS is not required for intestinal survival (Yildiz & Schoolnik, 1998).

This difference has been attributed to strain variation within *V. cholerae*, which will require further study (Merrell *et al.*, 2000).

The last phase of *Vibrio* infection when cells detach from epithelial layers is termed the mucosa escape response, and this phase requires the expression of RpoS (Nielsen *et al.*, 2006). The expression of genes required for motility and chemotaxis is up-regulated by RpoS in the mucosa escape response and in stationary phase (Nielsen *et al.*, 2006). Under *in vitro* virulence-inducing conditions, production of cholera toxin is 10- to 100-fold higher in the *rpoS* mutants than in the wild type, and virulence genes, including *aphA*, *toxT* and *vpsA,* are expressed significantly higher in the *rpoS* mutants (Nielsen *et al.*, 2006). Thus, it is likely that, during the last phase of infection, RpoS represses virulence gene expression and stimulates motility to facilitate transmission (Nielsen *et al.*, 2006).

Vibrio vulnificus is a human pathogen that can cause wound infections and septicemia. RpoS protects cells from many stress conditions except for heat shock (Hulsmann *et al.*, 2003). RpoS positively regulates the production of extracellular enzymes, such as albuminase, caseinase and elastase, which may be required for survival of bacteria under many environmental conditions and for host adaptation (Hulsmann *et al.*, 2003). RpoS is also required for full motility (Hulsmann *et al.*, 2003). Interestingly, the catalase HPI is controlled by RpoS in *V. vulnificus*, while the gene encoding catalase HPII, which is highly RpoS-dependent in *E. coli*, is not expressed (Park *et al.*, 2004).

Vibrio anguillarum is the causative agent of vibriosis in fish (Lopez & Crosa, 2007). An essential virulence factor EmpA, encoding metalloprotease, is positively

regulated by RpoS (Denkin & Nelson, 2004). The virulence of *rpoS* mutants is severely reduced in zebra fish (Ma *et al.*, 2009). Similar to *V. vulnificus*, the *V. anguillarum rpoS* mutants are also impaired in production of extracellular enzymes, including phospholipase, diastase, lipase, caseinase, hemolysin, catalase and protease (Ma *et al.*, 2009).

Yersinia enterocolitica is an invasive enteropathogen that causes gastroenteritis in human. Adherence and invasion of *Y. enterocolitica* initiate at the terminal ileum. RpoS positively regulates the expression of Yst enterotoxin (Iriarte *et al.*, 1995), but does not control the expression of *inv* and *ail*, two virulence genes that are also required for invasion (Badger & Miller, 1995). RpoS has little effect in invasion in cell culture and in virulence of *Y. enterocolitica* in mouse models (Badger & Miller, 1995; Iriarte *et al.*, 1995).

Shigella flexneri infection causes severe dysentery in humans (Schroeder & Hilbi, 2008). After adherence and invasion of the colon mucous epithelial layer, *S. flexneri* is engulfed in phagocytic vacuoles (Schroeder & Hilbi, 2008). Following the lysis of these vacuoles, *S. flexneri* replicates and spreads to adjacent cells (Schroeder & Hilbi, 2008). As expected, RpoS is critical for resistance to acidic and oxidative stress in *S. flexneri* (Mogull *et al.*, 2001). When an *rpoS* mutant allele of *E. coli* was introduced to *S. flexneri* by P1 transduction, the resultant mutant exhibited no defect in invasion and formation of plaques on cultured Henle 407 cell monolayers, indicating that RpoS is not required for intercellular proliferation and spreading (Mogull *et al.*, 2001). However, the invasive ability of *S. flexneri rpoS* mutants has yet to be tested in animal models.

Overall, RpoS and its regulated genes are important for stress resistance and adaptation in enteric pathogens. Though RpoS plays an unequivocal role in the virulence of *Salmonella* species, the requirement for RpoS in the virulence and/or host adaptation in other species remains elusive. Nevertheless, given the importance of RpoS in adaptation, mutants of RpoS may be impaired in transmission to hosts due to reduced survival under adverse conditions. However, this has yet to be confirmed in animal models.

