

STUDIES ON THE CONTROL OF GENE EXPRESSION
OF *RPOS* AND ITS REGULON
IN *ESCHERICHIA COLI*

By

GUOZHU CHEN, B.Sc., M.Sc.

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McMaster University

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McMaster University
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Abstract

Many factors regulate the expression of *rpoS* at the levels of transcription, translation, proteolysis and RpoS activity. However, the relative importance of different levels of control is not well understood. By manipulating *rpoS* expression, we will better understand environmental adaptation responses. The first part of the study suggests that RpoS mutants can be selected using minimal media supplemented with succinate. We isolated 20 mutants that had the ability to grow on succinate as the sole carbon source. All mutants exhibited lower levels of RpoS function or *osmY* transcription. Sequencing and alignment analyses confirmed that all isolated succinate utilizing mutants possessed mutated alleles of the *rpoS* gene. Succinate transversion mutant can revert to an *rpoS* wild type phenotype under sub-optimal conditions.

The second part of the study has demonstrated that inducible *rpoS* antisense RNA complementary to *rpoS* mRNA can inhibit the expression of RpoS in both exponential and stationary phases and can attenuate expression of the *rpoS* regulon in *E. coli*.

The final part of the study artificially expressed RpoS protein and its regulon in both exponential and stationary phases. *rpoS* mRNA can be induced at an early stage of cell growth when RpoS levels are normally extremely low (i.e., in early exponential phase) and can activate the expression of the RpoS regulon in exponential phase and stationary phase. Our results showed that controllable levels of RpoS could regulate the expression of *rpoS* and RpoS-dependent genes indicating RpoS expression is sufficient for expression of the regulon in exponential phase.

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Preface

This thesis is organized in a series of chapters in the form of journal articles. The three manuscripts that I have prepared here have been formatted to comply with the thesis regulations. All the work presented in this thesis has been carried out by the author.

Table of Contents

Descriptive Note	ii
Abstract	iii
Acknowledgments	iv
Preface	vii
Table of Contents	viii
List of Tables	xii
List of Figures	xiii
List of Abbreviations	xv
CHAPTER 1: INTRODUCTION	1
Overview	2
Regulation of RpoS	3
Transcriptional control of RpoS	3
The <i>rpoS</i> promoter regions	4
CAMP-CRP	4
BarA	5
ppGpp and polyphosphate	5
Homoserine lactone (HSL)	6
Weak acids	6
Translational control of RpoS	6
The <i>rpoS</i> mRNA secondary structure	7
The RNA binding protein Hfq (HF-I)	7
Histone-like protein H-NS	7
Small regulatory RNAs: OxyS, DsrA and RprA	8
The LysR-like regulator LeuO	8
UDP-glucose	9
RpoS proteolysis	9
ClpXP protease	10
Two-component response regulator RssB	10
DnaK	10
Control of RpoS activity	11
Crl	11
The RpoS regulon	11
Antisense regulation in bacteria	12
Overview	12
Naturally occurring antisense RNAs	12
Control of gene expression by antisense RNAs	13
Project outlines and objectives	13

References	16
CHAPTER 2: METHODS AND MATERIALS	23
Bacterial strains and plasmids	24
Chemicals, enzymes and reagents	24
Growth media	24
Supplements and antibiotics	25
Working solutions and buffers	25
Growth and storage of cultures	27
Preparation of glycerol stocks	28
Centrifugation	28
Transformation with plasmid DNA	28
Preparation of fresh <i>E. coli</i> competent cells	28
Preparation of frozen <i>E. coli</i> competent cells	29
Transformation of fresh and frozen <i>E. coli</i> competent cells and selection of transformants	30
Preparation of DNA from <i>E. coli</i>	30
Small scale preparation of <i>E. coli</i> plasmid DNA	30
Method 1, alkaline lysis	30
Method 2, Wizard™ plus mini preparation DNA purification system	31
Large scale preparation of <i>E. coli</i> plasmid DNA	31
Alkaline lysis	31
Qiagen plasmid midi purification	32
Preparation of <i>E. coli</i> chromosomal DNA	32
<i>In vitro</i> manipulation of DNA	33
Purification and concentration of DNA	33
Purification of DNA	33
Concentration of DNA	34
Agarose gel electrophoresis of DNA	34
Size and concentration estimation of DNA on agarose gels	34
Cloning of DNA	35
Restriction digestion of plasmid DNA	35
Dephosphorylation of 5' ends of plasmid DNA	36
Partial restriction endonuclease digestion of plasmid DNA	36
blunt-end DNA	37
Isolation of DNA fragments from agarose gels	37
QIAEX II gel extraction system	37
Ligation of DNA	37
Ligation of cohesive termini	37
Ligation of blunt-end DNA	38
Identification of bacterial colonies that contain recombinant plasmid DNA	38
Method 1, restriction analysis of small scale preparations of plasmid DNA	38

Method 2, α -complementation	38
Methods 3, rapid screening of transformants for inserts using the polymerase chain reaction (PCR)	39
Rapid cloning in plasmid DNA	40
PCR Reactions	41
Transduction	42
Preparation of P1 vir transduction lysates	42
P1 vir mediated transduction	43
λ DE3 Lysogenization	44
Lysogenization	44
Verification of λ DE3 lysogens	45
β -Galactosidase assay	45
Northern blot analysis of RNA	46
Diethylpyrocarbonate treatment of solutions and consumables	46
Preparation of labeled probe	46
Preparation of total RNA from <i>E. coli</i>	47
Preparation of RNA formaldehyde agarose gel	48
Northern blotting	48
Fixing RNA to the membrane	49
Hybridization in tubes	49
Signal detection	49
Enzyme assays	49
Preparation of cultures	49
Determining the protein content of cell extracts	50
Polyacrylamide gel electrophoresis of protein and catalase	50
Determination of catalase activity	51
Western blotting analysis	52

CHAPTER 3:

POSITIVE SELECTION FOR LOSS OF RPOS FUNCTION IN <i>ESCHERICHIA COLI</i>	53
Preface	54
Summary	56
Introduction	57
Results	58
Discussion	63
Experimental procedures	67
References	72

CHAPTER 4:

CONTROLLED EXPRESSION OF AN RPOS ANTISENSE RNA CAN INHIBIT RPOS FUNCTION IN <i>ESCHERICHIA COLI</i>	91
Preface	92
Summary	94

Introduction	95
Materials and methods	98
Results	105
Discussion	112
References	116
CHAPTER 5:	
CONTROLLED INDUCTION OF THE RPOS REGULON IN <i>ESCHERICHIA COLI</i> USING AN RPOS-EXPRESSING PLASMID	144
Preface	145
Abstract	147
Introduction	148
Materials and methods	150
Results	156
Discussion	161
References	164
CHAPTER 6: SUMMARY AND THESIS REFERENCES	183
Summary	184
Thesis references	186

List of Tables

CHAPTER 3

Table 1. Bacterial strains used in this study 75

Table 2. Oligonucleotides used in this study 76

Table 3. RpoS-dependent gene expression based on β -galactosidase activity and catalase activity of Suc⁺ mutants. 77

Table 4. Types of mutations, predicted effects on protein structure, and reversion rates of Suc⁺ mutant strains. 78

CHAPTER 4

Table 1. Bacterial strains used and constructed in this study 122

Table 2. Plasmids used and constructed in this study 123

CHAPTER 5

Table 1. Bacterial strains used and constructed in this study 167

Table 2. Plasmids used and constructed in this study 168

List of Figures

CHAPTER 3

- Figure 1. Expression of RpoS-dependent *osmY-lacZ* in three isolated Suc⁺ mutants (HS1091-A to HS1091-C) and control strains HS1091 and HS1091P. 79
- Figure 2. Catalase activity in three isolated Suc⁺ mutants (HS1091-A to HS1091-C) and control strains HS1091 and HS1091P in LB broth culture. 81
- Figures 3. Catalase activity in polyacrylamide gel containing protein extracted from *rpoS* wild type strain HS1091 and mutant strains HS1091-A, HS1091-B and HS1091-C grown to exponential phase (E) and stationary phase (S) in LB media. 83
- Figure 4. Western blot analysis of RpoS protein levels in *E. coli* wild type strain HS1091, *rpoS* null mutant HS1091P and three representative Suc⁺ mutants: HS1091-B (deletion mutant), HS1091-H (transversion mutant), and HS1091-I (insertion mutant). 85
- Figure 5. Western blot analysis of RpoS protein levels in five succinate transversion revertants (transversion revertants: HS1091-H/C, HS1091-N/H and HS1091-R/E, as well as nonsense revertants: HS1091-A/F and HS1091-J/D). 87
- Figure 6. *rpoS* reversion rates for Suc⁺ mutants. 89

CHAPTER 4

- Figure 1. Schematic representation of the *rpoS* gene in the *E. coli* chromosome (top) and in antisense expression plasmids pSOPR and pSOPRL. 124
- Figure 2. Antisense RNA plasmid pSOPR. 126
- Figures 3. Northern blot analysis of *rpoS* antisense RNA induced with IPTG in MC4100DE3 series in exponential (A) and stationary (B) phases showing the effect of IPTG on *rpoS* antisense RNA expression. 128
- Figures 4. Northern blot analysis of short and large fragments of *rpoS* antisense RNAs induced by IPTG in BL21DE3 and MC4100DE3 in (A) exponential phase and (B) stationary phase. 130

Figures 5. Northern blot analysis of <i>rpoS</i> antisense RNA induced by 0.3 mM IPTG in MC4100DE3 series in exponential and stationary phases showing the optimization of induction of <i>rpoS</i> antisense RNA expression of short and large fragments of <i>rpoS</i> antisense RNA.	132
Figure 6. Western blot analysis of RpoS levels in <i>E. coli</i> MC4100 derivatives using anti- σ^S -antiserum.	134
Figure 7. Catalase specific activity of <i>rpoS</i> wild type strain MC4100DE3 containing control plasmid pET21 and antisense construct pSOPR grown in the absence and presence of 0.3 mM IPTG in LB media.	136
Figure 8. Catalase activity in polyacrylamide gels containing protein extracted from <i>rpoS</i> wild type strain MC4100DE3 carrying control vector pET21 and antisense construct pSOPR grown in the absence and presence of 0.3 mM IPTG in LB media.	138
Figure 9. Glycogen accumulation of strains during growth on Kornberg medium plates.	140
Figure 10. The proposed mechanism of <i>rpoS</i> antisense RNA inhibition of <i>rpoS</i> expression.	142
 CHAPTER 5	
Figure 1. Structure of <i>rpoS</i> -encoding plasmid pRPOS.	169
Figures 2. Northern analysis of <i>rpoS</i> mRNA induced by IPTG.	171
Figure 3. <i>rpoS</i> mRNA levels quantified from a Northern blot (See Figure 2).	173
Figure 4. Western blot analysis of RpoS levels using anti- σ^S -antibody.	175
Figure 5. Induced <i>rpoS</i> mRNA activates RpoS-dependent catalase expression.	177
Figure 6. Induction of <i>rpoS</i> activates expression of RpoS-dependent catalase HP11.	179
Figure 7. Glycogen accumulation in RpoS-expressing strains HS1600DE3pRPOS and wild type strain MC4100DE3.	181

List of Abbreviations

bp	base pair
°C	Degrees Celsius
CFU	Colony Forming Unit
DEPC	diethylpyrocarbonate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2'-deoxynucleotide-5'-triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
g	gram
h	hour (s)
IPTG	isopropylthio- β -D-galactoside
λ	Lambda
kb	kilobase
LB	Luria-Bertani
M	molar
mg	milligram
min	minute (s)
ml	millilitre
mM	millimolar
mRNA	messenger RNA
ng	nanogram
nm	nanometer
OD ₆₀₀	Optical Density at 600 nm
ONPG	0-nitrophenyl- β -D-galactopyranoside
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
s	second (s)
SDS	Sodium Dodecyl Sulfate
Suc	succinate
UV	Ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside

CHAPTER 1: INTRODUCTION

Overview

Gram-negative bacteria have evolved cellular mechanisms to respond to environmental stresses. Gram-negative bacteria such as *Escherichia coli* and *Salmonella typhimurium* undergo physiological changes during stationary phase and have the ability to survive prolonged periods of starvation (Hengge-Aronis, 1996b). In *E. coli*, seven sigma factors have evolved to regulate gene expression in response to various conditions; σ^D (σ^{70}), the vegetative sigma factor, is constitutively expressed and directs transcription of the housekeeping genes. The σ^N (σ^{54}) regulon includes genes important for adaptation to nitrogen limitation, σ^H (σ^{32}) for heat shock, σ^E (σ^{24}) for extracytoplasmic stress, σ^F (σ^{28}) for flagellin synthesis, FecI (σ^{19}) for iron transport and σ^S (σ^{38}) for stationary-phase-specific stress response (Lonetto and Gross, 1996).

The stationary-phase-specific sigma factor, σ^S (referred to as RpoS) is encoded by the *rpoS* gene (Lange and Hengge-Aronis, 1991; Mulvey and Loewen, 1989). *rpoS* was originally identified as the gene, *nur* (important for near-UV resistance) (Tuveson and Jonas, 1979), and later as the gene, *katF* (a regulator for the *katE*-encoding catalase hydroperoxidase) (Loewen and Triggs, 1984), *appR* (a regulator of acid phosphatase) (Touati *et al.*, 1986) and *csi-2* (a central regulator for carbon starvation-inducible gene)(Lange and Hengge-Aronis, 1991). They were subsequently found to be alleles of the same gene, *rpoS* (Lange and Hengge-Aronis, 1991; Touati *et al.*, 1991). It is now known that when *E. coli* cells enter stationary phase, RpoS up-regulates the expression of more than 100 genes (Ishihama, 2000) in response to a diverse number of environmental stresses; including oxidative stress, near-UV irradiation, nutrient limitation, high osmolarity, low pH, and cold shock (Hengge-Aronis, 2002).

Escherichia coli cells undergo physiological and morphological changes to survive for long periods of time in stationary phase. Cells tend to be small and spherical in shape in stationary phase, compared to the large rod shape characteristic of *E. coli* in exponential phase (Kolter *et al.*, 1993).

Regulation of RpoS

The cellular level of RpoS is low in early exponential phase and increases when cells enter stationary phase. Multiple stress signals affect different levels of RpoS regulation. Expression of *rpoS* is controlled at the levels of transcription, translation, proteolysis and RpoS activity (Hengge-Aronis, 2002). The role of RpoS and the genes of the *rpoS* regulon can be found in several recent reviews (Hengge-Aronis, 1996a; Loewen *et al.*, 1998; Hengge-Aronis, 2002; Eisenstark *et al.*, 1996). However, the nature of RpoS regulatory mechanisms and the relative importance of different control levels are not completely characterized.

Transcriptional control of RpoS

Studies utilizing *rpoS-lacZ* transcriptional fusions reveal that *rpoS* transcription begins in early exponential phase ($OD_{600} = 0.3$) and reaches its maximal level as the cells enter stationary phase (Mulvey *et al.*, 1990; Schellhorn and Stones, 1992). Although protein levels of RpoS are undetectable during exponential phase, relatively high levels of *rpoS* mRNA are present throughout the exponential and stationary phases. Several transcriptional factors are involved in the regulation of *rpoS*, including *rpoS* promoter regions (Tanaka *et al.*, 1993; Tanaka *et al.*, 1995), cAMP-CRP (Lange and Hengge-Aronis, 1991; Lange and Hengge-Aronis, 1994a), the histidine sensor kinase BarA (Mukhopadhyay *et al.*, 2000), guanosine tetraphosphate (ppGpp), polyphosphate,

homoserine lactone (HSL) (Huisman and Kolter, 1994) and weak acids (Schellhorn and Stones, 1992). These factors are discussed below.

The *rpoS* promoter regions

The *rpoS* gene is located downstream of the *nlpD* gene, which encodes a lipoprotein with a potential cell wall formation function (Lange and Hengge-Aronis, 1994b). Two weak promoters (*nlpDp1* and *nlpDp2*) upstream of the *nlpD* gene direct expression of both *nlpD* and *rpoS*, resulting in the basal levels of *rpoS* mRNA seen in exponential cells (Ichikawa *et al.*, 1994; Lange and Hengge-Aronis, 1994b). The main promoter (*rpoSp*) is located within the *nlpD* gene and drives high levels of *rpoS* mRNA during transition into stationary phase (Lange *et al.*, 1995).

Sigma factors begin the process of transcription initiation by recognizing the promoter sequences at the -10 and -35 regions relative to the transcription start site (Helmann and Chamberlin, 1988). Analysis of 33 RpoS-dependent promoters reveals that RpoS recognizes a promoter consensus sequence of CTATACT located in the -10 region (Espinosa-Urgel *et al.*, 1996). However, no homologous -35 consensus sequence is found. The -10 consensus promoter sequence and DNA structure of the regions are recognized specifically by either RpoS or σ^D while some promoters are recognized by both RpoS and σ^D (Tanaka *et al.*, 1993; Tanaka *et al.*, 1995).

CAMP-CRP

In strains carrying a *cya* (encoding adenylate cyclase) or *crp* (encoding the cyclic AMP receptor protein CRP) mutation, the expression of *rpoS* increases in exponential phase. Also, the addition of exogenous cAMP to the strain carrying *cya* mutation decreases *rpoS* expression (Lange and Hengge-Aronis, 1991; Lange and

Hengge-Aronis, 1994a) suggesting the cAMP-CRP complex represses *rpoS* transcription.

BarA

BarA is a hybrid histidine sensor kinase in *E. coli*. UvrY is the cognate response regulator in the BarA-UvrY two-component system (Pernestig *et al.*, 2001). A strain with a *lacZ* insertion in the chromosomal copy of *barA* exhibits a hydrogen peroxide-sensitive phenotype (Mukhopadhyay *et al.*, 2000), and has lower *rpoS* transcript and protein levels in exponential phase (Mukhopadhyay *et al.*, 2000). This study suggests that BarA may be a positive regulator of *rpoS* transcription in exponential phase (Mukhopadhyay *et al.*, 2000).

ppGpp and polyphosphate

Guanosine tetraphosphate (ppGpp) is the effector of stringent response during amino acid limitation and carbon starvation (Cashel *et al.*, 1996). The accumulation of ppGpp is dependent on the expression of two genes: *relA*, encoding ppGpp synthase I, and *spoT*, encoding ppGpp synthase II. ppGpp increases in response to amino acid limitation and other starvation conditions. The accumulation of ppGpp is impaired in *relAspoT* double mutant (Gentry *et al.*, 1993). Strains with a mutant have decreased expression of *rpoS-lacZ* transcriptional fusions, demonstrating that ppGpp positively affects *rpoS* transcription. Overexpression of exopolyphosphatase in *E. coli* results in abolished polyphosphate production and results in increased sensitivity to hydrogen peroxide. Expression of exopolyphosphatase in the above strain carrying a transcriptional *rpoS-lacZ* fusion results in decreased *rpoS* expression. The strain is also sensitive to oxidative stress (Shiba *et al.*, 1997). These results indicate that

polyphosphate is a positive regulator of *rpoS* transcription (Kuroda *et al.*, 1997).