7.5 Respiratory pathogens

Pseudomonas aeruginosa is an opportunistic pathogen that causes chronic-lung infection (Oliver *et al.*, 2000). RpoS is highly expressed in *P. aeruginosa* isolated from sputum samples of cystic fibrosis (CF) patients with chronic lung infection (Foley *et al.*, 1999). As the case in *E. coli*, RpoS is critical for survival of *P. aeruginosa* under osmotic shock, heat shock, and oxidative stress conditions (Suh *et al.*, 1999). The effect of RpoS on expression of known virulence factors varies. For example, RpoS positively regulates the production of Exotoxin A that inhibits eukaryotic protein synthesis, and alginate, an important factor in the persistence of *P. aeruginosa* in CF lung and evasion of phagocytosis (Sonnleitner *et al.*, 2003; Suh *et al.*, 1999). The secreted protease activities of Elastase and LasA are also reduced in *rpoS* mutants (Suh *et al.*, 1999). However, the production of pyocyanin, a virulence secondary metabolite that interferes with host immune defense response (Ulmer *et al.*, 1990), is enhanced in *rpoS* mutants (Suh *et al.*, 1999). In a rat-chronic-infection model that specifically assesses the effect of extracellular secreted virulence proteins, *rpoS* mutants survive as well as wild type but

cause more damage to lung tissues, which may be attributable to excess pyocyanin production (Suh *et al.*, 1999). RpoS is required for full motility of *Pseudomonas* and thus has been suggested to be important for colonization (Suh *et al.*, 1999).

The RpoS-translational regulator Hfq is critical for virulence of *P. aeruginosa* O1 in the wax moth *Gelleria mellonella* and in mice (Sonnleitner *et al.*, 2003), while RpoS only has a moderate virulence effect in *G. mellonella* (Sonnleitner *et al.*, 2003). Production of pyocyanin is negatively controlled by Hfq and RpoS (Sonnleitner *et al.*, 2003). RpoS has little effect on motility of *P. aeruginosa* (Sonnleitner *et al.*, 2003), which differs from previous results (Suh *et al.*, 1999), probably due to differences in testing conditions.

The role of RpoS in quorum sensing of *P. aeruginosa* remains elusive. It has been shown that transcription of *rpoS* is controlled by quorum sensing regulators, LasR and RhlR (Latifi *et al.*, 1996), while another study reports that quorum sensing has little effect on expression of RpoS and is in fact repressed by RpoS (Whiteley *et al.*, 2000). The basis for these conflicting effects is unknown. Nonetheless, there certainly is overlapping regulation between regulons of quorum sensing and RpoS in *P. aeruginosa*. For example, the production of cytotoxic lectins is controlled by both RpoS and the quorum sensing regulator RhlR (Winzer *et al.*, 2000).

Legionella pneumophila is a facultative intracellular pathogen that can cause severe pneumonia, named the Legionnaires' disease (Swanson & Hammer, 2000). A natural reservoir for *L. pneumophila* is a wide range of amoebae living in soil and water sources (Fields, 1996). *L. pneumophila* is transmitted to the human respiratory tract

through contaminated water aerosols (Swanson & Hammer, 2000). During phagocytosis, *L. pneumophila* engulfed in phagosomes initially suppresses virulence traits until entry into stationary phase, when virulence and transmission traits are activated to stimulate transmission to adjacent cells (Swanson & Hammer, 2000).

RpoS plays a critical role in regulation of transmission and virulence of *L. pneumophila* (Abu-Zant *et al.*, 2006; Bachman & Swanson, 2004). Transcription of *rpoS* peaks in exponential phase while the protein level of RpoS reaches maximum in post-exponential phase (Bachman & Swanson, 2004). RpoS is important for survival in osmotic shock but not other stress conditions in exponential phase (Hales & Shuman, 1999). In stationary phase, though cells become more stress resistant, RpoS is dispensable (Hales & Shuman, 1999).