Homoserine lactone (HSL)

Strains with an *rspA* (encoding a putative lactonizing enzyme) mutation repress the expression of a *bolA-lacZ* transcriptional fusion. Overexpression of RspA reduces *rpoS-lacZ* transcription (Huisman and Kolter, 1994). RspA may reduce the levels of a starvation signal molecule (lactone) responsible for *rpoS* induction indicating that homoserine lactone maybe an inducer of *rpoS* expression (Huisman and Kolter, 1994), however confirmation of its role has not been reproduced (Hengge-Aronis, 2002; Loewen *et al.*, 1998).

Weak acids

Prior studies have shown that *rpoS* expression is induced when cells in exponential phase are resuspended in the culture supernatant from a stationary phase culture. Acetate, a fermentation by-product, may be the inducing signal at the onset of stationary phase (Schellhorn and Stones, 1992). Other non-fermentable weak acids, such as benzoate and propionate, with a pKa similar to that of acetate show the same inducing effect indicating that internal proton flux may provide an inducing signal (Mulvey *et al.*, 1990; Schellhorn and Stones, 1992).

Translational control of RpoS

The most notable control of *rpoS* gene expression occurs at the translation level. RpoS mRNA translation is stimulated upon the transition to stationary phase. Many input signals are involved, including high osmolarity, low temperature, low pH (pH 5.0) and a high cell density during late exponential phase.

The key factors in the control of *rpoS* translation include *rpoS* mRNA secondary

structure, Hfq and H-NS proteins, and small regulatory RNAs such as OxyS, DsrA and RprA. Additional factors that influence *rpoS* translation include the LysR-like regulator LeuO (Klauck *et al.*, 1997) and UDP-glucose (Bohringer *et al.*, 1995).

The *rpoS* mRNA secondary structure

The *rpoS* mRNA secondary structure contains a ribosome-binding site (RBS), the initiation codon and a downstream sequence with complementarity to a 3' region in 16s rRNA, all factors that control the translation of *rpoS* (Lange and Hengge-Aronis, 1994a; Loewen *et al.*, 1998). This region of self-complementary secondary structure prevents ribosome binding to the RBS (Lange and Hengge-Aronis, 1994a). Deletion of the 5' nontranslated leader region reduces *rpoS* translation, suggesting a role in post-transcriptional control (Cunning *et al.*, 1998).

The RNA binding protein Hfq (HF-I)

Hfq (HF-I) is an RNA binding protein that positively regulates *rpoS* translation by promoting a conformational change of the secondary structure of *rpoS* mRNA that allows access to the ribosome (Muffler *et al.*, 1997). Hfq can also bind DsrA, a small regulatory RNA, at low temperature (Sledjeski *et al.*, 2001), however its physiological function in *E. coli* is not known (Muffler *et al.*, 1997).

Histone-like protein H-NS

H-NS is a nucleoid histone-like protein. Mutations in *hns*, but not absence of Hfq increase RpoS levels. This suggests that H-NS affects *rpoS* translation in an Hfq dependent manner (Muffler *et al.*, 1997). H-NS is a negative regulator of *rpoS* translation but it is not clear if H-NS binds directly to *rpoS* mRNA or how H-NS interacts with Hfq.

Small regulatory RNAs: OxyS, DsrA and RprA

OxyS is a 109-nucleotide untranslated RNA whose expression is dependent on OxyR (Altuvia *et al.*, 1997). RpoS protein levels are reduced when *oxyS* is overexpressed (Altuvia *et al.*, 1997). OxyS represses induction of RpoS except in an *hfq* mutant background, indicating that OxyS requires a functional Hfq (Zhang *et al.*, 1998). OxyS binds to Hfq with an A-rich 26 bp linker region and prevents Hfq from activating RpoS in response to oxidative stress (Zhang *et al.*, 1998).

While OxyS represses *rpoS* translation, DsrA and RprA stimulate RpoS function. DsrA is an 87-nucleotide regulatory RNA that increases *rpoS* translation at 20°C relative to growth at 37°C (Lease and Belfort, 2000; Sledjeski *et al.*, 1996). DsrA interacts with and stabilizes *rpoS* mRNA resulting in increased RpoS protein levels (Sledjeski *et al.*, 1996). Hfq form a tertiary complex with DsrA to alter the secondary structure of the 5' –untranslated region of the *rpoS* mRNA and DsrA RNA. Hfq have RNA-binding sites on DsrA. (Sledjeski *et al.*, 1996). The stem-loop 1 of DsrA binds to the 5' -untranslated leader sequence of *rpoS* mRNA, resulting in increased translation efficiency by opening the secondary structure of the 5' –untranslated region of *rpoS* mRNA (Majdalani *et al.*, 1998).

RprA is a small, untranslated RNA involved in *rpoS* translation. Mutations in *rprA* in the *dsrA* mutant background reduce *rpoS* translation, indicating that RprA promotes *rpoS* translation (Majdalani *et al.*, 2001). However, the mode of action of RprA is unknown.

The LysR-like regulator LeuO

LeuO is a transcriptional regulator in the LysR-like family (Klauck *et al.*, 1997).

Mutations in *leuO* in a *dsrA-lacZ* transcriptional fusion result in reduced transcriptional activity at low temperature, indicating that LeuO can repress *dsrA* transcription.

Overexpression of LeuO in the presence of DsrA reduces *rpoS* translation, especially at low temperature (Klauck *et al.*, 1997), however, the physiological function of LeuO is unknown.

UDP-glucose

UDP-glucose is a metabolite that inhibits *rpoS* translation (Bohringer *et al.*, 1995). Mutations in *pgi* (encoding phosphoglucose isomerase), *pgm* (encoding phosphoglucomutase) or *galU* (encoding UDP-glucose pyrophosphorylase) result in increased RpoS levels in exponential phase cells. These mutants are deficient in UDP-glucose production, it suggests that UDP-glucose is involved in *rpoS* translation by an unknown mechanism (Bohringer *et al.*, 1995).

RpoS proteolysis

Regulation of *rpoS* transcription and translation can be mediated by many environmental stress conditions. RpoS activity further increases due to the inhibition of proteolysis by high osmolarity (Muffler *et al.*, 1996c), low pH (Lee *et al.*, 1995), temperature (Repoila and Gottesman, 2001) and carbon starvation (Takayanagi *et al.*, 1994). In exponential phase, proteolysis maintains a low basal level of RpoS (Schweder *et al.*, 1996). The half-life of RpoS in exponential phase is 1.4 min. Upon entry into stationary phase, greater RpoS stability increases the half-life to 16.5 min (Lange and Hengge-Aronis, 1994a). In response to osmotic challenge, RpoS half-life increases to 45 min (Lange and Hengge-Aronis, 1994a). The key factors implicated in the control of RpoS degradation are discussed below.

ClpXP protease

ClpXP protease is a key regulator that degrades RpoS. RpoS levels in exponential phase increase to levels comparable to those seen in stationary phase with mutations in *clpX* or *clpP* (Schweder *et al.*, 1996). RpoS protein regions between amino acids residues 173 and 188 (Schweder *et al.*, 1996) or sequences between residues 127 and 247 (Muffler *et al.*, 1996b) are the target for ClpXP. However, it is unclear which region is the target.

Two-component response regulator RssB

The two-component response regulator RssB, also known as SprE (Pratt and Silhavy, 1996), when phosphorylated, binds to RpoS. This complex promotes ClpXP binding and enhances RpoS sensitivity and the rate of proteolysis (Zhou *et al.*, 2001). A tertiary complex between RpoS, phosphorylated RssB, ClpX and a quaternary complex, also with ClpP, have been observed *in vitro* (Zhou *et al.*, 2001). Mutations in RssB result in a ten-fold increase in RpoS levels during exponential phase, indicating that RssB promotes RpoS degradation (Muffler *et al.*, 1996a). Acetyl phosphate is a phosphate donor that activates RssB activity and therefore increases the rate of RpoS proteolysis (Bouche *et al.*, 1998). In addition, binding of RssB to RpoS results in reduced expression of RpoS-dependent genes suggesting that the response regulator RssB can antagonize RpoS activity (Zhou and Gottesman, 1998; Becker *et al.*, 2000).

DnaK

DnaK is a heat shock chaperone that protects RpoS from degradation. Deficiency in DnaK results in reduced levels of RpoS in the stationary phase (Rockabrand *et al.*, 1998).

Control of RpoS activity

Transcriptional initiation of an RpoS-dependent gene requires RNA polymerase holoenzyme (σ^S and RNA polymerase core enzyme ($\alpha_2\beta\beta'$)) (Ishihama, 2000). RpoS has one of the lowest affinities compared to other six sigma factors for the core enzyme *in vitro* (Maeda *et al.*, 2000). Anti- σ^{70} factor Rsd is a sigma 70 regulator and may play a role in the competition of sigma factors between σ^{70} and RpoS for the core enzyme (Maeda *et al.*, 2000).

Crl

Crl (a subunit of curli fimbriae) is found in the cell surface and formed in stationary phase, during periods of low temperature or osmolarity (Olsen *et al.*, 1993). Crl stimulates RpoS-dependent *katE* and *bolA* expression by modulating the association of RpoS with the RNA polymerase core (Pratt and Silhavy, 1998). A *crl* and *rpoS* double mutant shows no cumulative affect on *katE-lacZ* expression suggesting that Crl and RpoS are in the same metabolic pathway (Pratt and Silhavy, 1998).

The RpoS Regulon

RpoS is a global stress response regulator in *E. coli* (Hengge-Aronis, 1996a). The RpoS regulon and the function of its many genes have not been completely characterized. Over 50 RpoS-dependent genes have been identified thus far (Loewen *et al.*, 1998). It is estimated that over 200 genes of various functions are under the control of RpoS (Schellhorn *et al.*, 1998). These functions include DNA protection and repair, thermotolerance, osmoprotectance, glycogen synthesis, and virulence (Hengge-Aronis, 1996a).

Antisense Regulation in Bacteria

Overview

Antisense RNA technology can be used to repress protein production and reduce target mRNA levels (Coleman *et al.*, 1984; Desai and Papoutsakis, 1999; Kernodle *et al.*, 1997). During the last decade, antisense RNA technology has focused on several aspects, including: efficiency of different RNA construct, investigation of unknown gene function under certain conditions when gene disruption is not possible, and manipulation of cell metabolic pathways. Often, antisense RNA can be advantageous over gene inactivation. When the gene under study is essential for growth, antisense RNA can be used to modulate expression of the gene (Casqueiro *et al.*, 1999). Perhaps the greatest advantage of antisense RNA, is that expression of the antisense, and thus the target, is controllable (Yatzkan *et al.*, 1998).

Naturally occurring antisense RNAs

Naturally occurring antisense regulation of gene expression has been demonstrated in both eukaryotic and bacterial species, (Majdalani *et al.*, 1998; Tamm *et al.*, 2001) by blocking ribosome binding or reducing mRNA stability (Daugherty *et al.*, 1989; Desai and Papoutsakis, 1999; Gill *et al.*, 1999). For example, OxyS RNA controls the OxyR regulon while MicF RNA inhibits OmpF in *E. coli*. The antisense RNAs DsrA and RprA positively regulate *rpoS* translation (Lease and Belfort, 2000; Majdalani *et al.*, 1998; Majdalani *et al.*, 2001) while OxyS RNA inhibits *rpoS* translation (Zhang *et al.*, 1998).

Control of gene expression by antisense RNAs

Antisense RNA technology has been successfully used to control gene expression in bacteria (Nellen and Lichtenstein, 1993; Guerrier-Takada *et al.*, 1997; Good and Nielsen, 1998) (For review, see (Wagner and Simons, 1994)) and phage (Walker and Klaenhammer, 2000; Sturino and Klaenhammer, 2002). For example, expression of an *hla* antisense RNA was used to inhibit alpha-toxin production *in vitro* and *in vivo* in *Staphylococcus aureus* to attenuate virulence (Kernodle *et al.*, 1997). A *vanR* antisense RNA was used to inhibit the expression of vancomycin resistance operon *vanHAX* (Torres *et al.*, 2001). Antisense RNA technology studies revealed that *nre* encodes an activator in nitrogen metabolism in *Penicillium chrysogenum* (Zadra *et al.*, 2000). In addition, *mar* antisense RNA was used to inhibit the multiple antibiotic resistance (*mar*) operon in *E. coli*, indicating that expression of multiple antisense RNAs complementary to polycistronic mRNA can downregulate the products of multiple genes (White *et al.*, 1997). Antisense RNA complementary to initiation start codon, the Shine-Dalgarno sequence and just upstream of the protein is sufficient to inhibit gene expression. When the entire, or just part, of the coding region was used, antisense construct yielded variable results to downregulate mRNA (Lucchini *et al.*, 2000).

Project Outlines and Objectives

Transition to stationary phase causes changes in gene expression and protein synthesis of RpoS. We have previously found that control of the expression of RpoS-regulated genes occurs not only in stationary phase but also in exponential phase (Schellhorn *et al.*, 1998). This result suggests that transcriptional and translational

controls on expression of *rpoS* are critical for induction of the RpoS regulon in exponential and stationary phases (Schellhorn *et al.*, 1998). The expression of stationary phase genes is important to help bacteria adapt to adverse environments. In addition, RpoS plays a role in regulating the expression of several virulence factors in *Salmonella typhimurium*. The ability of *Salmonella typhimurium* strains containing RpoS-dependent-Spv virulence plasmids to respond to starvation stress in host tissues is an essential component of virulence (Coynault *et al.*, 1996). The physiological functions of most *rpoS*-regulated genes are not known.

The overall aim of this study is to control gene expression of RpoS and control its regulon's gene expression in *E. coli*. The hypothesis is that *rpoS* antisense RNA, complementary to *rpoS* mRNA, can inhibit the RpoS protein translation and attenuate expression of the *rpoS* regulon in *E. coli*. Also, *rpoS* sense mRNA can be artificially induced at an early stage of cell growth when RpoS levels are normally extremely low (i.e., in the early exponential phase) and can activate the expression of the RpoS regulon in exponential phase and stationary phase in *E. coli*. In order to test these hypotheses, the *rpoS* sense and antisense were cloned into a pET expression vector and the expression of *rpoS* and previously identified RpoS-dependent genes were assayed for activity.

Selection and identification of succinate mutants helped to explain why *rpoS* mutants are common in different laboratory strains under certain growth conditions. We have identified one of these conditions by selecting *rpoS* mutants on minimal agar plates containing succinate as sole carbon and energy source. Some succinate mutant

can revert to an *rpoS* wild type phenotype suggesting that expression of the *rpoS* regulon can be switched on again under sub-optimal growth conditions.

Induced *rpoS* antisense RNA can be used to attenuate expression of RpoS and its regulon. It not only provides a greater understanding of the bacterial environmental adaptation responses, but also offers a model for translational regulation of a sigma factor in bacteria. Induction of a plasmid-encoded *rpoS* gene offers a useful tool for the study of RpoS-dependent functions required for the expression of the environmental stress functions. This is particular useful for identifying RpoS-dependent function in exponential phase.

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CHAPTER 2:

METHODS AND MATERIALS

Bacterial strains and plasmids

All of the *E. coli* strains or bacteriophage used and constructed in this study are listed in Chapters 3, 4 and 5. All of the plasmids that were used and constructed are given in Chapters 3, 4 and 5.

Chemicals, enzymes and reagents

Chemicals, media and molecular biology reagents were supplied by Sigma Chemical Co. (St. Louis, MO), Roche Diagnostics Corporation Ltd., Invitrogen Canada Inc. (Burlington, ON), Fisher Scientific Ltd. (Toronto, ON), MBI Fermentas Inc. (Burlington, ON), Bio-Rad Laboratories (Philadelphia, PA), Promega Corporation (Madison WI), Qiagen Inc. (Mississauga, ON), Stratagene (La Jolla, CA), and Novagen Inc. (Madison, WI).

Growth media

All growth media listed below were prepared with distilled water and autoclaved at 121°C (15 lbs.sq.in.) for 20 min, unless stated otherwise.

2TY agar contained per litre; 16 g tryptone, 10 g yeast extract, 5 g NaCl, 22 g agar. To make ampicillin plates, the autoclaved agar stock was melted in a microwave and, when temperature is below 45°C, the required amount of antibiotic was added.

2TY broth contained 16 g tryptone, 10 g yeast extract and 5 g NaCl per litre.

LB Agar contained, per litre; 10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar.

Distilled water was added to 950 ml and adjusted to pH7 with 5 M NaOH

(approximately 0.2 ml). The volume of solution was then adjusted to one litre with distilled water.

LB broth contained, per litre; 10 g tryptone, 5 g yeast extract, 10 g NaCl. Distilled water was added to 950 ml and adjusted to pH7 with 5 M NaOH (approximately 0.2 ml). The volume of solution was then adjusted to one litre with distilled water.

M9 Salts (5 x) was per litre; 64 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 15 g KH_2PO_4 , 2.5 g NaCl and 5.0 g NH_4Cl . After autoclaving, MgSO_4 and CaCl_2 were added at 10 $\mu\text{g}/\text{ml}$.

Supplements and antibiotics

All supplements and antibiotics were prepared in sterile distilled water and filter-sterilized with sterile filter (pore size, 0.45 μm) unless stated otherwise.

<u>Supplement</u>	<u>Stock concentration</u>	<u>Use concentration</u>
Glucose	20 mg/ml	0.4%
Succinate	10%	0.5%
X-gal	25 mg/ml in dimethylformamide	50 $\mu\text{g}/\text{ml}$

<u>Antibiotic</u>	<u>Stock solution</u>	<u>Store at</u>	<u>Use concentration</u>
	(mg/ml)	(°C)	($\mu\text{g}/\text{ml}$)
Ampicillin	25	-20	100
Chloramphenicol	50 in ethanol	-20	100
Kanamycin	100	-20	50
Streptomycin	50	-20	50
Tetracycline	50 in ethanol	-20	12.5

Working solutions and buffers

Denhardt's solution was made as 100x concentrate and contained per 50 ml; 2 g bovine serum albumin, 2 g Ficoll 400 and 2 g polyvinylpyrrolidone.

Ethidium bromide solution was prepared as a stock solution of 10 mg/ml in water and stored in the dark at room temperature.

Gel loading buffer was made as 5x concentrate and contained per 10 ml; 5 g sucrose, 1 ml 10xTBE and 0.01 g bromophenol blue.

Glucose-Tris-EDTA (GTE) contained per litre; 9.0 g glucose, 1.25 ml 2 M Tris-HCl (pH8) and 2 ml 0.5 M EDTA.

IPTG was made as 1.0 g in 46.7 ml H₂O. The solution was then filter sterilized, aliquoted and stored at -20°C.

10x MOPS buffer contained per litre: 41.2 g 3-(N-morpholino)-propanesulphonic acid (MOPS), 10.9g sodium acetate, 3-hydrate and 3.7g EDTA. The pH was adjusted to 7 and the solution was filter sterilized.

Phenol/Chloroform/Isoamyl alcohol (PCI) the following solutions were added in a 25:24:1 ratio; phenol, chloroform, isoamyl alcohol.