In exponential phase, RpoS down-regulates the transcription of *L. pneumophila* virulence genes *csrA*, *letE*, *fliA* and *flaA* and represses motility, infectivity, and cytotoxicity (Bachman & Swanson, 2001; Bachman & Swanson, 2004). However, in post-exponential phase, RpoS is critical for the transcription of flagella genes *fliA* and *flaA* (Bachman & Swanson, 2004). The repression of traits for transmission and cytotoxicity by RpoS in exponential phase may be important to allow cell replication to a high level, while in stationary phase, RpoS repression is relieved and the transmission traits are up-regulated by RpoS (Bachman & Swanson, 2004).

The pathogenesis of *L. pneumophila* requires the virulence factor Mip, a peptidyl-prolyl isomerase, for invasion and replication within both amoebae and macrophages (Bachman & Swanson, 2004). The transcription of *mip* is severely impaired in post-

exponential phase *rpoS* mutants (Bachman & Swanson, 2004). Production of phospholipase and lipophospholipase, two virulence factors, is also under positive control of RpoS (Broich *et al.*, 2006). In addition, RpoS positively regulates expression of ProA, a secreted virulence protease that is cytotoxic to macrophage and is important for virulence in a guinea pig model (Broich *et al.*, 2006). RpoS also regulates the expression of the ankyrin genes that play a critical role in intracellular growth within amoebae hosts and human macrophages (Habyarimana *et al.*, 2008). A pleiotropic regulator LqsR is RpoS-dependent (Tiaden *et al.*, 2007). LqsR-regulated genes are involved in virulence, motility and cell division, and mutations in *lqsR* result in attenuated growth in macrophages and protozoan hosts, *A. catellanii* and *D. discoideum* (Tiaden *et al.*, 2007). RpoS may also contribute to blocking phagolysosome formation by preventing the accumulation of LAMP-1, a phagolysomal protein (Bachman & Swanson, 2001). RpoS is crucial for the pore forming activity of *L. pneumophila* and adaptation to phagosomal intracellular environments during infection (Abu-Zant *et al.*, 2006).

The expression of the *icm* and *dot* genes, encoding the Icm/Dot type IV secretion system in *L. pneumophila*, is required for cytotoxicity and intracellular replication within macrophages and for intracellular growth in the protozoan host *Acanthamoeba castellanii* (Zusman *et al.*, 2002). RpoS only has a minor effect on the expression of the Icm/Dot genes (Hovel-Miner *et al.*, 2009; Zusman *et al.*, 2002). However, many genes encoding Icm/Dot secreted proteins require RpoS for full expression (Hovel-Miner *et al.*, 2009).

The potential involvement of RpoS in invasion of cell cultures likely depends on the characteristics of macrophages (Bachman & Swanson, 2001). The intracellular

environment is likely more deleterious to bacteria in primary macrophages than that in macrophage-like cells (Bachman & Swanson, 2001). *L. pneumophila* requires RpoS for efficient replication in protozoan hosts, *A. castellanii* (Hales & Shuman, 1999) and *A. polyphaga* (Abu-Zant *et al.*, 2006), and in murine bone marrow-derived macrophages (Bachman & Swanson, 2001) and human monocyte-derived macrophages (Abu-Zant *et al.*, 2006). However, RpoS is not required for replication in cultured human macrophage-like HL-60 and THP-1-derived cells (Hales & Shuman, 1999). In murine bone marrow-derived macrophages, most *rpoS* mutants, except for a small subpopulation (~5%), cannot replicate within infected vacuoles during initial infection in the first 48h (Bachman & Swanson, 2001). However, *rpoS* mutants can grow to wild type levels after 72h (Bachman & Swanson, 2001).