5 M KAc, pH 4.8 29.44 g KAc was added to 60 ml distilled water. 11.5 ml glacial acetic acid was then added and 28.5 ml of distilled water to bring the final volume to 100 ml. The solution was then 3 M with respect to potassium and 5 M with respect to acetate. The solution was stored at 4°C.

Potassium phosphate buffer was per litre: 8 g NaCl, 1.2 g K₂HPO₄ and 0.34 g KH₂PO₄. The pH was adjusted to 7.3 with HCl and the solution was autoclaved.

Protein blocking buffer was prepared in TBS-T and contained per litre: 50 g albumin bovine Fraction V (0.5%) and 5 g skim milk (0.5%).

Protein transfer buffer contained per litre: 3.03 g Tris-base, 14.4 g glycine and 200 ml of methanol added before use.

RNase prepared free from DNases was made as 10 mg/ml stock in TE buffer. The mixture was heated at 100°C for 10 min and then allowed to cool to room temperature. RNase was then dispensed in 0.2 ml aliquots to sterile eppendorfs and stored at -20°C.

3 M NaAc contained 408 g sodium acetate per litre.

Tris-borate-EDTA (TBE) buffer was made at 10x concentration and contained per litre; 108 g Tris base, 55 g boric acid, 9.3 g EDTA. The pH was adjusted to 8.3 with HCl.

Tris buffer saline (TBS) contained per litre: 2.42 g Tris-base and 8 g NaCl₂. The pH was adjusted to 7.6 and the solution was autoclaved.

TBS-T was made as a 0.1% (v/v) Tween 20 in TBS and stored at 4°C.

Tris-EDTA (TE) buffer was made from stock solutions and contained per litre; 5 ml 2 M Tris-HCl (pH8), 4 ml 0.25 M sodium-EDTA (pH8) to give final concentrations of 10 mM Tris-HCl and 1 mM EDTA.

X-Gal was made as a 25 mg/ml stock. 50 mg was dissolved in 2 ml dimethylformamide and stored at -20°C.

Z Buffer contained per litre: 16.1 g Na₂HPO₄·7H₂O (60 mM), 0.75 g KCl anhydrous (10 mM), 0.246 g MgSO₄·7HO (1 mM) and 2.7 ml of β-mercaptoethanol (0.05 M) added just before use. The pH was adjusted to 7 and the solution was not autoclaved.

Growth and storage of cultures

Strains in current use were kept on agar plates at 4°C. Cultures were kept as glycerol stocks that were held at -80°C for long term storage. Two identical tubes were labeled and placed in an -80°C freezer.

All the above strains were grown overnight in LB media containing the appropriate antibiotics at 37°C. Liquid cultures were grown in 15 ml test tubes, 250 ml conical flasks or 2 litre flasks and incubated in an orbital shaker at 200 rpm.

Preparation of glycerol stocks

These were prepared in one of two ways. A 5 ml culture of an overnight suspension was centrifuged and the pellet resuspended in 20% glycerol in an eppendorf, or to a streak plate 5 ml 20% glycerol was added and cells were scraped using sterile pipette tip and the resultant mixture was then aliquoted into screw-cap tubes. The aliquots could be frozen at -20°C if storing for 1 year, or at -80°C if to be kept in excess of 1 year.

Centrifugation

Small scale preparation of plasmid DNA in eppendorfs were centrifuged using a bench top microcentrifuge at 12,000xg and large-scale preparation of plasmid DNA, in 500 ml bottles, were centrifuged in a Sorvall RC-5B refrigerated superspeed centrifuge with a GSA rotor. For volumes of 25-50 ml a Sorvall SA-600 rotor or a bench top refrigerated centrifuge was used.

Transformation with plasmid DNA

Preparation of fresh *E. coli* competent cells

The method of Hanahan (1983) was used.

A single colony of *E. coli* DH5 α cells was inoculated into 10 ml 2TY broth and grown at 37°C overnight. A 300 μ l amount of the overnight culture of cells was added to 30 ml 2TY broth in a 250 ml flask and grown with shaking at 37°C until an OD₅₉₅ is 0.4-0.6. The cells were transferred to a 50 ml disposable centrifuge tube and

centrifuged at 2,000xg for 10 min at 4°C. The pellet was resuspended gently in 2.5 ml ice-cold TFB (10 mM MES, 100 mM KCl, 45 mM MnCl₂·4H₂O, 10 mM CaCl₂·2H₂O, 3 mM hexamine cobaltic chloride, filter sterilized and stored at 4°C) then kept on ice for 15 min. 100 µl of DMSO was added and the cells returned to ice for 15 min. 100 µl DTT/KAc (2.25 M DTT, 40 mM KAc, pH6) was then added and placed on ice for 10 min before use. 100 µl of DMSO was added and the cells were placed on ice for at least 5 min before use. Optimum competence was reached at 1 h. After 3 h the cells could not be used.

Preparation of frozen *E. coli* competent cells

A single colony was grown in 1 ml 2TY at 37°C, 200 rpm overnight. The culture was used to inoculate 80 ml of 2TY in a 500 ml flask and the flask was incubated for a further 2.5-3 h at 37°C, 200 rpm until an OD₅₉₅ is 0.4-0.6. The culture was then separated to 30 ml centrifuge tubes and incubated on ice for 10 min. The cells were harvested by centrifugation at 4,000xg for 10 min at 4°C in a Sorvall SA-600 rotor, and resuspended in a tube in 15 ml (total volume) ice cold RF1 (100 mM RbCl; 50 mM MnCl₂·4H₂O; 30 mM potassium acetate; 10 mM CaCl₂·2H₂O; glycerol 15 % (v/v); the pH was adjusted to 5.8 with 0.2 M acetic acid, the solution was filter sterilized and stored at room temperature). The cells were incubated on ice for 15 min and centrifuged at 4,000xg for 10 min at 4°C in a Sorvall SA-600 rotor. The cell pellets were resuspended in 7.5 ml (total volume) RF2 (10 mM MOPS; 10 mM RbCl; 75 mM CaCl₂·2H₂O; glycerol 15 % (v/v); the pH was adjusted to 6.8 with 1 M NaOH, the solution was filter sterilized and stored at -20°C), and incubated on ice for a further 15 min. The cells were then divided into 200 µl aliquots in chilled microcentrifuge tubes

and flash frozen in liquid nitrogen for 10 s. The competent cells were stored at -70°C and thawed as required.

Transformation of fresh and frozen *E. coli* competent cells and selection of transformants

A 200 μl aliquot of *E. coli* competent cells was stored on ice in a 10 ml plastic sterile tube. Plasmid DNA was added to 200 μl competent cells. The mixture was left on ice for 20 min then heat shocked at 42°C for 90 s and then rapidly transferred and replaced on ice for 2 min. 2 ml 2TY was added to each tube then incubated with shaking at 37°C for 30-40 min. Cells were harvested by centrifugation at 2,000xg for 10 min at 4°C and resuspended in 200 μl 2TY broth. 200 μl aliquots spread on 2TY agar containing selective antibiotics. A control of competent cells without DNA, treated as above, was used to check for viability of the competent cells.

Preparation of DNA from *E. coli*

Small scale preparation of *E. coli* plasmid DNA

Method 1, alkaline lysis.

This method was based on that of Ish-Horowicz and Burke (1981).

A single colony on an agar plate was used to inoculate 10 ml LB broth containing the appropriate antibiotic, and grown overnight at 37°C with shaking (200 rpm). 1 ml of culture was dispensed into an eppendorf tube and centrifuged at 12,000xg for 1 min, resuspended in 100 μl ice-cold GTE containing 4 mg/ml lysozyme and stored for 5 min at room temperature. 200 μl of a freshly prepared alkaline solution (1% SDS, 0.2 M NaOH) was added and the tube was closed and inverted several times then transferred to ice for 5 min. 150 μl ice-cold 3 M KAc, pH 4.8 or 3 M NaAc, pH5.2 was

added, then the tube was mixed for 10 s and returned to ice for 15 min. The solution was centrifuged at 6500xg for 5 min in the eppendorf and the supernatant was transferred to a fresh tube. An equal volume of phenol chloroform isoamyl alcohol was added and the solution was mixed by vortexing, then the upper aqueous layer was transferred to a fresh tube. Two volumes of ethanol were added, and the tube was placed at room temperature for 3 min. DNA was precipitated by centrifugation at 12,000xg for 5 min at 4°C, and the pellet was washed in 1 ml 70% ethanol, vortexed then centrifuged at 12,000xg for 3 min at 4°C. The pellet was dried then resuspended in 50 µl TE (pH 8.0) containing the appropriate RNase and the solution was incubated at 37°C for 15 min.

Method 2, Wizard™ plus miniprep preparation DNA purification system

This was the method of choice when preparing DNA for sequencing. The kit, supplied by Promega, gave 2 µg of DNA from a 1 ml overnight culture. A protocol was used according to the manufacturer's instructions.

Large scale preparation of *E. coli* plasmid DNA

Alkaline lysis

The method used was a modification of that of Birnboim and Doly (1979). 10 ml 2TY containing the appropriate antibiotic was inoculated with a single colony from a fresh plate culture and grown during the day. This culture was then used to inoculate 500 ml 2TY containing the appropriate antibiotic that was grown overnight at 37°C with shaking. The culture was then split into 2x 250 ml bottles and centrifuged for 10 min at 6,000xg. The pellet was resuspended in 25 ml GTE with lysozyme (2 mg/ml) and left at room temperature for 5 min. 50 ml fresh alkaline solution (1% SDS, 0.2 M

NaOH) was added, mixed gently, and the suspension was then placed on ice for 2 min. 37.5 ml ice-cold 5 M KAc (pH 4.8) was added, then the suspension was inverted sharply several times and placed on ice for 15 min. Protein, SDS and chromosomal DNA were then removed by centrifugation at 12,000xg for 10 min and the supernatant was transferred to a fresh bottle through a sterile Pasteur pipette with a cotton wool plug. An equal volume of phenol chloroform isoamyl alcohol was added and the solution was shaken for 8 min. Phases were separated out by centrifugation at 12,000xg for 10 min. The upper aqueous phase was transferred to a fresh tube. An equal volume of ethanol was added and the solution was placed at room temperature for 3 min. Precipitated DNA was pelleted by centrifugation at 12,000xg for 15 min; the pellet was washed in 70% ethanol then resuspended in 10.5 ml TE.

QIAGEN plasmid midi purification

This kit, supplied by QIAGEN, allowed 100 µg of plasmid DNA to be isolated from 25-100 ml culture. *E. coli* was grown in 25-100 ml of 2TY or LB containing the appropriate antibiotic overnight and then centrifuged in a 50 ml sterile tube for 10 min at 6,000xg. The remaining procedures were used according to the manufacturer's instructions.

Preparation of *E. coli* chromosomal DNA

A single colony of *E. coli* cells was inoculated into 10 ml 2TY broth and grown at 37°C overnight with shaking. 1 ml of the culture was centrifuged at 5,000xg for 10 min then washed twice in solution I (25 mM Tris-HCl, 25 mM Na-EDTA). The pellet was resuspended in solution I containing 10 mg/ml lysozyme. The suspension was incubated at 37°C for 30 min and 500 µl of 5 M NaCl added. The suspension was then

vortexed until it became viscous. 1.2 ml 10% SDS was added and the suspension vortexed again until the viscosity was reduced. The suspension was incubated at 65°C for 15 min, then cooled to room temperature and 2.4 ml 5 M KAc, pH7 added. The solution was vortexed and left on ice for 20 min. The suspension was centrifuged at 6,000xg for 30 min at 4°C. The supernatant was transferred to a 30 ml centrifuge tube then recentrifuged at 6,000xg for 15 min at 4°C. 5 ml isopropanol was added and the solution was mixed, then placed at -20°C for 30 min. The solution was then centrifuged at 6,000xg for 15 min at 4°C, the supernatant discarded and the pellet left to dry at room temperature. The pellet was redissolved in 700 µl solution II (50 mM Tris-HCl, pH8; 10 mM EDTA), and transferred to a 1.5 ml eppendorf tube and 75 µl 3 M NaAc, pH7 and 500 µl isopropanol added and mixed. The DNA was then centrifuged at 12,000xg for 3 min in a cold room. The pellet was washed in 0.5 ml 80% ethanol, dried and resuspended in 200 µl TE containing the appropriate amounts of RNase and then the solution was incubated at 37°C for 15 min.

***In vitro* manipulation of DNA**

Purification and concentration of DNA

Purification of DNA

Contaminated protein was removed from DNA by extraction with phenol/chloroform according to the method of Sambrook et al. (1989). The volume of DNA solution was made up to 0.5 ml with TE. An equal volume of phenol chloroform isoamyl alcohol was added and the solution was vortexed. The samples were centrifuged at 6500xg for 3 min. The upper aqueous layer was transferred to a fresh tube. The aqueous layer was then ethanol precipitated.

Concentration of DNA

DNA samples were purified in order to remove any components that might inhibit further reactions. DNA was precipitated out of solution by the addition of 1/10 volume 3 M NaAc, pH 5.2 and 2 volumes of 100 % ethanol. The solution was placed at room temperature for 3 min then centrifuged at 12,000xg for 10 min at 4°C. The pellet was washed in ice-cold 70 % ethanol then resuspended in TE.

Agarose gel electrophoresis of DNA

Agarose gels were prepared using agarose at 0.8-1.5% in 1x TBE or 1x TAE depending upon the size of DNA fragments to be run, and the method by which they were to be isolated. Agarose was melted in a 1,000W microwave on high power for 1 min, cooled and added to a final volume of 50 or 100 ml using distilled water. Ethidium bromide (10 mg/ml) was added to the molten agarose at a final concentration of 0.2 µg/ml. Gels were run at 100 volts for the appropriate period using 1x TBE or 1x TAE as running buffer. Gel loading buffer was added to DNA samples (1 µl buffer to 3 µl DNA sample) before loading the wells of the gel. Molecular markers were 1 kb ladder or Hind III-cut λ DNA. Once the marker front had migrated far enough the power was switched off, the gel unloaded and examined under UV-illumination.

Size and concentration estimation of DNA on agarose gels

This was done by comparing the brightness of the sample bands to those of the markers included on agarose gel electrophoresis of DNA samples.

A sample of DNA mixed with gel loading buffer was run on an agarose gel with aliquots of markers of known concentration. A photograph was taken under UV light. Fluorescence and intensity of bands on the photograph, are proportional to the mass of

DNA. The amount of the sample was estimated by comparing the intensities of the sample DNA and marker bands.

An alternative method was to calculate the absorbance ratios at 260 and 280 nm in order to determine the amount and purity of the DNA sample. 10-20 μl of the DNA was dissolved in 2 ml of water and placed in a quartz cuvette. The absorbance was then measured at 260 and 280 nm, the ratio 260:280 giving the purity of the DNA sample. DNA concentration alone is calculated at 260 nm with an OD of 1.0 being equivalent to 50 $\mu\text{g/ml}$ double stranded DNA (Sambrook *et al.*, 1989).

Cloning of DNA

Restriction digestion of plasmid DNA

The basic technique described by Sambrook *et al* (1989) was used for restriction endonuclease digestion.

1 μl of restriction enzyme (10 units/ μl), 1 μl of 10 \times React buffer, DNA and ddH₂O to 10 μl were mixed together. This mixture was then incubated at the recommended temperature (e.g. 37°C) for 1-2 h. At the end of the digestion, an aliquot of the DNA was checked by gel electrophoresis through a 1% agarose using undigested plasmid DNA as a marker. If digestion was incomplete, more restriction enzyme was added and then incubated again. The completely digested DNA was purified.

If two enzyme digestions were performed, and the two enzymes required different buffers, the DNA was first digested with the enzyme whose buffer had the lower concentration of salt, this enzyme was then heat inactivated at 65°C for 15 min and buffer components were added in order to meet the requirements of the second enzyme.

In cases where the enzyme could not be heat inactivated, it was necessary to first clean up the DNA before cutting with the second restriction enzyme in the appropriate buffer. Where partial digests were necessary, less enzyme was used over a much shorter incubation times. A more reproducible method was to dilute out the restriction enzyme in appropriate buffer using doubling dilutions and to incubate the same amount of DNA with the varying concentrations of enzyme. This was done at the appropriate temperature for 30 min. The extent of digestion was checked by running the DNA on an agarose gel.

Dephosphorylation of 5' ends of plasmid DNA

The shrimp alkaline phosphatase (SAP) prevents recircularisation of plasmid DNA, by removing 5' terminal phosphate groups from linear vector DNA. Plasmid DNA, 1.5 μ l of SAP, 2.5 μ l SAP dilution and appropriate 10 \times SAP buffer were mixed together. The mixture was incubated for 30 min at 37°C. The reaction was stopped by incubating the solution at 65°C for 15 min. The treated DNA was purified.

Partial restriction endonuclease digestion of plasmid DNA.

The DNA sample was purified and concentrated first. Reactions were carried out in volumes of 20-100 μ l. Appropriate plasmid, 2 μ l 10 \times React buffer, 1 μ l enzyme (10 units/ μ l) and ddH₂O to 20 μ l, were mixed together. The incubation time was much shorter than the complete digestion. The reaction was stopped by incubating the solution at 65°C for 15 min. The treated DNA was purified. Following the incubation period, an aliquot of the digested DNA was electrophoresed and photographed. The solution of digested DNA was purified and concentrated.

Blunt-end DNA

Some restriction enzymes naturally produce blunt ends when cutting DNA. Where such an enzyme was not available it was necessary to create blunt ends using an appropriate enzyme. The Klenow fragment of DNA polymerase I is capable of filling in 3' recessed ends of DNA to produce such blunt ends under the correct conditions. To purified DNA in a minimum volume of TE, the following reagents (stored at -20°C) were added: 5 µl nick translation buffer (0.5 M Tris, pH 7.2; 0.1 M MgSO₄; 1 mM DTT; 500 µg/ml BSA), 2 µl dNTP mixture (0.5 M with respect to dATP, dGTP, dCTP and dTTP), and 3 units of Klenow. The volume was made up to 50 µl with water. To inactivate the enzyme, the mixture was then incubated at 37°C for 10 min and then at 65°C for 5 min. The DNA could then be treated with SAP if necessary to remove phosphate ends.

Isolation of DNA fragments from agarose gels.

QIAEX II gel extraction system

The DNA band to be extracted was excised from the agarose gel, weighed, and placed in a 1.5 ml eppendorf tube. A kit for QIAEX II gel extraction system from Qiagen Inc. (Mississauga, ON) was used according to the manufacturer's instructions.

Ligation of DNA

Ligation of cohesive termini

Ligations were performed with a molar ratio of insert : vector of 3:1.

Calculation of insert : vector ratio used the following equation.

$$\text{ng of vector} \times \text{kb size of insert} \div \text{kb size of vector} \times \text{insert : vector molar ratio} = \text{ng of insert}$$

1 μ l of 10x ligation buffer (30 mM Tris-HCl, pH 7.5; 6 mM MgCl₂; 10 mM DTT; 50 μ g/ml BSA) and 1 μ l T4 DNA ligase (5 units/ μ l) were added to the vector and fragment DNA mix, ddH₂O to 10 μ l. The reaction was incubated at 4°C overnight. If insufficient DNA was available, as much foreign DNA as possible was added to the plasmid DNA per ligation without increasing the volume of the ligation reaction to greater than 10 μ l. Two additional control reactions were set up, e.g. the plasmid vector alone and the fragment of foreign DNA.

Ligation of blunt-end DNA

PEG 4000 stimulated blunt-end ligation of synthetic oligomers as short as 8 nucleotides in length. The reaction mixtures were prepared at 0°C by mixing all components containing 1 μ l of 10x ligation buffer, 1 μ l T4 DNA ligase (5 units/ μ l), the appropriate amounts of vector, insert DNA and ddH₂O. Two μ l 50% PEG 4000 was added to the above reaction mixtures at room temperature. The reaction was incubated at 22°C for 1 h.

Identification of bacterial colonies that contain recombinant plasmid DNA

Method 1, restriction analysis of small scale preparations of plasmid DNA

A number of independently transformed bacterial colonies are picked and grown in small-scale cultures. Plasmid DNA was then analyzed by digestion with restriction enzymes and gel electrophoresis.

Method 2, α -complementation

Many of the vectors (e.g., the pUC series and pGEM®-T Easy vector) carry a short fragments containing the coding information for the first 146 amino acids of the β -galactosidase gene (*lacZ*). The *lac*⁺ bacteria that result from α -complementation form

blue colonies in the presence of X-gal. However, insertion of DNA fragments into the polycloning site of the plasmid interrupted the coding region that is not capable of α -complementation. Bacteria carrying recombinant plasmids formed white colonies.

100 μ l of each transformation culture was plated onto duplicate antibiotic plates, then incubated overnight (16-24 h) at 37°C. Longer incubations or storage of plates at 4°C (after 37°C overnight incubation) may be used to facilitate blue/white screening. White colonies generally contain inserts, however, inserts may also be present in blue colonies.

Method 3, rapid screening of transformants for inserts using the polymerase chain reaction (PCR)

The use of PCR to screen colonies for plasmids containing inserts was preferred to the use of a time-consuming plasmid miniprep screening method. This screening method used PCR to amplify DNA inserted into the polylinker. Colonies were tooth picked from LB plates firstly into 1.5-ml tube containing 50 μ l of sterile water and then into a corresponding well of a microtitre plate containing LB medium containing the appropriate antibiotics. The microtitre plates containing tooth picked colonies were then stored at -20°C for future use. The cells in 1.5 ml tubes were boiled at 100°C for 5 min. 30 μ l of the resulting mixture was transferred to a fresh 0.5-ml tube for PCR.

PCR conditions were as follows: the reactions were hot started at 94°C for 3 min before adding Taq polymerase. This was then followed by a series of cyclic heating (35 cycles) at 94°C for 1 min, 61°C for 1 min and 72°C for 2 min. At the end of 35 cycles there was a further elongation period of 72°C for 10 min and then the samples were

stored at 4°C. PCR products were checked by agarose gel electrophoresis, and those found to be of the required size were used for mini preparation.

Rapid cloning in plasmid DNA

In the protocol given below, ligation of plasmid and foreign DNA was carried out directly in the melted slabs of agarose recovered from the gel used for purification. The method works for ligation of cohesive termini (see Sambrook *et. al.*, 1989).

The foreign DNA was digested with the appropriate restriction enzyme(s). The amount of foreign DNA digested was sufficient to yield approximately 0.2 µg of the target fragment. The digestion was carried out in a volume of 20 µl or less. In a separate tube, 0.5 µg of the vector DNA was digested with the appropriate restriction enzyme(s) in a total reaction volume of 20 µl or less. When digestion with the restriction enzyme(s) was complete, appropriate phosphatase was added and incubated for 30 min at 37°C. The desired fragments were separated by electrophoresis on an agarose gel. The gel was examined by UV illumination. From the relative fluorescent intensities of the desired fragments, the amounts of DNA were estimated and cut out using a razor in the smallest possible volume of agarose and the excised slices of gel were placed in separate, labelled microcentrifuge tubes and heated to 70°C for 10-15 min to melt the agarose. Aliquots of the melted gel slices were combined in a fresh tube prewarmed to 37°C. The final volume of the combined aliquots was 10 µl or less, and the molar ratio of foreign DNA: plasmid vector was approximately 2:1. In separate tubes, two additional ligations were set up as controls, one containing the plasmid vector alone and the other containing only the fragment of foreign DNA. The three tubes were incubated for 10-15 min at 37°C, and then 10 µl of T4 DNA ligase mixture

was added to each tube and the contents were mixed well before the agarose hardened and incubated the reactions for 12-16 h at 16°C. At the end of the ligation, three tubes containing 200 µl of frozen competent *E. coli* each were removed from storage at -70°C. The agarose in the ligation mixtures was remelted by heating them to 70°C for 10-15 min. 5 µl of one of the ligation mixtures were immediately added to 200 µl of competent *E. coli* and the contents of the tube were mixed quickly by gentle shaking. The transformation protocol in section 2.9.3 was followed.

PCR reactions

PCR reactions generally required optimization to obtain a good yield of product. However some basic procedures are included here. All samples and reagents were kept on ice while not in use, fresh tips for each reagent were used while accurate volumes of each reagent were pipetted. Gloves were worn to avoid contamination when working with PCR samples.

The PCR mix consisted of 1 µl of 25 pmole primers, 5 µl of 10x PCR buffer, 5 µl of template DNA at the appropriate dilution (neat, 1/10, 1/100 and 1/1,000), 1 µl of dNTP mixture (containing dATP, dCTP, dGTP and dTTP at 10 mM final concentration) and water to 50 µl. Also included in each set of reactions was a minus template control and a minus primer control. The reagents were mixed by gently tapping and the tubes were centrifuged for a few seconds to bring reagents down to the bottom. PCR reaction was run in the PCR machine under pre-set optimal conditions for the appropriate time. The cycle of temperature settings was as follows: the reaction was hot started at 94°C for 3 min before adding *Taq* DNA polymerase (Invitrogen Corporation, Carlsbad, CA). This was then followed by a series of cyclic heating (25 cycles) at 94°C for 45s, 61°C

for 30s and 72°C for a further 90 s. At the end of 25 cycles there was a further elongation period of 72°C for 7 min and then the samples were stored at 4°C until they were required for use. 4 µl PCR products were mixed with 1 µl of loading dye to the tube. The entire PCR sample as well as DNA markers were loaded into the assigned well of the agarose gel and electrophoreses at 100 volts for 45-60 min. The photograph was taken under UV light.

Transduction

Preparation of P1vir transduction lysates

To prepare a phage lysate from log phase cultures, *E. coli* cultures were incubated overnight in 5 ml LB broth containing 5 mM CaCl₂ at 37°C. The phage requires CaCl₂ for infection. Cells were subcultured the next morning into 20 ml of fresh LB at 1/100 dilution and grown to an OD₆₀₀ of 0.3. 2x 5 ml cultures were then transferred into 2x 15 ml polypropylene tubes (one tube served as control), and 10⁷ PFU phage was added to one tube and incubation was continued until lysis was apparent. The cultures were incubated at 37°C for an additional 1 h to allow complete lysis of the cells. 100 µl of chloroform was added and centrifuged at 5500xg for 10 min at 4°C. The top lysate was transferred to a new tube and mixed thoroughly by inversion. 100 µl of chloroform was added to kill any remaining viable cells. The lysed cultures were recentrifuged at 5500xg for 10 min at 4°C. Lysates were then stored at 4° in 15 ml tubes. To determine the phage titre, the lysate was diluted in 1:10 series in LB broth. 100 µl of a diluted phage was mixed with 100 µl of a log phase *E. coli* culture and plated on LB plate. After 15 min incubation at room temperature, 2.5 ml soft agar (47 °C) was added to the plate. The mixture was immediately poured on the surface of the

plate and then the plate was incubated overnight at 37°C. The lysate should give a titre of 10^{10} - 10^{11} PFU/ml and can be stored at 4°C for a few years.

P1vir mediated transduction

To measure the effect of *rpoS* antisense RNA on the expression of the highly RpoS-dependent *osmY* gene (Lange *et al.*, 1993), an *osmY-lacZ* operon fusion was transduced into an *rpoS* wild type strain. A DNA fragment can be transferred from one strain to another using a transducing phage P1 that can package random fragments of host DNA. The genetic marker usually is an antibiotic resistance gene for selection of transductants. P1 lysate was prepared from the donor strain containing the selective marker to be transduced to the recipient strain. *E. coli* cultures were incubated overnight in 5 ml LB broth containing 5 mM CaCl₂ at 37°C. The cultures were centrifuged for 5 min at 5,500xg and resuspended in 250 µl of LB broth containing 5 mM CaCl₂. 100 µl of P1 lysate was added into 100 µl cells and incubated for 10 min at 37°C water bath to allow adsorption. 12 ml of LB containing 0.1 M sodium citrate was added and the cells were centrifuged at 5,500xg for 5 min. The supernatant was removed and the cells were washed once with 12 ml of LB containing 0.1 M sodium citrate. The pellets of cells were resuspended in 12 ml of LB containing 0.1 M sodium citrate and incubated for 30 min at 37°C. Cultures were centrifuged again at 5,500xg for 5 min and the pellets were collected. The pellets were resuspended in LB containing 0.1 M sodium citrate. 100 µl was plated on LB plate supplemented with the appropriate antibiotics. As a control, the diluted phage lysate and the recipient cells were also plated on this selective media.

λ DE3 lysogenization

Lysogenization

λ DE3 (*imm*²¹ Δ *in5 Sam7*) encodes the T7 RNA polymerase gene that is under control of *lacUV5* promoter. λ DE3 cannot combine to the chromosome by itself due to the inactive *int* gene in phage λ D69. The helper phage (λ B10) can offer *int* function for the λ DE3 but cannot integrate into *E. coli* chromosome by itself due to the lack of the phage repressor *cI*. The selection phage (λ B482) can kill λ DE3 mutants but can not neither kill true λ DE3 lysogens nor integrate into *E. coli* chromosome because the selection phage has the same immunity as λ DE3.

The protocol used was a modification of that of the λ DE3 lysogenization manual, which was supplied by Novagen Inc., Madison, WI. A single colony of *E. coli* cells was used to inoculate 10 ml LB broth containing 0.2% maltose, 10 mM MgSO₄ and grown overnight at 37°C with shaking (200 rpm). A 300 μ l of the overnight culture of cells was added to 30 ml LB broth containing 0.2% maltose, 10 mM MgSO₄ and grown with shaking at 37°C until an OD₆₀₀ was 0.5. A 50 μ l of the cells was mixed with 10⁸ pfu of λ DE3, 10⁸ pfu of λ B10 and 10⁸ pfu of λ B482. The resulting mixture was incubated at 37°C for 20 min with shaking (200 rpm) to allow the phage to adsorb to the cells. 1 ml LB containing 0.2% maltose, 10 mM MgSO₄ was added to each tube and 200 μ l aliquots of 1/200 dilution spread onto LB agar containing selective antibiotics. A control of cells mixed with 10⁸ pfu of λ B482, treated as above, was used to check for viability of λ DE3 lysogen.

E. coli strains that were used to integrate λ DE3 into the chromosomal DNA included HS1600, MC4100, GC4468, GC122, HS1098, HS1098P, HS1095 and HS1095T. The cells were mixed with λ DE3 phage lysate, the helper phage and the selection phage.

Verification of λ DE3 lysogens

In the presence of inducer IPTG, T7 RNA polymerase gene is induced. The tester phage makes large plaques on true λ DE3 lysogen plates. In the absence of IPTG, smaller clear plaques are observed. A single colony of *E. coli* cells was used to inoculate 10 ml LB broth containing 0.2% maltose, 10 mM MgSO₄ and grown overnight at 37°C with shaking (200 rpm). A kit for verification of λ DE3 lysogens was used according to the manufacturer's instructions.

β -Galactosidase assay

β -galactosidase assays were performed by the method described by Miller (1992). Single colony isolates were inoculated into LB media supplemented with appropriate antibiotics and incubated overnight at 37°C at 200 rpm. Growth was monitored by measuring OD₆₀₀. Overnight cultures were serially subcultured 1:100 twice to an OD₆₀₀ of 0.2, before inoculation into fresh media to an initial OD₆₀₀ of 0.02-0.05. Aliquots of culture were removed every 30 min –1h, placed on ice with chloramphenicol to stop further protein synthesis, and assayed for β -galactosidase activity.

0.5 ml of cells (for which OD₆₀₀ was measured) was mixed with 0.5 ml of Z Buffer. 50 μ l chloroform and 25 μ l 0.1%SDS are added. The tubes were vortexed and equilibrated at 30°C for 5 min. The reaction was started by adding 0.2 ml of 4 mg/ml

ONPG dissolved in Buffer Z. When the solution turned yellow, the reaction was stopped by adding 0.5 ml 1 M Na₂CO₃. The tubes were centrifuged 5 min at 12,000xg and the optical density at 420 nm was measured. The β-galactosidase activity = (1,000 x OD₄₂₀) / (OD₆₀₀ x (cell volume) x Time) Miller units. All assays were done in duplicate.

Northern blot analysis of RNA

Diethylpyrocarbonate treatment of solutions and consumables

DEPC was added to solutions at 0.1% concentration, shaken vigorously, incubated at 37°C overnight and then autoclaved. Glassware was cleaned with detergent, thoroughly rinsed, filled with fresh made 0.1% DEPC and left overnight at 37°C, and then autoclaved for 15 min to remove residual DEPC. Cleaned plasticware was thoroughly rinsed with 0.1 M NaOH, 1 mM EDTA followed by autoclaved 0.1% DEPC-treated water. Tips were placed in a beaker containing 0.1-0.2% DEPC in 50% ethanol. After leaving overnight, the solution was decanted off and the tips autoclaved. Microcentrifuge tubes and tips were submerged in 0.1% DEPC and left overnight to soak. The solution was decanted off before autoclaving. Alternatively, microcentrifuge tubes and tips were double autoclaved before using to ensure that they were RNase-free.

Preparation of labeled probe

DNA was labeled by random priming (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Plasmid DNA was digested with an appropriate restriction enzyme and run out on a 1% agarose gel along with appropriate controls. Alternatively, double-strand PCR products were used. PCR was carried out using *Taq* polymerase with primers that are complementary to the target gene. The PCR product was purified and

diluted to a concentration of 2-25 ng/ μ l in TE buffer. The DNA sample was denatured by heating for 10 min in a boiling water bath and chilled on ice in a volume of 20 μ l. A 1.5 ml eppendorf tube was placed on ice and the appropriate volume of each reagent added in the following order: DEPC-treated water to a final reaction volume of 20 μ l; 3 μ l dATP, dGTP, dTTP mixture (1 μ l 0.5 mM each), 5 μ l [α -³²P]-dCTP (50 μ Ci), 25-50 ng denatured DNA and 1 μ l Klenow enzyme (2 units/ μ l). The solution was gently mixed by pipetting up and down and centrifuged at 12,000xg for 10 s to collect the contents at the bottom of the tube. The reaction was incubated at 37°C for 30 min. The reaction could be terminated by heating at 65°C for 10 min or adding 2 μ l 0.2 M EDTA (pH8.0). The probes were purified using ProbeQuant G-50 micro columns (Amersham Biosciences, Inc., Piscataway, NJ). The protocol was designed to remove unincorporated radioactive or non-radioactive nucleotides and other reaction components from DNA fragments. Incorporated oligonucleotides are retained on the membrane and are therefore efficiently purified from contaminants. To determine the cpm/ μ l of labeled probe, two μ l probe was added to 5 ml of scintillant and mixed by inversion, cpm was determined and % label incorporation was calculated. The probes could then be used or stored at -20°C in the dark.

Preparation of total RNA from *E. coli*

The *E. coli* culture was harvested and total RNA was extracted using RNeasy Mini and Midi Kits from Qiagen Inc. (Mississauga, ON). The average yields of total RNA were 250 μ g and 50 μ g for midi preparation and mini preparation, respectively.

The purity and concentration of the RNA could be calculated from the absorbance ratios at 260 and 280 nm. For pure RNA a good absorbance ratio lies

between 1.8 and 2.1. If the ratio was above 2.1 this indicates that significant degradation had occurred.

Preparation of RNA formaldehyde agarose gel

RNA was separated using the formaldehyde/formamide procedures. RNA formaldehyde agarose gels were prepared as follows: 1.2 g agarose was melted in 10 ml 10x MOPS buffer and 90 ml nuclease free water in a 1,000 W microwave on high power for 1.5 min, cooled and added to preheated 1.8 ml 37% formaldehyde. If necessary, ethidium bromide (10 mg/ml) was added to the molten agarose at a final concentration of 0.01 µg/ml.

The mixture of 40 µl of RNA sample and 10 µl of 5x RNA loading buffer was incubated for 15 min at 55°C, chilled on ice, and loaded onto the equilibrated RNA gel. The gel was run at 50 volts for the appropriate time using 10x MOPS buffer. The gel was unloaded and examined under UV light. The gels were washed in DEPC-treated water and 10x SSC to remove any adhering agarose and formaldehyde.

Northern blotting

A platform was placed in a container of blotting buffer (20x SSC) and covered with a wick made from a strip of Whatman 3 MM filter paper and saturated in this buffer. The RNA formaldehyde agarose gel was placed well down on the platform and a piece of Hybond-N⁺ membrane (cut to the same size as the gel) was placed over the top. Three more filter sheets were then placed on top of this and a stack of paper towels, weighted on top (750 g), the RNA transfer procedure was allowed to continue for 12-21 h. When completed, the apparatus was dismantled and the membrane washed in DEPC-treated water and 10x SSC to remove any adhering agarose and formaldehyde.

Fixing RNA to the membrane

The Hybond-N⁺ membrane was placed, RNA side up, in a DEPC-treated water treated container and then fixed by baking at 80°C for 2 h or by using optimized UV crosslinking procedure.

Hybridization in tubes

Hybridization buffer, Denhardt's buffer, was preheated to 60°C, the blot added and prehybridized for 30-60 min, with agitation in a hybrid oven. The probe was heated to 100°C for 10 min, allowed to cool on ice and added to 1 ml of hybridization buffer taken from the blot. This was mixed and added to the blot to prevent hotspots.

Hybridization occurred 12-21 h. Stringency washes were performed, first with 250 ml 2x SSC, 0.1% SDS, second with 1x SSC, 0.1% SDS, and third with 0.1x SSC, 0.1% SDS. The first for 2x 5 min, the second for 15 min and the third for 2x 10 min. These were all performed at 60°C with agitation.

Signal detection

The membrane was placed on a piece of SaranWrap cling film and wrapped. The blot was put in an autoradiography cassette. In a darkroom, X-ray film was laid over the blot and left to develop for the appropriate time. The film was then developed. The membrane was kept at -80°C if it was to be probed. Once ready, the film was removed, developed, and examined. Alternatively, the membrane was laid over the top of PhosphoImager Screen to develop at room temperature for 1 to 10 min.