Burkholderia pseudomallei, a member of the beta-proteobacteria, is the causative agent of melioidosis. *B. pseudomallei* can invade host cells and induce the formation of a multinucleated giant cell (MNGC) by cell fusion (Utaisincharoen *et al.*, 2006). *B. pseudomallei* requires RpoS for resistance to stresses including starvation, oxidative stress and acidic conditions, but not to osmotic shock and heat exposure (Subsin *et al.*, 2003). RpoS is not involved in invasion of cultured human epithelial cells HEp-2 and murine macrophage RAW264.7 (Subsin *et al.*, 2003). However, another study reports that RpoS is important in invasion of RAW264.7 cells but not required for intracellular replication after invasion (Utaisincharoen *et al.*, 2006). The reason for this difference is not known. Survival of *rpoS* mutants in IFN-gamma interferon activated macrophages is severely impaired in comparison to wild type cells (Utaisincharoen *et al.*, 2006). RpoS

mutants cannot induce MNGC formation that is important for *B. pseudomallei* to spread to neighboring cells (Utaisincharoen *et al.*, 2006).

To summarize, in respiratory pathogens *L. pneumophila* and *B. pseudomallei*, RpoS regulated genes are important for survival within intracellular environment, though this appears to be also dependent on cell lines. In *P. aeruginosa*, the virulence effect of RpoS is not conclusive. RpoS positively regulates expression of extracellular enzymes but negatively affects production of the virulence factor pyocyanin. Whether RpoS controls colonization and virulence needs to be further tested in animal models.

7.6 Lyme disease spirochaete

Borrelia burgdorferi, the Lyme-disease-causing bacterium, is readily transmitted between arthropod and mammalian hosts. In contrast to proteobacteria, RpoS in *B. burgdorferi* is not important for resistance under most stress conditions except for hyperosmolarity (Elias *et al.*, 2000) and low pH (Caimano *et al.*, 2004). RpoS is induced in stationary phase, low pH, and during temperature shift from 23°C to 37°C (Caimano *et al.*, 2004; Yang *et al.*, 2000). The induction of RpoS is controlled by RpoN and an associated activator Rrp2 (Burtnick *et al.*, 2007; Hubner *et al.*, 2001; Smith *et al.*, 2007; Yang *et al.*, 2003). Two dimensional gel analysis reveals that RpoS controls the expression of a group of proteins in stationary phase (Elias *et al.*, 2000). RpoS is essential for virulence of *B. burgdorferi* in mouse models (Caimano *et al.*, 2004). RpoS positively regulates expression of the *ospC* gene (Gilbert *et al.*, 2007; Hubner *et al.*, 2001), encoding an outer surface lipoprotein critical for virulence (Schwan *et al.*, 1995). The expression of RpoS regulon *in vivo* is modulated by mammalian host signals, since

transcriptome analysis shows that many genes regulated by RpoS are only expressed *in vivo* within dialysis chambers (Caimano *et al.*, 2007).

7.7 Insect pathogens

Serratia entomophila is a soilborne pathogen that causes amber disease and general septicemia lethal to the grass grub, *Costelytra zealandica* (Giddens *et al.*, 2000). *S. entomophila* appears to have only one catalase, whose expression is RpoS-independent (Giddens *et al.*, 2000). Both wild type and *rpoS* mutants are sensitive to acid conditions (Giddens *et al.*, 2000). RpoS positively regulates the expression of *anfA1*, an important virulence factor during the development of larvae infection (Giddens *et al.*, 2000).

Xenorhabdus nematophilus, a member of gamma proteobacteria, is mutualistic to *Steinernema carpocapsae* nematodes but pathogenic to many insects (e.g., *Manduca sexta*). RpoS is important for survival upon exposure to H_2O_2 but not to osmotic stress (Vivas & Goodrich-Blair, 2001). In addition, *rpoS* mutants survive longer that WT in long-term batch cultures. The *rpoS* gene is required for colonization in the mutualistic host, *S. carpocapsae* nematodes, but not for virulence in insects (Vivas & Goodrich-Blair, 2001).