Enzyme assays

Preparation of cultures

The overnight cultures were diluted 1/1,000 into 200 ml LB fresh media and

monitored to OD₆₀₀ of 0.01. Fifty ml aliquots of cells were transferred and added with IPTG. Fifteen ml samples were taken as soon as OD₆₀₀ reaches 0.25 (exponential phase) and OD₆₀₀ reaches 1.5 (stationary phase). As a control, cells were grown in the absence of IPTG were also harvested.

Determining the protein content of cell extracts

Protein content of cell extracts was determined using the Bio-Rad protein assay kit. The method was based on that of Bradford (1976). Bio-Rad reagent contained phosphoric acid, methanol and Coomassie brilliant blue G-250. The dye binds to proteins under acidic conditions. A standard curve was constructed using bovine serum albumin over a 0 to 10 µg/ml range and the OD at 595 nm was measured. 0.2 ml of Bio-Rad reagent was added to 0.8 ml of standard or test samples. The contents were mixed and left to stand for 5 min before reading the absorbances at 595 nm.

Polyacrylamide gel electrophoresis of protein and catalase

The glass plates, spacers and combs were cleaned with soap and rinsed in deionised water and dried. The surfaces were polished with ethanol and dried. The plates and spacers were then assembled in the casting module. Polyacrylamide gels were prepared at 10%. The gel was prepared as follows: 6 ml solution A mix (acrylamide 30 g and 0.8 g bisacrylamide dissolved in 100 ml distilled water), 4 ml solution B mix (Tris 18.15 g dissolved in 24 ml 1 M HCl, 0.4 ml TEMED), and 8 ml 0.4% ammonium persulphate were mixed then poured between two glass plates using a pastuer pipette. An appropriate comb was inserted and the gel left to set for 1 h. Once ready, the gel was placed in the Bio-Rad holder, the upper buffer was added and the lower buffer was used as running buffer. The wells were gently washed out and

samples were loaded from left to right. Samples were normally run on a gel for 2-3 h at 100 volts. When the dye front had got out of the bottom the power was switched off. The gel plates were disassembled. For staining catalase gels, the gel was transferred to a container containing 50 ml of 50 mM phosphate buffer, pH 7.0 (50 µg/ml horseradish peroxidase). The gel was left to soak for 15 min before draining off the solution. The gel was transferred to a second solution in 5 mM H₂O₂ (27 µl of 30% H₂O₂ dissolved in 50 ml of 50 mM phosphate buffer, pH 7.0). The gels were drained off and rinsed twice with distilled water. Finally 50 ml of 0.5 mg/ml diaminobenzidine was added for at least 30 min until clear bands were distinct. The third solution was decanted into toxic waste container and gels were stored in 10% glacial acetic acid and catalase gels were photographed. For staining protein gels, the gels were transferred to stained with Coomassie Brilliant Blue R250 solution dissolved in methanol/acetic acid and placed on a slowly rotating platform for at least 4 h. The gel was destained and soaked in the destaining solution (methanol/acetic acid solution) overnight. The solution was changed several times until distinct bands appeared. The gels were then photographed.

Determination of catalase activity

Catalase activities in protein content of cell extracts were assayed spectrophotometrically. One ml of hydrogen peroxide (0.5 ml of 30% H₂O₂ freshly diluted in 250 ml 50 mM potassium phosphate buffer, pH 7.0) was added to each sample and mixed quickly. The assays were done in duplicate and the decrease in OD₂₄₀ was monitored (molar extinction coefficient at OD₂₄₀ (ml/mol/cm)). The specific activity of catalase (µmol/min/mg) was calculated as $(1,000 \times \text{OD}_{240}/\text{time}) / (43.6 \times \text{mg of protein/ml of reaction mixture})$.

Western blotting analysis

The appropriate amounts of proteins were separated on 10% SDS polyacrylamide gels overnight using Vertical Slab Gel Unit, Model SE400 (Hoefer Scientific Instruments, San Francisco, CA) and then transferred to a Hybond-P membrane (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The membranes were stained with Ponceau S to confirm efficient transfer. Following transfer, the blots were placed into blocking buffer overnight. The blots were then incubated with blocking buffer containing primary antibody for 2 h at room temperature. Following washing three times with TBS-T or blocking buffer, the blots were placed into blocking buffer containing secondary antibody, and shaken for 1 h. The blots were again washed three times and then incubated in 10 ml of the ECL staining solution (1:1 detection reagents mixture, Amersham Pharmacia Biotech Inc., Piscataway, NJ) and exposed to X-ray film (Kodak X-OMAT AR, Eastman Kodak Company, Rochester, N.Y.) for 10 s to 10 min.

CHAPTER 3:

**POSITIVE SELECTION FOR LOSS OF RPOS FUNCTION
IN *ESCHERICHIA COLI***

Preface

Chapter three, entitled “Positive selection for loss of RpoS function in *Escherichia coli*”, was written as a manuscript that has been formatted to comply with the thesis regulations. All the work presented in this chapter has been carried out by the author.

Positive Selection for Loss of RpoS Function
in *Escherichia coli*

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Summary

RpoS, the alternative vegetative sigma factor, plays an important role in regulating global gene expression for survival and adaptation of *Escherichia coli* under stress conditions. We have found that wild type strains grow poorly in succinate minimal media compared with isogenic strains carrying RpoS null mutations. We tested if this differential growth characteristic could be used to selectively isolate mutants that have lost RpoS function. Using an *rpoS*⁺ parent strain harboring an operon *lacZ* fusion to the highly-RpoS dependent *osmY* promoter, we isolated 20 Suc⁺ mutants. All isolated mutants exhibited attenuated β -galactosidase expression on indicator media suggesting a loss in either RpoS or *osmY* promoter function. Because all Suc⁺ mutants were also defective in catalase activity, an OsmY-independent RpoS-regulated function, it was likely that RpoS activity was affected. To confirm this, we sequenced the PCR-amplified *rpoS* gene from the 20 mutants. All isolated mutants were found to possess *rpoS* mutant alleles. Types of mutations detected included single or multiple base deletions, insertions, and transversions. There were no transition mutations. RpoS mutations that allowed cells to grow on succinate included Leu-128-->stop codon TAG, Asn-136-->Tyr, Ala-158-->Glu, Glu-186-->stop codon TAG, and Ile-210-->Ser. These results suggest that RpoS function may be selected against some conditions and may help explain why independent laboratory strains have acquired mutations in this important regulatory gene. In addition, some succinate revertant colonies have been restored to RpoS wild type phenotype.

Introduction

RpoS, the alternative vegetative sigma factor, also referred to as σ^s or σ^{38} , controls a large number of stress related genes in *Escherichia coli*. Many RpoS-dependent genes including *katE* encoding catalase HPII, are induced during entry into stationary phase (Ishihama, 2000). RpoS plays an important role in regulating global gene expression for survival and adaptation under starvation condition (Hengge-Aronis *et al.*, 1993; Ishihama, 1997; Jishage and Ishihama, 1997; Lange and Hengge-Aronis, 1991).

It has long been known that some *E. coli* laboratory strains carry mutations in the *rpoS* gene including strains AB1157, W3110, JC7623, W1485 and a Shiga-like toxin-producing strain (SLTEC) (Visick and Clarke, 1997; Waterman and Small, 1996). *rpoS* mutants may have a growth advantage in stationary phase and can outgrow wild type cells during prolonged incubation (Finkel and Kolter, 1999; Zambrano *et al.*, 1993; Zambrano and Kolter, 1996) suggesting that mutations in the *rpoS* gene may confer a selective advantage under certain circumstances.

During the course of isolating *rpoS-lacZ* operon mutants (Schellhorn and Stones, 1992), we found that *rpoS* mutants were able to grow better on succinate minimal medium plates than wild type strains. This suggests that use of this carbon source may select for strains that have lost RpoS function. In this study, we isolated mutants that had acquired the ability to grow on succinate (Suc⁺) as sole carbon source by spontaneous chromosomal mutation. We then examined the properties of the Suc⁺ mutants to determine the mutational frequency and the exact nature of the mutations by DNA sequencing. Some transversion mutant revertants were also isolated.

Results

Selection of Suc⁺ mutants

The mutational frequency to Suc⁺ phenotype of *E. coli* cells plated on succinate minimal media was found to be 10⁻⁸ mutants per cell plated, consistent with the frequency of spontaneous loss of function of an average gene in *E. coli* (Stanley *et al.*, 1987). All Suc⁺ colonies examined (several hundred) were white to light blue due to lack of expression of β -galactosidase indicating loss of *osmY* expression. All white or light blue colonies initially detected exhibited reduced gas development when plates were flooded with 30% hydrogen peroxide consistent with loss of catalase HPII function. Twenty Suc⁺ mutants, exhibiting varying levels of reduced *osmY* expression, were selected for further study.

rpoS expression in Suc⁺ mutants

Because their expression is highly dependent on RpoS, catalase HPII and OsmY were used as reporters to evaluate expression in Suc⁺ mutants. Both *osmY* gene expression (measured as β -galactosidase activity) and catalase enzyme activity were substantially reduced in all 20 isolated Suc⁺ mutants, particularly in stationary phase cultures (Table 3; Figures 1 and 2). Levels of *osmY* gene expression and catalase enzyme activity in the Suc⁺ mutants reduced to levels in *rpoS* null mutant HS1091P.

HPII and HPI catalase activity

To confirm that catalase levels in Suc⁺ mutants were reduced relative to those of an RpoS null mutant HS1091P, proteins were separated in native polyacrylamide gels and stained for catalases HPII and HPI. In exponential and stationary phases,

representative results are shown for Suc⁺ mutants, HS1091A-C (Figure 3, data for HS1091-D to HS1091-T are not shown). In all cases, HPII activity during exponential phase was too low to be detected. HPII activity during stationary phase was found to be absent in Suc⁺ mutants. As expected, HPII activity was present in *rpoS* wild type strain HS1091 in stationary phase while no HPII activity was observed in *rpoS* null mutant HS1091P in stationary phase (Figure 3).

Identification of rpoS mutations

To confirm that the phenotypic changes in the 20 Suc⁺ mutants were indeed caused by sequence changes in the *rpoS* gene, the *rpoS* gene of each of the mutants was amplified by PCR and sequenced. Putative mutations were confirmed by sequencing using primers flanking the mutation (Table 2) and RpoS mutant sequences were aligned with sequence of the wild type *rpoS* gene. As shown in Table 4, among the 20 mutants, 19 mutations could be readily characterized by sequencing PCR products while one mutant yielded no PCR product. HS1091-C did not yield *rpoS* PCR product using several sets of primers but it did yield a control *rrnA* PCR product. Although it was not characterized further, the mutation in HS1091-C is a probably a deletion spanning the *rpoS* gene. Types of mutations found in the other mutants included single or multiple base deletions, insertions, transversions and many base changes. Ten mutants acquired base deletions within *rpoS*, of which one, HS1091-D, was found to possess a 159 bp deletion extending from Glu-284 to Glu-337 with no resulting frame shift. HS1091-M possessed an 11 bp deletion and HS1091-T had a 10 bp deletion, the remaining 7 mutants had only a 1 bp deletion. Two mutants were found to have acquired base insertions: HS1091-I had an 8 bp insertion and HS1091-P had a 1 base insertion. Five

mutants had single amino acids substitutions (transversions or nonsense). RpoS mutations included Leu-128-->stop codon TAG, Asn-136-->Tyr, Ala-158-->Glu, Glu-186-->stop codon TAG, and Ile-210-->Ser. There were 2 mutants HS1091-G and HS1091-L that had acquired many base changes. Of the 19 mutants, 13 had acquired frame shift mutations (Table 4). In summary, all Suc⁺ strains were found to have acquired mutations in the *rpoS* gene.

Selection and identification of Suc⁺ mutant revertants

To determine whether some succinate mutants can revert to *rpoS* wild type phenotypes, Suc⁺ mutants were streaked onto minimal media containing lactose and X-gal. Colonies with restoration to RpoS function are blue, while *rpoS* mutants are white or pale blue. Five transversion mutant revertants including two nonsense mutant revertants were isolated. These transversion mutant revertants were confirmed to restore to *rpoS* gene by sequencing using primers flanking the mutation (Table 2).

RpoS protein levels in Suc⁺ mutants and revertants

In stationary phase, RpoS protein was absent in *rpoS* null strain HS1091P. RpoS protein was not present in *rpoS* deletion strain HS1091-B and insertion strain HS1091-I. Western blotting was repeatedly performed to confirm that higher RpoS protein levels were detected in *rpoS* transversion strain HS1091-H compared to wild type strain HS1091 (Figure 4). One interpretation may be that a single amino acid change causes a conformational change in the protein such that it has a higher affinity for the primary antibody. Another interpretation may be that the mutation is more resistant to ClpXP protease (Schweder *et al.*, 1996), resulting in increased stabilization of the sigma factor. In exponential phase, the results were expected to be the same

pattern as those of the stationary phase. As expected, RpoS protein was not detectable in any mutant strains in exponential phase (data not shown), likely because it is rapidly turned over by the protease ClpXP (Schweder *et al.*, 1996). Western blot was performed to confirm that five succinate revertant colonies have been restored to RpoS wild-type phenotype. In stationary phase, RpoS protein was presented in all succinate revertants strains including transversion mutant revertants (Figure 5). Interestingly, two nonsense succinate revertants regained RpoS protein levels as missense strains although nonsense succinate mutants did not produced RpoS protein. In exponential phase the results were expected to be the same pattern as those of the stationary phase. As expected, RpoS protein was not detectable in any strains in the exponential phase (data not shown). As control, stationary phase sample of wild type strain HS1091 produced RpoS protein (data not shown). Therefore, RpoS protein was presented in succinate mutant revertants.

Reversion rate tests of rpoS mutant and wild type strains

Every fast growing Suc⁺ colony was found to have impaired RpoS activity suggesting that growth on succinate selects for loss of RpoS function. Since several identified mutations in *rpoS* were transversion and nonsense mutations, the phenotype (Suc⁺) of the mutants should be readily revertible to the wild type phenotype (Suc⁻) if the change in *rpoS* is responsible. If other, unidentified, second site mutations were responsible for the observed Suc⁻ phenotype of the mutants, reversion would not restore RpoS activity (Suc⁻ phenotype). To test the hypothesis that *rpoS* mutants that carry transversion mutations can readily revert to the wild type HS1091 phenotype, the Suc⁺ mutants were plated on M9 minimal media containing lactose and X-gal to select for

restoration of RpoS-dependent *osmY-lacZ* expression (Lac⁺, blue colonies). After 36 hours of growth, many presumptive RpoS⁺ colonies were found for strains HS1091-A, HS1091-H, HS1091-J, HS1091-N, and HS1091-R, mutants that are predicted to carry revertible mutations. Of the mutants able to grow on M9 minimal lactose plates, most of these colonies were dark blue and had little catalase activity. Five of the *rpoS* transversion mutant revertants were dark blue and had regained catalase activity. Only these were likely true revertants. Though blue in color, indicating restoration of *osmY* expression, none of the isolated *rpoS* deletion or insertion revertants was found to have regained wild type levels of catalase activity. These strains likely acquired secondary mutations that activated *osmY* expression (e.g. *osmY* promoter mutation). The determined reversion rates are shown in Table 4 and Figure 6.

Discussion

In this study, we showed that fast growing mutants on succinate minimal media have invariably acquired mutations in the *rpoS* gene indicating that *rpoS* mutations can be selected by growing cultures on a defined media containing succinate as the sole source of carbon. All isolated mutants possessed properties associated with *rpoS* deficiency including changes in sequence of the *rpoS* gene, reduced RpoS protein levels, and deficiencies in expression of RpoS-dependent genes. Since all mutants examined were found to have mutations in *rpoS*, these results strongly suggest that it is mutation in *rpoS* itself and not some secondary mutation that is responsible for the observed growth advantage. Previous studies have shown that *rpoS* mutations accumulate in stationary phase cells (Notley-McRobb *et al.*, 2002; Zambrano *et al.*, 1993) suggesting that a loss in expression of this important regulator may, under some conditions, confer some selective advantage. RpoS mutations may indirectly increase the expression of sigma 70 regulated transporter genes by increasing the availability of core polymerase for expression of non-RpoS regulated genes (Notley-McRobb *et al.*, 2002) or may increase amino acid scavenging ability of stationary phase cells (Finkel and Kolter, 1999). Either of these changes would confer a competitive advantage to the cell under starvation conditions where nutrient availability is limited. Mutants in *rpoS* function exhibit enhanced biofilm formation (Corona-Izquierdo and Membrillo-Hernandez, 2002) and, surprisingly, efficiently compete with wild strains in colonizing the mouse intestine (Krogfelt *et al.*, 2000).

These observations may explain, in part, why laboratory strains of *E. coli* have, paradoxically, acquired mutations in a gene that controls the expression of many

functions required under sub-optimal growth conditions. The fact that, in this study, all isolated Suc⁺ mutants had acquired independent mutations in *rpoS*, rather than other genes, indicates that the loss of this dispensable regulator may be sufficient for increased fitness under certain conditions. Others have also observed that *rpoS* deficiency may be advantageous under conditions of nutrient deprivation. Kolter and co-workers have described the GASP (growth advantage in stationary phase) phenotype in which mutants that accumulate in stationary phase have an increased ability to scavenge nutrients, particularly amino acids and are better able to out grow wild type strains (Zinser and Kolter, 1999). Interestingly, one of these is *rpoS*-deficient (Zinser and Kolter, 1999). Similarly, we have found that both the Suc⁺ mutants isolated in this work and *rpoS* null mutants are generally better able to use some amino acids, particularly γ -aminobutyrate and glutamine as sole nitrogen sources (Schertzberg and Schellhorn, unpublished data). In the studies cited above, selection occurred in liquid cultures making it difficult to characterize individual mutants as they arise in the culture. The selection observed in this study, in contrast, could be examined on solid media.

The types of mutations that accumulate in cultures, both in this study and previous studies (Atlung *et al.*, 2002) are mainly transversions and small insertions and deletions. Larger changes can also occur including large duplications (Kolter *et al.*, 1993) and the presumed deletion in strain HS1091-D (this study). An insertion in the *mutS-rpoS* intergenic region may affect pathogenicity (Culham and Wood, 2000) through changes *rpoS* expression or polar effects on downstream genes (Atlung *et al.*, 2002). An intriguing aspect of variability in “wild type” sequences is the suggestion that

the expression of the individual RpoS regulon members may be slightly different therefore dramatically increasing the complexity of study of RpoS regulation.

An evolutionary advantage of selection for loss of RpoS function may be that such mutations allow the cell to “switch off” expression of the regulon when it is not required and, in those mutants that have acquired revertible mutations (for example transversion and nonsense mutants), switch it back on by reversion when the regulon is really required. Many K12 strains (up to 50%) possess common amber mutation in codon 33 of the *rpoS* gene (Atlung *et al.*, 2002). Examination of the evolutionary relationships among many laboratory strains of *E. coli* has revealed that this mutation has been suppressed, reverted or pseudoreverted in several strains. This “restoration” of RpoS function by mutation may be responsible for sequence variability observed in this region of the *rpoS* gene (Atlung *et al.*, 2002).

It is likely that, given the large predicted size of the RpoS regulon (about 200 members (Schellhorn *et al.*, 1998)), control of RpoS-dependent expression is critical since inappropriate expression of such large regulon is likely detrimental to the cell. This may partially explain why there are so many redundant controls on RpoS that function at levels of transcription, translation and protein stability (Hengge-Aronis, 2002). The possibility that the regulon may be modulated by mutation, as suggested by the result of this study, may represent yet another form of control of expression that operates at the population rather than at the single cell level. Accordingly, loss of *rpoS* in a subpopulation within the stationary phase culture may allow these survivors to efficiently scavenge nutrients released from dead cells (Finkel and Kolter, 1999). A significant fraction of these cells carrying attenuated mutant alleles (primarily those

carrying transversion and nonsense mutations) could then revert when selected for under defined conditions.

While the results of this study clearly show that growth on succinate can select for loss of RpoS function, the mechanism for this selection is not understood. We are currently examining accumulation of metabolites in *rpoS* mutants relative to wild type strains to identify the physiological changes that occur in spontaneous *rpoS* mutants.

Acknowledgements

This work was supported by grants from the Natural Sciences and Engineering Research Council (NSERC) of Canada and the Canadian Institutes of Health Research (CIHR) to H.E.S. We thank R. Hengge-Aronis for the RpoS antibody used in these studies. We also thank members of the Schellhorn lab for reading the manuscript and Lily C. Chang and Xiaoli Zhao for excellent technical assistance in conducting preliminary experiments.

Experimental procedures

Media and growth conditions

Liquid and solid media were prepared as described by Miller (1992). All bacterial strains were grown on Luria Bertani (LB) or on M9 minimal media containing succinate (1%) or lactose (1%) as a carbon source supplemented with the appropriate antibiotics and/or X-gal (50 µg/ml, final concentration). Typically, a single colony of each strain was inoculated into 5 ml of media containing appropriate antibiotics and grown overnight at 37°C. An aliquot of overnight culture was subcultured into fresh media. Fifteen ml samples were taken as soon as OD₆₀₀ reaches 0.25 (exponential phase) and OD₆₀₀ reaches 1.5 (stationary phase).

Selection of Suc⁺ mutants and Suc⁺ mutant revertants

All bacterial strains used and mutants selected are listed in Table 1. To isolate and readily identify Suc⁺ mutants that have lost RpoS function, we employed a strategy that uses an indicator strain (HS1091) carrying an operon fusion to *osmY*, a highly RpoS-dependent gene (Lange *et al.*, 1993). Use of this strain in mutation studies allowed convenient monitoring of loss of RpoS-dependent function on X-gal-containing indicator plates: colonies with functional RpoS are blue, while *rpoS* mutants are white or pale blue.

Single colonies were inoculated into LB broth, grown overnight and then washed with M9 minimal salts and plated (~10⁹ cells) onto succinate minimal plates containing X-gal. Several fast growing Suc⁺ colonies from each plate was purified and further characterized by testing for loss of catalase activity (an RpoS-regulated function that is independent of OsmY). To ensure that isolated mutants were produced as the

result of independent mutational events, each mutant was isolated from an independently grown and plated culture. To test the hypothesis that succinate transversion mutants can revert to *rpoS* wild type phenotypes, Suc⁺ mutants were streaked onto minimal media containing lactose and X-gal. Colonies with restoration to RpoS function are blue, while *rpoS* mutants are white or pale blue.

β-Galactosidase activity assays

To quantitatively assess the effect of the *rpoS* alleles on the expression of RpoS-dependent gene *osmY*, β-galactosidase activity was assayed using ONPG as a substrate (Miller, 1992).

Polyacrylamide gel electrophoresis of crude protein extracts

Samples of cells were washed in 0.05 M phosphate buffer and centrifuged at 3300xg for 10 min. The pellets were resuspended in 0.05 M phosphate buffer and sonicated using a Heat Systems sonicator (Misonix Inc., Farmingdale, New York) equipped with a cup horn. Cell debris was removed by centrifugation at 4°C for 15 min at 12,000xg. Protein concentration was determined by using bovine serum albumin as the standard (Bradford, 1976). Proteins were resolved by electrophoresis in a 10% non-denaturing polyacrylamide gel (Schellhorn and Stones, 1992). To detect catalase activity, non-denaturing gels were stained using horseradish peroxidase and diaminobenzidine (Clare *et al.*, 1984). To confirm equal protein loading, parallel gels were stained with Coomassie Blue R-250 (Sambrook *et al.*, 1989).

Catalase activity assays

Colony catalase activity on plates was qualitatively determined by adding a drop of 30% hydrogen peroxide to a colony and observing gas evolution. Catalase activity in

cell extracts was quantified spectrophotometrically (Beers and Sizer, 1952). Ten μ l cell extract was added to 1 ml of hydrogen peroxide (0.5 ml of 30% H_2O_2 freshly diluted in 250 ml 50 mM potassium phosphate buffer, pH 7.0), and the decrease in OD_{240} was monitored. The specific activity of catalase was calculated as $(1,000 \times OD_{240}/\text{time of incubation})/(43.6 \times \text{mg of protein/ml of reaction mixture})$ (Beers and Sizer, 1952).

Western blotting analysis

Protein (30 μ g) samples were separated on 10% SDS polyacrylamide gels overnight using a Vertical Slab Gel Unit, Model SE400 (Hoefer Scientific Instruments, San Francisco, CA). Resolved proteins were transferred to a Hybond-P PVDF membrane (Amersham Pharmacia Biotech Inc., Piscataway, NJ) in the cold room at 4°C. The membranes were stained using Ponceau S to check efficiency of protein transfer. The blots were then placed into blocking buffer (0.5% albumin bovine Fraction V, 5% skim milk in TBS-T) and blocked overnight. Following blocking, the membranes were incubated with blocking buffer containing primary antibody (anti- σ^S antibody, a gift from R. Hengge-Aronis, University of Konstanz) for two hours at room temperature. The blots were washed three times with TBS-T (0.1% Tween 20 in TBS, pH 7.6) or blocking buffer and then placed into blocking buffer containing the secondary anti-rabbit antibody (Rabbit Immunoglobulin, horseradish peroxidase-linked whole antibody from donkey, 1:1,000 dilution) for 1 hour at room temperature with gentle shaking. The blots were again washed three times and incubated in 10 ml of the ECL staining solution (1:1 detection reagents mixture, Amersham Pharmacia Biotech Inc., Piscataway, NJ). The bands were visualized by exposure of Kodak X-OMAT AR

or BioMax MR film (Eastman Kodak Company, Rochester, New York) for 10 s to 10 min.

PCR amplification of the rpoS gene

Suc⁺ mutants that had lost both *lacZ* (*osmY*) and colony catalase activity were considered presumptive *rpoS* mutants and were characterized further. To determine base sequence changes in the putative mutant *rpoS* genes, the *rpoS* DNA of 20 Suc⁺ mutants was amplified using chromosomal DNA as template. PCR was performed using *Taq* DNA polymerase (Invitrogen Corporation, Carlsbad, CA) and primers AB12984 and AB12985 (Table 2). PCR conditions were as follows. Reactions were “hot started” at 94°C for 3 minutes before adding *Taq* polymerase. This was then followed by a series of cyclic heating (25 cycles) at 94°C for 45 s, 61°C for 30 s and 72°C for 90 s. At the end of 25 cycles, there was a further elongation period of 72°C for 7 min and then the samples were stored at 4°C. PCR products were purified using QIAquick PCR Purification Kit (Qiagen Inc., Mississauga, Ontario). Two independent PCR products were sequenced for each mutant from two independent colonies (see Table 2 for sequence primers). DNA sequencing was performed at MOBIX lab, McMaster University, and analyzed using Chromas 1.41 software (C. McCarthy, Griffith University, Brisbane, Australia). To determine base sequence changes in the putative succinate mutant revertants, the procedures described here were also used.

Confirmation of sequences of complementary strands

To confirm identified mutations in the *rpoS* sequence, complementary strands were sequenced using primers (Table 2) that flank the mutations. Complementary

strand sequences were aligned and analyzed using the program Gene Runner 3.04 (Hastings Software Inc., Moraga, CA).

Reversion rate tests of *rpoS* mutants and wild type strains

As identified transversion mutations should be readily revertible, we tested if we could reisolate wild type derivatives by selecting strains for Lac⁺ colonies (*osmY*⁺) on M9 minimal media with lactose plates. Single colonies from LB with X-gal plates of strains HS1091, HS1091P, HS1091-A-T, were inoculated into 5 ml of LB broth in triplicate. The tubes were incubated overnight in a shaker at 37°C at 200xg. The cells were then washed in 5 ml M9 minimal media and resuspended in 200 µl M9 minimal media. 100 µl of cells were plated onto M9 minimal media with lactose and X-gal plates. The plates were incubated at 37°C overnight. Presumed reverted colonies (blue in colour) were purified. These colonies, as well as two original colonies from each of the 22 strains (20 mutants, wild type and *rpoS*::Tn10 strain), were inoculated into LB media in 96 well microtiter plates and grown overnight at 37°C. The resulting cultures were replica plated onto selective media and incubated overnight for two days. Catalase activity was assayed on LB media using 30% hydrogen peroxide. Colonies that were blue and evolved gas (catalase positive) were considered true revertants. The reversion rate was calculated as the average number of confirmed reverted mutant colonies divided by the total number of plated cells.

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Table 1. Bacterial strains used in this study

<i>E. coli</i> strain	Genotype/Phenotype	Source/References
GC4468	<i>ΔlacU169 rpsL</i>	(Schellhorn and Stones, 1992)
GC122	As GC4468 but <i>rpoS13::Tn10</i>	(Schellhorn and Hassan, 1988)
HS1091	As GC4468 but <i>osmY-lacZ</i>	(Schellhorn <i>et al.</i> , 1998)
HS1091P	As HS1091 but <i>rpoS::Tn10</i>	(Schellhorn <i>et al.</i> , 1998)
HS1091-A to HS1091-T	As HS1091 but Suc ⁺ mutants	This study

Table 2. Oligonucleotides used in this study

Oligonucleotide	Sequence
AB12179	5' -GTGAGGCCAATTCACGACCTA-3'
AB12984	5' -CTTGCATTTTGAAATTCGTTACA-3'
AB12985	5' -TTAACGACCATTCTCGGTTTTAC-3'
AB16789	5' -GTCAAAC TTCTCTACCGCGC-3'
AB16790	5' -ATCCAGTTGCTCTGCGATCT-3'
AB18752	5' -AGCTTATGGGACA ACTCACG-3'
AB18753	5' -ATCTTGCGTGGTATCTTCCG-3'
AB18754	5' -AGGAACCCAGTGATAACG-3'
AB18755	5' -AAGATACCACGCAAGATG-3'
AB18756	5' -GCGGTAGAGAAGTTTGAC-3'

Table 3. RpoS-dependent gene expression based on β -galactosidase activity, and catalase activity of Suc⁺ mutants. All cultures were grown in triplicate from independent colony isolates.

Strain	β -galactosidase (Miller units)		Relative RpoS- dependent expression* (%)	Catalase (μ mol/min/mg)	
	Exponential phase	Stationary phase		Exponential phase	Stationary phase
HS1091	62.1	183.7	100	5.0	37.7
HS1091P	3.2	5.6	3.1	1.3	3.5
HS1091-A	3.8	5.1	2.8	2.2	4.4
HS1091-B	4.2	4.9	2.7	2.6	4.0
HS1091-C	4.4	5.2	2.8	1.6	5.4
HS1091-D	4.2	4.9	2.7	1.8	2.9
HS1091-E	4.5	5.0	2.7	1.7	2.8
HS1091-F	4.8	5.1	2.8	1.0	3.0
HS1091-G	4.8	5.1	2.8	3.2	9.0
HS1091-H	5.2	9.5	5.2	2.2	3.8
HS1091-I	5.0	5.8	3.2	1.4	3.6
HS1091-J	4.8	5.4	2.9	2.9	6.6
HS1091-K	4.7	5.1	2.8	1.4	2.7
HS1091-L	4.0	6.2	3.4	2.0	4.1
HS1091-M	4.1	6.4	3.5	1.7	2.9
HS1091-N	4.3	6.0	3.3	1.9	2.4
HS1091-O	4.4	6.1	3.3	1.5	2.8
HS1091-P	4.6	5.1	2.8	1.8	2.5
HS1091-Q	4.8	6.9	3.8	1.6	2.2
HS1091-R	4.6	9.4	5.1	2.7	3.6
HS1091-S	4.2	6.1	3.3	1.7	2.7
HS1091-T	4.1	5.8	3.2	2.4	5.7

* Relative *rpoS* expression is the percentage of β -galactosidase activity assayed in mutant strains relative to the wild type strain in stationary phase.

Table 4. Types of mutations, predicted effects on protein structure, and reversion rates of Suc⁺ mutant strains.

Strain	Type of mutation	Effect of mutation (Frame shift +/-)**	Missense mutation +/-	Reversion rate (x10 ⁻⁸)
HS1091	wild type	-	-	NA
HS1091P	<i>rpoS</i> null mutant	NA***	NA	ND****
HS1091-A	nonsense	-, Leu to stop codon TAG, short protein	+	0.2
HS1091-B	1 base deletion	+, short protein	-	<10 ⁻⁸
HS1091-C	ND	ND	ND	<10 ⁻⁸
HS1091-D	159 bp deletion	-, short protein	-	<10 ⁻⁸
HS1091-E	1 base deletion	+, short protein	-	<10 ⁻⁸
HS1091-F	1 base deletion	+, short protein	-	<10 ⁻⁸
HS1091-G	many base changes	+	-	<10 ⁻⁸
HS1091-H	transversion	-, Ile to Ser	+	1.6
HS1091-I	8 base insertion	+, short protein	-	<10 ⁻⁸
HS1091-J	nonsense	-, Glu to stop codon TAG, short protein	+	1.1
HS1091-K	1 base deletion	+, short protein	-	<10 ⁻⁸
HS1091-L	many base changes	+	-	<10 ⁻⁸
HS1091-M	11 base deletion	+, short protein	-	<10 ⁻⁸
HS1091-N	transversion	-, Asn to Tyr	+	0.1
HS1091-O	1 base deletion	+, short protein	-	<10 ⁻⁸
HS1091-P	1 base insertion	+, short protein	-	<10 ⁻⁸
HS1091-Q	1 base deletion	+, short protein	-	<10 ⁻⁸
HS1091-R	transversion	-, Ala to Glu	+	1.0
HS1091-S	1 base deletion	+, short protein	-	<10 ⁻⁸
HS1091-T	10 bp deletion	+, short protein	-	<10 ⁻⁸

*— +, yes, **— -, no, ***— NA, not applicable, **** — ND, not detected.

Figure 1. Expression of RpoS-dependent *osmY-lacZ* in three isolated Suc⁺ mutants (HS1091-A to HS1091-C) and control strains HS1091 and HS1091P in LB broth culture (Note: activity is reported on a logarithmic scale to more clearly show reduced activity in isolated mutants).

Chen and Schellhorn, Figure 1.

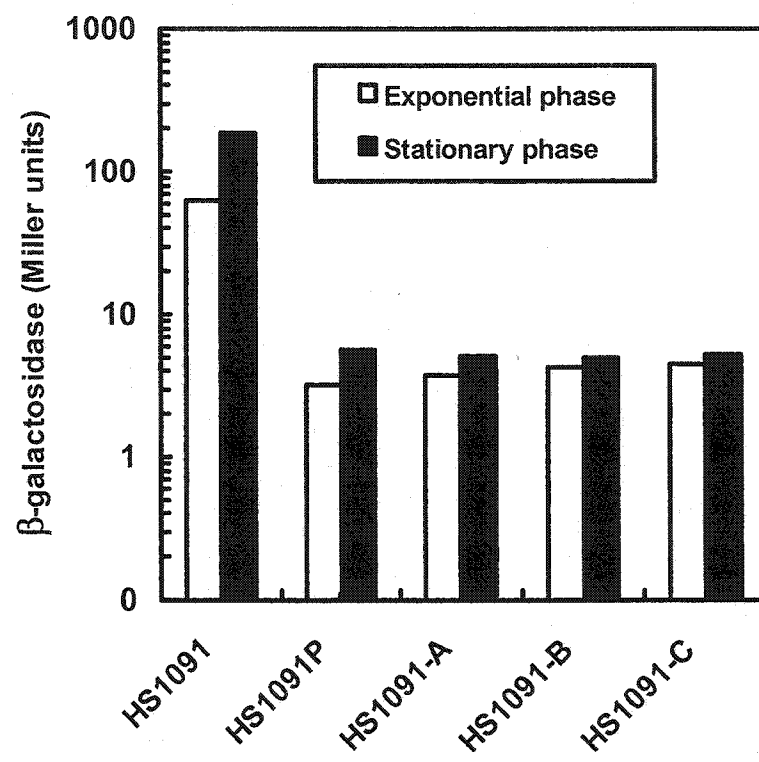
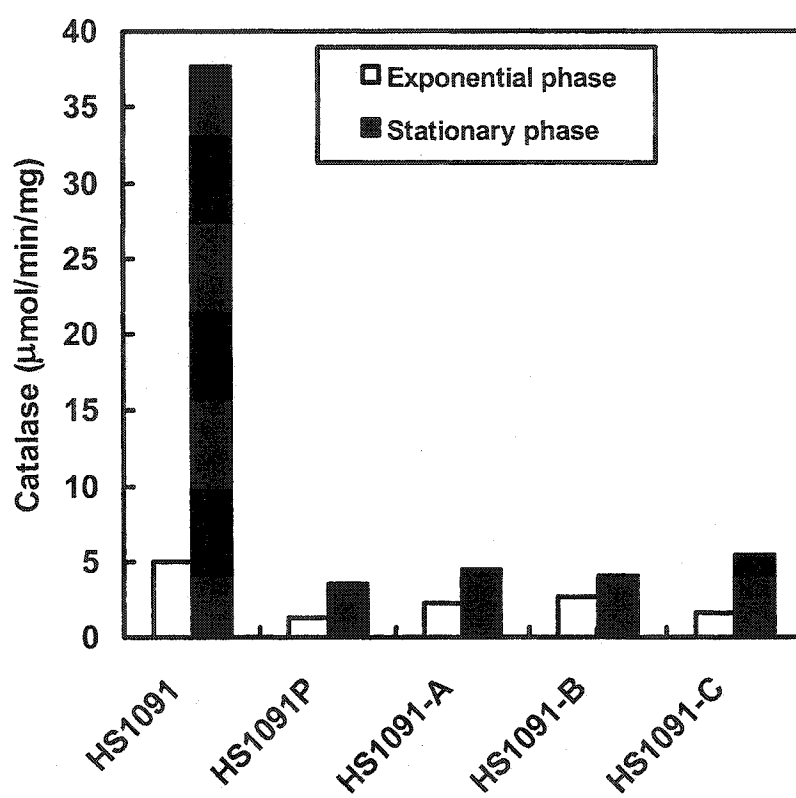


Figure 2. Catalase activity in three isolated Suc⁺ mutants (HS1091-A to HS1091-C) and control strains HS1091 and HS1091P in LB broth culture. Catalase activity in cell extracts was quantified spectrophotometrically as described in Experimental procedures (Table 3).