7.8 Plant pathogens

The plant pathogen ***Erwinia carotovora*** requires RpoS for survival under stresses including starvation, acidic pH, and exposure to H_2O_2 (Mukherjee *et al.*, 1998). RpoS mutants are more virulent during infection in celery and tobacco but not potato (Andersson *et al.*, 1999; Mukherjee *et al.*, 1998). The expression of a virulence factor,

Nip (necrosis inducing protein), is enhanced in *rpoS* mutants (Mattinen *et al.*, 2004; Mukherjee *et al.*, 1998). RpoS also negatively regulates the production of extracellular enzymes, pectate lyase, polygalacturonase, and cellulase, which are important for degradation of plant cell wall during infection (Mattinen *et al.*, 2004; Mukherjee *et al.*, 1998). This negative regulation is probably mediated through the RpoS-dependent gene *rsmA*, encoding a repressor for extracellular enzymes (Mukherjee *et al.*, 1998). A competition study shows that *rpoS* mutants cannot outcompete wild type *in vitro* or *in planta* in tobacco (Andersson *et al.*, 1999).

Burkholderia plantarii is a plant pathogen that can cause rice seedling blight, and its *rpoS* mutants show severe defect in pathogenesis (Solis *et al.*, 2006). Since RpoS mutants colonize rice leaves as well as wild type cells, this virulence defect is likely due to control of virulence traits by RpoS (Solis *et al.*, 2006). However, these RpoS-regulated virulence traits have not been identified.

Ralstonia (previously ***Pseudomonas***) ***solanacearum***, is a soilborne phytopathogen that can cause lethal vascular wilt disease in plants with a wide host range (Genin & Boucher, 2004). Survival of *rpoS* mutants is impaired in acid and starvation but not in heat, oxidative or high osmolarity conditions (Flavier *et al.*, 1998). The production of extracellular polysaccharide and activity of endogluconase, two known virulence factors (Kao *et al.*, 1992; Roberts *et al.*, 1988), are attenuated in *rpoS* mutants while the polygalacturonase (PGL) activity is elevated in *rpoS* mutants (Flavier *et al.*, 1998). Tomatoes infected with *rpoS* mutants show delayed wilting of leaves compared with plants infected with wild type *P. solanacearum*, indicating attenuation in virulence

(Flavier *et al.*, 1998). RpoS is also important for the production of the quorum sensing autoinducer acylhomoserine lactone (Flavier *et al.*, 1998).

7.9 RpoS as a niche-adaptation regulator

Given that RpoS is found in bacteria that occupy very different environments, a natural question arising is "Are there common requirements for gene expression amongst bacteria that invade different hosts?" Common requirements during host adaptation include slow growth as the pathogen adapts to new nutrient sources and possible exposure to host defense mechanisms including oxidative and acid stress components. These factors are controlled by RpoS regardless of the nature of the host. However, there are also many specific functions that may only be required on an episodic basis for host adaptation and colonization that are not required in other environments. Thus adhesion factors, extracellular enzymes including lipases, proteases, and sugar metabolizing functions, also may be dependent on RpoS when bacteria are experiencing suboptimal growth conditions. While RpoS is conserved across several genera of the Proteobacteria, the species-specific nature of the regulon can vary considerably. RpoS-controlled genes, though important for full virulence in specific cases, are invariably non-essential genes and thus likely do not have to be expressed under conditions in which RpoS itself is at basal levels, as is the case during exponential growth in nutrient rich environments. As a result, the regulatory environment in which RpoS-controlled genes exist is fairly permissive. It is possible that horizontally-transferred genes, that may enhance host adaptation, might easily integrate into the suboptimally-expressed regulon controlled by

RpoS. Phylogenetic studies examining the evolutionary relationship of RpoS to the broad class of genes that it controls will be necessary to resolve this question.

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Table 7.1 Effects of RpoS on virulence of specific pathogens.