Chen and Schellhorn, Figure 2.



Figures 3. Catalase activity in polyacrylamide gel containing protein extracted from *rpoS* wild type strain HS1091 and mutant strains HS1091-A, HS1091-B and HS1091-C grown to exponential phase (E) and stationary phase (S) in LB media.

Chen and Schellhorn, Figure 3.

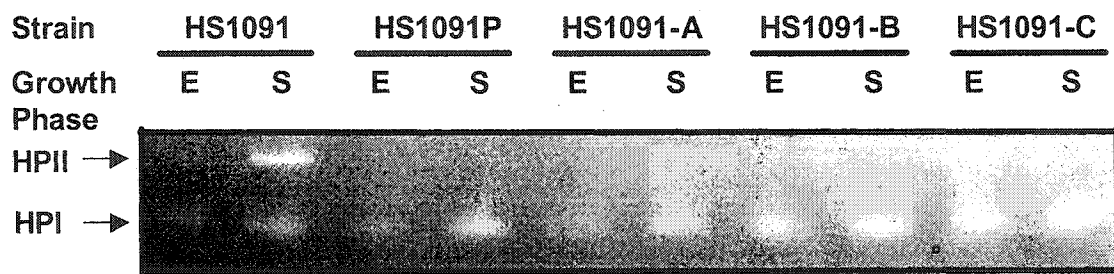


Figure 4. Western blot analysis of RpoS protein levels in *E. coli* wild type strain HS1091, *rpoS* null mutant HS1091P and three representative Suc⁺ mutants: HS1091-B (deletion mutant), HS1091-H (transversion mutant), and HS1091-I (insertion mutant).

Chen and Schellhorn, Figure 4.

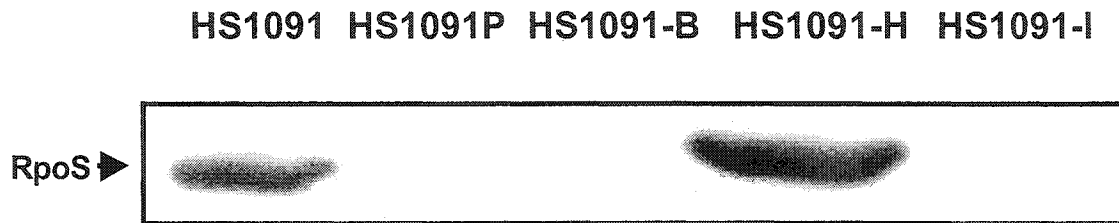


Figure 5. Western blot analysis of RpoS protein levels in five succinate transversion revertants (transversion revertants: HS1091-H/C, HS1091-N/H and HS1091-R/E, as well as nonsense revertants: HS1091-A/F and HS1091-J/D).

Chen and Schellhorn, Figure 5.

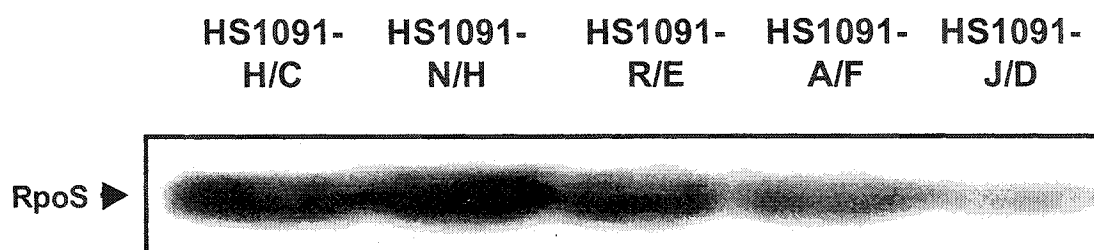
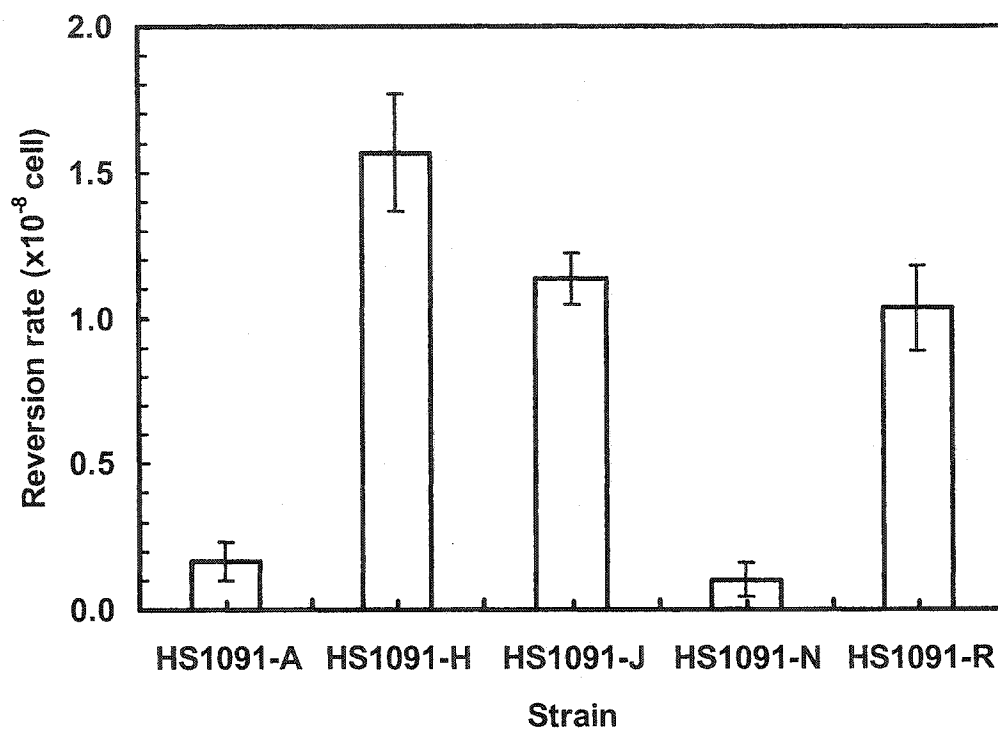


Figure 6. *rpoS* reversion rates for Suc⁺ mutants. Several transversion and nonsense mutations in *rpoS* (Suc⁺) were revertible to the wild type phenotype (Suc⁻).

Chen and Schellhorn, Figure 6.



CHAPTER 4:

**CONTROLLED EXPRESSION OF AN *RPOS* ANTISENSE RNA
CAN INHIBIT RPOS FUNCTION IN *ESCHERICHIA COLI***

Preface

Chapter four, entitled “Controlled expression of an *rpoS* antisense RNA can inhibit RpoS function in *Escherichia coli*”, was written as a manuscript that has been formatted to comply with the thesis regulations. All the work presented in this chapter has been carried out by the author.

**Controlled expression of an *rpoS* antisense RNA can inhibit RpoS function
in *Escherichia coli***

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Running title: *rpoS* antisense inhibition of RpoS function.

SUMMARY

In this report, we show that an inducible *rpoS* antisense RNA, complementary to *rpoS* message, can inhibit expression of RpoS in both exponential and stationary phases and can attenuate expression of the *rpoS* regulon in *E. coli*. Plasmids containing *rpoS* antisense DNA expressed under the control of the *T7lac* promoter and T7 RNA polymerase were constructed, and expression of the *rpoS* antisense was optimized in the pET expression system. *rpoS* antisense levels could be manipulated to effectively control expression of RpoS and RpoS-dependent genes. RpoS expression was inhibited by the expression of *rpoS* antisense in both exponential and stationary phases in *E. coli*. RpoS-dependent catalase HPII was also downregulated as determined by catalase activity assays and native polyacrylamide gels stained for catalase. Induced RpoS antisense expression also reduced RpoS-dependent glycogen synthesis. These results demonstrate that controlled expression of antisense RNA can be used to attenuate expression of a regulator required for the expression of host adaptation functions and may offer a basis for designing effective antimicrobial agents.

INTRODUCTION

Naturally-occurring antisense RNA can be an important regulator of gene expression in eukaryotic (Tamm *et al.*, 2001) and in bacterial cells (Majdalani *et al.*, 1998), by either blocking ribosome binding (Daugherty *et al.*, 1989) or reducing mRNA stability (Desai and Papoutsakis, 1999; Gill *et al.*, 1999). Antisense RNA technology has been successfully used to manipulate gene expression in bacteria (Nellen and Lichtenstein, 1993; Guerrier-Takada *et al.*, 1997; Good and Nielsen, 1998) (for review, see Wagner and Simons, 1994). For example, *mar* antisense RNA can inhibit expression of the multiple antibiotic resistance (*mar*) operon in *E. coli* (White *et al.*, 1997) thus increasing the sensitivity of the cell to antibiotics. Expression of an *hla* antisense RNA inhibits alpha-toxin production in *Staphylococcus aureus* and thereby attenuates virulence (Kernodle *et al.*, 1997). Antisense RNAs have also been used to manipulate metabolism in *Clostridium acetobutylicum* (Desai and Papoutsakis, 1999), *Enterococcus faecalis* (Torres *et al.*, 2001) and *Penicillium chrysogenum* (Zadra *et al.*, 2000). Selectively-expressed antisense RNA can effectively inhibit the growth of bacteriophage (Walker and Klaenhammer, 2000; Sturino and Klaenhammer, 2002).

RpoS (σ^S) is an alternative sigma subunit of bacterial RNA polymerase (Tanaka *et al.*, 1993; Jishage *et al.*, 1996). In response to environmental stress and nutrient starvation, RpoS mediates increases in the expression of many genes (Hengge-Aronis, 1996). RpoS-dependent genes such as *katE*, encoding catalase HPII, are induced to help the cell survive in stationary phase and during entry into stationary phase (Loewen *et al.*, 1985; McCann *et al.*, 1991; Schellhorn and Hassan, 1988). Expression of the monocistronic gene *glgS*, required for glycogen synthesis, is also stimulated by RpoS

(Hengge-Aronis and Fischer, 1992; Liu *et al.*, 1995). *rpoS* mutants have a glycogen-negative phenotype (Lange and Hengge-Aronis, 1991b) and overexpression of *glgS* stimulates excess glycogen synthesis in early stationary phase (Hengge-Aronis and Fischer, 1992). Stationary phase cells are resistant to multiple environmental stresses and undergo changes in cell morphology and physiology, thus entry into stationary phase is accompanied by changes in gene expression and protein synthesis (Lange and Hengge-Aronis, 1991a).

Many factors regulate the expression of *rpoS* at the levels of transcription, translation and protein stability (for review, see (Hengge-Aronis, 1996)). At the translational level, both positive and negative regulators have been identified. The small untranslated RNA OxyS (Zhang *et al.*, 1998) represses RpoS while small untranslated RNAs, DsrA (Majdalani *et al.*, 1998) and RprA (Majdalani *et al.*, 2001) activate RpoS. The histone-like protein H-NS (Barth *et al.*, 1995) and the LysR-like regulator LeuO (Klauck *et al.*, 1997) repress RpoS, but the host factor HF-1 (Zhang *et al.*, 1998) and the histone-like protein HU (Balandina *et al.*, 2001) activates RpoS. At the post-translational level, protease ClpPX (Schweder *et al.*, 1996), the response regulator RssB (Becker *et al.*, 1999) and the DnaK chaperonin (Rockabrand *et al.*, 1998) negatively regulate RpoS stability. Posttranslational protein degradation has a major effect on RpoS levels during the course of growth (Schweder *et al.*, 1996; Schweder *et al.*, 2002). The net consequence of these controls is low exponential phase levels of RpoS but during the transition to stationary phase, levels increase and remain high (Schweder *et al.*, 1996). RpoS is an attractive target for new antimicrobial strategies because this regulator controls many genes that are important for adaptation

to the host environment including catalase HPII. Furthermore, RpoS controls a large regulon thus ablation of its action can be easily assessed by several means. In addition, natural antisense RNA regulators are known to inhibit translation of RpoS and this process can be mimicked by the use of antisense RNA.

The primary goal of this study was to block *rpoS* expression using plasmid-encoded, inducible *rpoS* antisense RNA. RpoS was chosen as a target because it controls a large regulon, has well-established effects on the phenotype of the cell and is a pathogenicity factor. We hypothesize that *rpoS* antisense RNA complementary to *rpoS* mRNA will inhibit RpoS function and attenuate expression of the *rpoS* regulon in *E. coli*. The efficacy of antisense RNA was evaluated by measuring glycogen accumulation and the expression of RpoS-dependent catalase HPII. The results indicate that the expression of the RpoS regulon in *E. coli* can be effectively modulated by antisense RNA.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions

The bacterial strains used and constructed in this study are listed in Table 1. The plasmids used and constructed are listed in Table 2. Luria Bertani (LB) liquid and solid media were prepared as described by Miller (Miller, 1992). For glycogen tests, *E. coli* strains were grown on Kornberg Medium agar plates to allow maximal glycogen synthesis (Hengge-Aronis and Fischer, 1992; Romeo and Preiss, 1989). All strains were grown in LB broth or 2TY broth containing the appropriate antibiotics in a shaker at 37°C and 200 rpm.

Selection of λ DE3 lysogens expressing the T7 RNA polymerase gene

To make an expression host for the pET expression system, a phage lambda derivative λ DE3 carrying the T7 RNA polymerase gene under the control of an IPTG-inducible *lacUV5* promoter (Dubendorff and Studier, 1991; Studier *et al.*, 1990) was integrated into the *E. coli* chromosome. A kit for lysogenization and verification of λ DE3 (Invitrogen, Mississauga, ON) was used according to the manufacturer's instructions. A T7 (RNA polymerase negative) tester phage, that can lyse cells only when supplied with T7 polymerase, was used to confirm successful integration of the T7 RNA polymerase-expressing phage. Both *rpoS*⁺ and *rpoS*⁻ lysogenic expression hosts were constructed.

Plasmid and genomic DNA isolation, manipulation and transformation

E. coli plasmid DNA was isolated by the alkaline lysis method (Sambrook *et al.*, 1989) or using a Midi plasmid preparation kit (Qiagen Inc., Mississauga, ON).

Genomic DNA isolation, endonuclease digestions, ligations, and transformations were performed using standard techniques (Sambrook *et al.*, 1989).

PCR-amplification and sequencing of *rpoS* antisense

Chromosomal DNA was isolated from *E. coli* MC4100 (Sambrook *et al.*, 1989). PCR was employed to amplify the *rpoS* fragments from *E. coli* DNA using several *rpoS* primers (the MOBIX lab, McMaster University, Hamilton, ON) as follows:

short *rpoS* fragment 5' primer 5'-CTTGCATTTTGAAATTCGTTACA-3'

3' primer 5'-GTGAGGCCAATTCACGACCTA 3'.

large *rpoS* antisense fragment, 5' primer 5'-CTTGCATTTTGAAATTCGTTACA-3'

3' primer 5'-TTAACGACCATTCTCGGTTTTAC-3'.

Annealing temperatures were 5°C below the lowest T_m of each primer pair. PCR was performed with *Taq* DNA polymerase (Invitrogen, Mississauga, ON) or *Expand* DNA polymerase (Roche Diagnostics, Laval, QC). PCR products were purified using QIAquick PCR purification kit (Qiagen Inc., Mississauga, ON) prior to further manipulation. All DNA used in cloning reactions and probe preparation were extracted from agarose gels using the QIAEX II DNA extraction kit (Qiagen, Inc., Mississauga, ON). All amplified products were sequenced by the MOBIX lab, McMaster University. Sequences of short and large *rpoS* antisense fragments were aligned with the complementary strand sequences and analyzed using the program Gene Runner 3.04 (Hastings Software, Inc., Moraga, CA).

Construction of *rpoS* antisense plasmids for *in vivo* experiments

Following PCR amplification and purification, a 1278 bp fragment containing 3'-A overhangs was ligated with compatible overhangs into plasmid pGEM®-T Easy

plasmid (Promega Corporation, Madison, WI) overnight (4°C) and the ligation mixture transformed into *E. coli* DH5 α . Transformants were selected on 2TY agar plates containing ampicillin (100 μ g/ml). Plasmids were isolated from several ampicillin-resistant colonies and digested with *EcoRI*, *HincII* and *AccI* to confirm the inserted fragment. The orientation of the cloned fragments was confirmed by DNA sequencing. One plasmid containing the entire 1278 bp sequence was designated pGC2. The *EcoRI* fragment of pGC2 was subcloned into the expression vectors pET21 and pET22b to yield pSOPRL (pET21 background) (Figure 1) and pGC226 (pET22b background). The orientation of the *rpoS* gene was reversed with respect to the *T7lac* promoter on the plasmids such that an antisense *rpoS* RNA will be expressed in cells treated with IPTG.

Similarly, a 1022 bp PCR fragment of DNA was ligated with compatible overhangs of pGEM $\text{\textcircled{R}}$ -T Easy plasmid to yield pGC2a. The 1042 bp *EcoRI* fragment of pGC2a was subcloned into pET21 and pET22b, resulting in pSOPR (pET21 background) (Figures 1 and 2) and pGC225a (pET22b background). The *rpoS* gene is in the reverse orientation with respect to the *T7lac* promoter.

Bacterial growth and sampling conditions

Single colonies were inoculated into 5 ml LB with streptomycin and ampicillin where appropriate. The cultures were incubated overnight in a shaker at 37°C at 200 rpm. The overnight cultures were diluted 1/500 into fresh LB media. After growth to an OD₆₀₀ of 0.25, the cultures were again diluted 1/500 and grown to an OD₆₀₀ = 0.01. The cultures were then divided into two flasks, one flask served as a control and 0.3 mM IPTG (final concentration) was added to the other flask. Cultures were grown to an OD₆₀₀ of 0.25 at which point 70 ml of the exponential phase cells from each flask were

collected. After further incubation to an OD₆₀₀ of 1.5, 25 ml of the stationary phase cells from each flask were collected.

Probe preparation, RNA extraction and Northern blot analyses

To produce probes for Northern blotting, a 1022 bp fragment of DNA corresponding to the short *rpoS* DNA sequence (above) was PCR-amplified from *E. coli* MC4100 chromosomal DNA. PCR products, purified using QIAquick PCR purification kit (Qiagen Inc., Mississauga, ON), were labeled using the random primer labeling method with [α -³²P]-dCTP to generate high specific activity ($\sim 10^9$ cpm/ μ g DNA) probes. Probes were purified using ProbeQuant G-50 micro columns (Amersham Biosciences, Inc., Piscataway, NJ).