Organism	RpoS-dependent phenotypes[a]						Virulence factors controlled by RpoS	Role of RpoS in virulence	Model	Reference
	Starvation	*Oxidative*	*Acid*	*Heat*	*Osmotic*	*Motility*				
Escherichia coli K12	+	+	+	+	+	-	NA	NA	NA	(Dong & Schellhorn, 2009a; Hengge-Aronis, 2002a; Patten *et al.*, 2004)
E. coli BJ4	NA	+	NA	+	NA	NA	NA	Not required for competitive colonization	Female mice: Ssc:CF1(streptomycin treated)	(Krogfelt *et al.*, 2000)
E. coli CFT073	NA	NA	NA	+	+	NA	NA	Not required for colonization in murine urinary tract	Mice, transurethral inoculation	(Culham *et al.*, 2001)
E. coli K1	NA	NA	+	+	+	NA	NA	Important for BMEC invasion	Cell culture: Brian Micro-vascular endothelial cells	(Wang & Kim, 2000)
E. coli O157:H7	+	NA	NA	NA	NA	NA	NA	Important for passage in mice and shedding in calves	ICR mice, Calves	(Price *et al.*, 2000)
Borrelia burgdorferi	NA	-	+	-	-/+	NA	OspC, DbpA	Essential	Female mice: C3H/HeJ, BALB/s, SCID	(Caimano *et al.*, 2004; Elias *et al.*, 2000; Hubner *et al.*, 2001)

Organism	RpoS-dependent phenotypes[a]						Virulence factors controlled by RpoS	Role of RpoS in virulence	Model	Reference
	Starvation	*Oxidative*	*Acid*	*Heat*	*Osmotic*	*Motility*				
Burkholderia plantarii	+	NA	NA	NA	NA	NA	NA	Important for rice seedling blight but not for colonization	Rice seedling leaves	(Solis *et al.*, 2006)
Burkholderia pseudomallei	NA	+	+	-	-	NA	NA	Not required for intracellular survival	Cell culture: HEp-2 and RAW264.7	(Subsin *et al.*, 2003)
Erwinia carotovora subsp. carotovora	+	+	+	NA	+	NA	Down-regulates extracellular enzymes and Nip	*rpoS* mutants are more virulent	Celery, Tobacco and Potato	(Andersson *et al.*, 1999; Mattinen *et al.*, 2004; Mukherjee *et al.*, 1998)
Legionella pneumophila	+	-	-	NA	+/-	+	Mip, FliA, Icm, ProA	Loss of *rpoS* results in impaired intracellular replication during the early stage of infection in murine primary and human monocyte-derived macrophages. RpoS is critical for growth in amoebae host and for pore-forming ability in erythrocytes. Not required for survival and cytotoxicity in macrophage-like cells.	Cell culture: Human macrophage-like cell U397, HL-60, monocyte- derived macrophage, THP-1 Murine bone marrow-derived macrophages Amoebae: *Acanthamoeba castellanii*, *A. polyphaga*	(Abu-Zant *et al.*, 2006; Bachman & Swanson, 2001; Bachman & Swanson, 2004; Broich *et al.*, 2006; Hales & Shuman, 1999; Hammer *et al.*, 2002; Zusman *et al.*, 2002)

Organism	RpoS-dependent phenotypes[a]						Virulence factors controlled by RpoS	Role of RpoS in virulence	Model	Reference
	Starvation	*Oxidative*	*Acid*	*Heat*	*Osmotic*	*Motility*				
Pseudomonas aeruginosa	+	+	NA	+	+	+	Exotoxin A, Alginate production	*rpoS* mutants are more virulent in rat-chronic lung infection, but RpoS has a moderate effect on virulence in *Galleria mellonella.*	Rat-chronic lung infection: Agar-beads embedded bacteria placed in the rat left lung; *Galleria mellonella* (wax moth)	(Sonnleitner *et al.*, 2003; Suh *et al.*, 1999)
Ralstonia solanacearum	NA	-	+	-	-	NA	EPS 1, EGL; but down-regulates PGL	RpoS has a minor effect in virulence	Tomato	(Flavier *et al.*, 1998)
Salmonella typhimurium	+	+	+	NA	NA	NA	SpvR, SpvABCD, and chromosome genes	Essential (oral lethal dose is 1000-fold higher for *rpoS* mutants and the CFU of WT infected spleen is 1,000 fold higher than that of mutants	Female BALB/c and C57BL/6 mice	(Fang *et al.*, 1992; Kowarz *et al.*, 1994)
Serratia entomophila	NA	-	-	NA	NA	NA	AnfA1	Important for control of antifeeding effect	*Costelytra zealandica*, larvae infection	(Giddens *et al.*, 2000)
Shigella flexneri	NA	+	+	NA	NA	NA	NA	Not required for invasion and plaque formation	Cell culture: Henle 407	(Mogull *et al.*, 2001)

Organism	RpoS-dependent phenotypes[a]						Virulence factors controlled by RpoS	Role of RpoS in virulence	Model	Reference
	Starvation	*Oxidative*	*Acid*	*Heat*	*Osmotic*	*Motility*				
V. cholerae	+	+	NA	NA	+	+	HA/protease is dependent on RpoS; but cholera toxin is down regulated by RpoS.	Mucosa escape response is RpoS-dependent; Not required for intra-intestinal survival in infant mice	Rabbit ileal loops, Infant (4-5 days) CFW mice	(Nielsen *et al.*, 2006; Yildiz & Schoolnik, 1998)
V. cholerae	NA	NA	NA	NA	NA	NA	NA	Required for efficient colonization	5-day-old suckling CD1 mice	(Merrell *et al.*, 2000)
Xenorhabdus nematophilus	+	+	NA	NA	-	-	NA	Required for growth in mutualistic hosts. Not required for virulence in insects.	Mutualistic to *Steinernema carpocapsae*; pathogenic to *Manduca sexta*	(Vivas & Goodrich-Blair, 2001)
Yersinia enterocolitica	+	+	+	+	+	NA	Yst (enterotoxin)	No effect in virulence and invasion; No difference in LD50 in mice	Cell culture: Hep-2; Female BALB/c mice	(Badger & Miller, 1995; Iriarte *et al.*, 1995)

Note: a. "+" positive effect; "-" negative effect; "+/-" either positive or negative depending on strain backgrounds or growth conditions, "NA"- information not available in the referred paper(s).

CHAPTER VIII

Conclusions

8.1 Concluding remarks

Why study stationary phase?

It has become clear that many genes important for host adaptation are expressed under poor growth conditions. Thus, identifying key processes in stationary phase may help identify new potential targets for novel antimicrobials. Industrially, many bacterial fermentation products are produced primarily in the stationary phase of growth, and a better understanding of important factors during this period may help improve production yields.

Studies on RpoS and stress response may have important applications in food safety and public health. Pathogenic *E. coli* strains can survive the high acidic conditions that they may encounter during food preparation or in human stomach fluids during infection. This survival ability largely depends on RpoS (for example, see (Dineen *et al.*, 1998)). The RpoS-controlled stress response systems enhance survival of *E. coli* under acidic, osmotic or high hydrostatic pressure conditions. In exponential phase when RpoS levels are low, cells are more sensitive to disinfectants, such as ClO_2 (Lisle *et al.*, 1998), UVC (Morton & Haynes, 1969), mild heat (Elliker & Frazier, 1938), and sunlight (Gourmelon *et al.*, 1997) compared with stationary-phase cells. RpoS is also important for cellular resistance to UVA light, sunlight and thermal disinfection (Berney *et al.*, 2006).

RpoS, a transient regulator in pathogenesis

Bacterial pathogenesis is a multifaceted process that requires concerted expression of not only specific virulence factors but also genes encoding other cellular

functions, including metabolism and adaptation. Being a transcription regulator, RpoS can mediate virulence either directly by controlling expression of virulence factors or indirectly by stimulating the general adaptation response to enhance survival of pathogens in hostile host environments. Since expression of RpoS is tightly controlled by environmental signals, including those specific to infection (e.g., intracellular infection of *Salmonella* and *Legionella*), RpoS may be viewed as a transient regulator that allows expression of specific genes to quickly respond to environmental stimuli. The RpoS regulons identified in different bacteria also vary substantially (Dong & Schellhorn, 2009a; Hovel-Miner *et al.*, 2009; Schuster *et al.*, 2004; Weber *et al.*, 2005). It is possible that, from an evolutionary point of view, RpoS has evolved to modulate temporal expression of specific genes whose expression is only transiently required, such as those genes for host adaptation or genes for adaptation to episodic environmental stresses (e.g., high osmolarity and oxidative stress).

RpoS-controlled gene expression, not a separate event

Given that RpoS expression and activity are regulated at multiple levels by a number of other regulatory factors (Hengge-Aronis, 2002a), expression of genes under control of RpoS should be considered not as an isolated event but rather a result of complex regulatory interaction between RpoS and other regulators, including H-NS (Barth *et al.*, 1995), ppGpp (Barth *et al.*, 1995; Gentry *et al.*, 1993), and Hfq (Muffler *et al.*, 1996b). For instance, H-NS, a nucleoid-associated DNA-binding protein (Fang & Rimsky, 2008; Navarre *et al.*, 2007), controls the expression of a large number of genes in *Salmonella* (Lucchini *et al.*, 2006; Navarre *et al.*, 2006) and *E. coli* (Grainger *et al.*,

2006; Hommais *et al.*, 2001; Oshima *et al.*, 2006). By direct binding to AT-rich regions, H-NS represses expression of virulence genes including the plasmid-borne *spv* virulence region and all five chromosomal pathogenicity islands in *Salmonella* (Fang & Rimsky, 2008; Navarre *et al.*, 2006), the LEE pathogenicity island in enteropathogenic *E. coli* (Bustamante *et al.*, 2001; Haack *et al.*, 2003), and all major virulence regions in uropathogenic *E. coli* (Muller *et al.*, 2006). Interestingly, H-NS controlled genes can be derepressed and transcribed by RpoS-associated RNA polymerase (Barth *et al.*, 1995; Fang & Rimsky, 2008; Navarre *et al.*, 2007; Olsen *et al.*, 1993; Shin *et al.*, 2005). Therefore, the episodic functions of RpoS may be required to allow transcription of genes repressed by H-NS or H-NS homologs, such as Ler (Torres *et al.*, 2007) and StpA (Lucchini *et al.*, 2009). In *Salmonella*, a third of StpA-repressed genes are under positive control of RpoS for expression (Lucchini *et al.*, 2009). In addition to the functional anti-repression relationship, H-NS and StpA also negatively control RpoS translation and stability (Barth *et al.*, 1995; Lucchini *et al.*, 2009; Yamashino *et al.*, 1995; Zhou & Gottesman, 2006).

8. 2 Future perspective

Recent insights from genomic expression profiling studies has expanded our understanding of RpoS from a particular stress regulator to a second vegetative sigma factor that has a much broader physiological function (Dong *et al.*, 2009a; Dong & Schellhorn, 2009a; Rahman *et al.*, 2006; Weber *et al.*, 2005). Nevertheless, we still do not know the function of a large number of RpoS-regulated genes, even in the well-studied model organism *E. coli*. In many pathogens known to require RpoS for full

virulence, the exact mechanism has not been identified. Characterization of the RpoS regulon in these pathogens may provide valuable insights. The RpoS regulon varies substantially between even closely-related pathogens, which may reflect modulation by other regulatory factors. One example of a known factor is the Crl protein, which regulates RpoS activity by direct interaction in both *E. coli* and *Salmonella* (Bougdour *et al.*, 2004; Lelong *et al.*, 2007; Robbe-Saule *et al.*, 2007; Typas *et al.*, 2007a). How Crl and similar regulatory factors may interact in other RpoS-expressing pathogens has not been examined. Therefore, both genomic and functional approaches are required to advance our understanding of the role that RpoS plays in bacterial pathogenesis and related cellular functions.

The interaction between a pathogen and its host is complex, having discrete infection stages including entry, attachment, colonization and dispersal. Regulators may have roles in one or more of these steps, and these must be each considered in a complete evaluation of RpoS as a potential virulence factor. In addition, choice of animal models to study regulatory factors may markedly affect results observed. Therefore, examination of RpoS involvement in virulence may require differentiation of temporal, locational, and host-specific effects.

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