Cells were harvested and RNA extracted using RNeasy Mini Kit (Qiagen Inc., Mississauga, ON). Total RNA concentration was determined by measuring absorbance at 260 nm (Sambrook *et al.*, 1989). Following denaturing electrophoresis, RNA was transferred to Hybond-N⁺ membranes (Amersham Pharmacia Biotech Inc., Piscataway, NJ) by capillary action (Sambrook *et al.*, 1989) and fixed by baking at 80°C for 2 h. Prehybridization and hybridization were performed at 60°C with gentle agitation. The membranes were hybridized with ³²P-labeled probes to the *rpoS* gene. The resulting blot was exposed to Kodak X-OMAT AR film (Eastman Kodak Company, Rochester, NY) or to a Storage Phosphor Screen (Molecular Dynamics Inc., Sunnyvale, CA) for quantitation.

Protein extraction and determination

Bacterial cell cultures were washed twice by centrifugation using 0.05 M phosphate buffer and sonicated using a Heat Systems sonicator (Misonix Inc.,

Farmingdale, NY) equipped with a cup horn (Schellhorn and Stones, 1992). Cell debris was removed by centrifugation at 4°C for 15 min at 12,000xg. Total protein concentration was determined by the method of Bradford (Bradford, 1976) (Bio-Rad Laboratories, Hercules, CA).

Western blotting analysis

Thirty µg proteins were separated on 10% SDS polyacrylamide gels overnight using a Vertical Slab Gel Unit, Model SE400 (Hoefer Scientific Instruments, San Francisco, CA) and then transferred to a Hybond-P membrane (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The membranes were stained with Ponceau S to confirm efficient transfer. Following transfer, the blots were placed into blocking buffer (0.5% albumin bovine Fraction V, 5% skim milk in TBS-T (0.1% Tween 20 in TBS, pH 7.6)) overnight. The blots were then incubated with blocking buffer containing primary antibody (anti- σ^S antibody, a gift from R. Hengge-Aronis, University of Konstanz, Konstanz, Germany) for 2 h at room temperature. Following washing (three times with TBS-T or blocking buffer, blots were placed into blocking buffer containing secondary anti-rabbit antibody (Rabbit Immunoglobulin, horseradish peroxidase-linked whole antibody from donkey, 1:1,000 dilution), and shaken for 1 h. Blots were again washed three times with TBS-T and incubated in 10 ml of ECL staining solution (detection reagent mixture, Amersham Pharmacia Biotech Inc., Piscataway, NJ) and exposed to X-ray film (Kodak X-OMAT AR or BioMax MR film, Eastman Kodak Company, Rochester, N.Y.) for 10 s to 10 min.

β -Galactosidase activity assays

β -Galactosidase activity was assayed using ONPG as the substrate (Miller, 1992).

Catalase activity assays and detection

Catalase activity on agar plates was qualitatively determined by adding a drop of 30% hydrogen peroxide to a colony and observing gas evolution. Catalase activity in cell extracts was assayed spectrophotometrically (Schellhorn and Stones, 1992) as follows. One ml of hydrogen peroxide (0.5 ml of 30% H₂O₂ freshly diluted in 250 ml 50 mM potassium phosphate buffer, pH 7.0) was added to 10 μ l of cell extracts, and the decrease in OD₂₄₀ was monitored. The specific activity of catalase was calculated as $(1,000 \times \text{OD}_{240} / \text{time of incubation}) / (43.6 \times \text{mg of protein/ml of reaction mixture})$ (Beers and Sizer, 1952). To assess catalase activity in gels, protein samples (10 μ g) were loaded into a 10% non-denaturing polyacrylamide gel for electrophoresis using a Bio-Rad Mini-PROTEAN II system (Bio-Rad Laboratories, Hercules, CA). To detect catalase activity, the gels were stained using horseradish peroxidase and diaminobenzidine (Clare *et al.*, 1984). To confirm equal protein loading, parallel gels were stained for protein using Coomassie Blue (Sambrook *et al.*, 1989).

Glycogen staining procedures

Glycogen accumulation in bacterial cells was tested on Kornberg medium agar plates (Hengge-Aronis and Fischer, 1992; Liu *et al.*, 1995) in the presence or absence of 0.3 mM IPTG. The plates were inverted on a 500 ml beaker containing 3.3% iodine, 6.6% potassium iodide solution. The colonies were stained with the iodine vapor by heating the solution for 3-5 min. Colonies in which glycogen had accumulated were

dark brown in colour while those with little or no glycogen production stained yellow.

RESULTS

Construction of *rpoS* RNA expression system in pET21 and pET22b

The DNA sequence for the *rpoS* gene (PubMed accession No. 8475100) was used to design primers to amplify *rpoS* fragments. The objective was to produce an antisense RNA with properties similar to those of other recombinant antisense RNAs (Majdalani *et al.*, 1998; Wagner and Simons, 1994). Thus, the *rpoS* antisense RNA constructed is complementary to a region including the translation initiation start codon, the Shine-Dalgarno sequence, other upstream untranslated sequences and all/part of the coding sequences (Figure 1). To test if *rpoS* antisense RNA can inhibit the expression of *rpoS* and RpoS-dependent genes, the *rpoS* PCR products were cloned into pET expression plasmids. The resulting plasmids in the antisense orientation with respect to the *T7lac* promoter, were designated pSOPR (short antisense fragment in pET21 background) and pSOPRL (large antisense fragment in pET21 background) (Figures 1 and 2). These plasmids were then transformed into various *E. coli* strains (Table 1) for *in vivo* expression of antisense RNAs.

Effect of IPTG on cell growth

IPTG is normally used at a concentration of 1.0 mM for induction of protein synthesis in a pET plasmid system with *T7lac* promoter, while 0.4 mM IPTG is used for a pET plasmid carrying the plain *T7* promoter (Novagen Inc., Madison, WI). However, we found that concentrations of IPTG greater than 0.5 mM inhibited the growth of *E. coli* strains carrying pET plasmids with *T7lac* promoter. To ensure that the results of expression studies were not affected by growth inhibition, 0.3 mM IPTG was used in all experiments. At this concentration, there was little effect of IPTG on cell growth (data

not shown).

Effect of IPTG on *rpoS* antisense RNA expression

To titrate the effect of IPTG concentration on *rpoS* antisense RNA expression, *rpoS* antisense RNA expressed from cultures grown in various concentrations of IPTG was probed in Northern blots. In exponential phase (Figure 3, A), *rpoS* antisense mRNA was expressed in the presence of IPTG (lanes 14-18). Expression was not detectable in the absence of IPTG (lane 13) or in strains carrying the control plasmid pET21 (lanes 7-12) or no plasmid (lanes 1-6). *rpoS* antisense RNA was also expressed in BL21DE3pSOPR in the presence of IPTG (lanes 19), which is a positive control. In stationary phase (Figure 3, B), in MC4100DE3pSOPR, *rpoS* antisense RNA was expressed only in the presence of IPTG (lanes 14-18). Expression was not detectable in the absence of IPTG (lane 13). Expression in MC4100DE3 and its transformants containing control plasmid pET21 was not detectable in the absence or presence of IPTG (lanes 1-12). The optimal concentration of IPTG for induction of *rpoS* antisense RNA was determined to be 0.3 mM in exponential phase and stationary phase, below the level that inhibits cell growth.

Induction of short and large fragments of *rpoS* antisense RNA expression in MC4100DE3 and BL21DE3

To increase the ratio of antisense RNA to *rpoS* mRNA, cultures were diluted enough (see experimental procedures) to reduce traces of products in the cultures and induced with IPTG at a very early stage of cell growth. Antisense *rpoS* RNA is then available to inactivate *rpoS* mRNA as it is generated during the cell growth. The level of antisense products expressed by the cells is high due to the high-copy-number nature

of the plasmid pET21 and the strong *T7lac* promoter.

In exponential and stationary phases (Figure 4, A and B), expression of *rpoS* antisense RNA was induced by IPTG in strains carrying the antisense transcriptional plasmids. In the exponential phase samples, the short and large fragments of *rpoS* antisense were highly induced from antisense expression plasmids pSOPR and pSOPRL, respectively, in the presence of IPTG, as indicated on Northern blots probed with *rpoS* DNA. The level of short fragment mRNA was higher than that of the large fragment RNA, especially in MC4100DE3. In stationary phase, antisense mRNA was also transcribed (data for large fragment mRNA not shown for MC4100DE3). In all cases, antisense RNA levels were not detectable in the absence of IPTG. Antisense RNA was also not detectable in *rpoS*⁺ or *rpoS*⁻ control strains without a plasmid or strains that harbor the control plasmid pET21 in the presence or absence of IPTG. The ratio of antisense RNA expressed by the cells to *rpoS* mRNA was high. The short fragment antisense construct pSOPR was selected for further study.

Optimization of *rpoS* antisense expression

To evaluate antisense RNA expression levels, Northern blotting analyses were performed. Results were shown in Figure 5. In exponential phase (Figure 5, A), *rpoS* antisense RNA was optimally expressed in the presence of 0.3 mM IPTG (lanes 10). Expression was not detected in the absence of IPTG (lane 9). Antisense RNA was not detected in the absence or presence of IPTG (lanes 1-8) in *rpoS*⁺ or *rpoS*⁻ control strains without a plasmid or strains that harbor the control plasmid pET21. In stationary phase (Figure 5, B), *rpoS* antisense RNA was highly expressed in the presence of 0.3 mM IPTG (lanes 10). The results were similar to those of exponential phase. As a positive

control, BL21DE3pSOPR was induced with IPTG (lane 11). All exponential and stationary phase samples were normalized to induce stationary phase sample value (MC4100DE3pSOPR in the presence of IPTG). In the presence of IPTG, antisense RNA levels were 32-fold induced in exponential phase and 21-fold induced in stationary phase (data not shown) as measured by image densitometry. These results demonstrate that antisense RNA expression can be controlled by IPTG. For maximal inhibition of RpoS expression, we found that antisense RNA must be induced in early exponential phase ($OD_{600} = 0.01$). In this way, antisense RNA is present in the cell before *rpoS* is transcriptionally activated and can thus effectively sequester *rpoS* mRNA before it is translated. However, antisense *rpoS* expressed from the pET translational plasmid pET22b in strains BL21DE3 and MC4100DE3 carrying pGC226 or pGC225a did not inhibit highly RpoS-dependent *katE* and *osmY* expression (data not shown). In this plasmid, the short and large fragments of *rpoS* antisense were probably translated into chimeric protein. pET22b contains its own RBS located downstream of *T7lac* promoter.

Antisense-repressed RpoS protein expression

In stationary phase (Figure 6), RpoS protein was produced in *rpoS*⁺ control cells (Figure 6, lanes 9 and 10), cells carrying control plasmid pET21 (lanes 13 and 14) and in uninduced *rpoS*⁺ cells carrying the antisense construct (lane 15). However, when antisense RNA was expressed (following induction with IPTG), RpoS protein was reduced (lane 16). RpoS protein was not produced in *rpoS*⁻ control strain in the presence or absence of IPTG (lanes 11 and 12). Levels of RpoS protein were higher in the MC4100DE3 strain than in MC4100DE3pET21. Proteins expressed from the

control plasmid pET21 (for example, β -lactamase and LacI) may interfere with RpoS protein synthesis.

The ClpXP protease rapidly degrades RpoS in exponential phase cells resulting in extremely low amounts of this sigma factor (Schweder *et al.*, 1996). Consistent with this, we found that in exponential phase cultures of all strains tested (Figure 6, lanes 1-8) RpoS protein levels were too low to be detected by Western blotting.

Inhibition of RpoS-dependent *osmY* expression by *rpoS* antisense

To measure the effect of *rpoS* antisense RNA on the expression of the highly RpoS-dependent *osmY* gene (Lange *et al.*, 1993), an *osmY-lacZ* operon fusion was transduced into MC4100DE3. Expression of *osmY* during growth of various strains (Table 1) was quantified by measuring β -galactosidase expression. OsmY expression was inhibited by the addition of IPTG to cultures containing *rpoS* antisense-expressing constructs and was normally expressed in control cultures incubated in the absence of IPTG or in strains containing only the pET21 plasmid vector (data not shown).

Inhibition of RpoS-dependent catalase HPII expression by *rpoS* antisense expression

E. coli catalase HPII, encoded by *katE*, can be used as a target protein for evaluating highly RpoS-dependent gene expression (Schellhorn and Hassan, 1988). Catalase activity was examined on LB and M9 agar plates supplemented with IPTG by adding 30% hydrogen peroxide to 24 h colonies (Schellhorn and Stones, 1992). The lack of significant gas evolution indicated that catalase activity was reduced in antisense expressing strains in the presence of IPTG. In addition to qualitative catalase assays, catalase enzyme activity was measured to confirm the effect of *rpoS* antisense

expression on catalase expression. As shown in Figure 7, A and B, catalase activity was greatly inhibited in *rpoS* antisense-expressing strains (compare open bars to filled bars in strain MC4100DE3pSOPR) in both exponential and stationary phases. The control strain MC4100DE3 and transformants containing control plasmid exhibited similar levels of activity and these were much higher than in the antisense producing strain (compare black bars in strains MC4100DE3pSOPR, MC4100DE3, and MC4100DE3pET21). Expression of the HPII catalase is known to be much more dependent on RpoS than that of the HPI catalase (Schellhorn and Stones, 1992). To test whether downregulation of catalase activity in exponential and stationary phases by induced *rpoS* antisense RNA is due to reduced expression of the RpoS-dependent catalase HPII, levels of HPII and HPI were assessed by non-denaturing PAGE followed by catalase staining. In strains expressing *rpoS* (MC4100DE3 and MC4100DE3pSOPR), HPII expression was normal: levels were low in exponential phase and high in stationary phase cultures (Figure 8, A and B). As expected, induction of *rpoS* antisense RNA inhibited RpoS-dependent HPII expression in stationary phase (Figure 8, B, lane 8) although HPII was not present in any sample in native catalase gel from exponentially grown cells (Figure 8, A). In addition, levels of catalase HPI in exponential and stationary phases were inhibited by induced *rpoS* antisense RNA (Figure 8, A and B).

Glycogen negative phenotype for *rpoS* antisense induced strain

Glycogen accumulation is stimulated by RpoS during entry into stationary phase (Hengge-Aronis and Fischer, 1992; Liu *et al.*, 1995) as expression of *glgS* is entirely dependent on RpoS in *E. coli*. RpoS deficient mutants do not produce GlgS and

overexpression of *glgS* stimulates higher levels of glycogen synthesis in early stationary phase (Hengge-Aronis and Fischer, 1992). Glycogen accumulation in bacterial colonies can be visualized on Kornberg Medium, a nitrogen-limited, glucose-rich medium (Hengge-Aronis and Fischer, 1992; Romeo and Preiss, 1989) by a dark brown stain in the presence of iodine, when little or no glycogen is present colonies are yellow in color (Liu *et al.*, 1995). As expected, MC4100DE3 cells (*rpoS*⁺) accumulated glycogen, while RpoS-deficient mutants (HS1600DE3) did not (Figure 9, A). However, induced RpoS antisense expression reduced glycogen synthesis resulting in yellow colonies following iodine staining (Figure 9, B).

DISCUSSION

During the last decade, antisense RNA research has focused on increasing the efficiency of antisense RNA, manipulating cell metabolic pathways, and investigating unknown gene functions when gene disruption is not possible under certain conditions (Casqueiro *et al.*, 1999). The use of antisense RNA to attenuate gene expression has several advantages over gene inactivation. Lethal mutations in essential genes can be avoided, genes can be inactivated when homologous recombination is weak (Casqueiro *et al.*, 1999), antisense expression is controllable (Yatzkan *et al.*, 1998) and therefore proteins can be transiently downregulated. Regulation of RpoS through plasmid-encoded *rpoS* antisense RNA can help us to understand bacterial pathogenesis and environmental adaptation responses. RpoS plays an important role during pathogenicity in enterobacteria and is induced under stress conditions. For example, RpoS is required for the virulence that is dependent on *spvABCD* by *Salmonella typhimurium* in a mouse model (Kowarz *et al.*, 1994; Coynault *et al.*, 1992; Coynault *et al.*, 1996). RpoS is also required for maintaining chronic lung infection in *Pseudomonas aeruginosa* in a rat model (Suh *et al.*, 1999). In addition, RpoS controls potential antimicrobial agent and drug efflux operon *yhiUV* in *E. coli* (Nishino and Yamaguchi, 2001; Schellhorn *et al.*, 1998).

Expression of antisense RNA from a multi-copy plasmid resulting in reduced mRNA translation has been successfully employed in previous studies. Introduction of a *marA* antisense-expressing plasmid into *E. coli* cells carrying a *marORA-lacZ* fusion reduces LacZ expression and subsequently increases multiple antibiotic susceptibility. Furthermore, the antisense RNA that most efficiently represses expression of *marORA*

is complementary to a region encompassing 20 bases of untranslated sequence upstream of *marR*, the AUG initiation codon and the first 92 bases of the *marA* coding sequence (White *et al.*, 1997) indicating that sequestration of mRNA control sequences can ablate translation. Expression of an antisense *hla* RNA from a plasmid reduces alpha-toxin virulence up to 17-fold relative to the wild type strain carrying a control plasmid in *Staphylococcus aureus* and, consequently, eliminates lethality in a mouse model (Ji *et al.*, 1999; Kernodle *et al.*, 1997). In this study, RpoS-dependent catalase HPII expression and glycogen accumulation were dramatically decreased in antisense-expressing cells during stationary phase compared to cells that did not express *rpoS* antisense RNA. Thus antisense technology can be used successfully to reduce levels of a transcriptional regulator and attenuate expression of a downstream target. Of the *rpoS* antisense RNAs used, one is complementary to a region that includes the 5'-untranslated region (93 bases) and part of the coding region of *rpoS* (907 bases) while another is complementary to a region that includes the 5'-untranslated region (93 bases) and all of the coding region of *rpoS* with the 156-bp transcription termination sequence. Expression of antisense RNA was sufficient to inhibit *rpoS* gene expression. The construct carrying the antisense sequence spanning the 5'-untranslated region and a part of the coding region of *rpoS* mRNA was found to be the most effective in the attenuating expression of the *rpoS* gene (Chen and Schellhorn, unpublished data).

Several natural antisense RNA regulators of *rpoS* gene expression, and the resulting phenotypes, have been identified in *E. coli*. In many cases their modes of action have been described. OxyS RNA represses *rpoS* translation by preventing RNA-binding protein Hfq from activating *rpoS* translation (Zhang *et al.*, 1998). OxyS RNA

is proposed to bind to Hfq protein through an A-rich linker region between two stem-loops in OxyS RNA (Zhang *et al.*, 1998), thereby resulting in an increase of *rpoS* translation by mediating the conformational change of the *rpoS* mRNA secondary structure (Lange and Hengge-Aronis, 1994). OxyS represses osmotic induction of RpoS in strains carrying *rpoS-lacZ* translational fusions treated with high salt (Zhang *et al.*, 1998). OxyS also represses the transcriptional activator FhlA through its ability to bind either the Shine-Dalgarno sequence or the coding region of *fhlA*, resulting in stable sense-antisense complex (Altuvia *et al.*, 1998; Argaman and Altuvia, 2000). DsrA stimulates *rpoS* translation at low temperature (20°C) (Lease and Belfort, 2000) by stabilizing *rpoS* mRNA (Sledjeski *et al.*, 1996). DsrA RNA acts in trans by RNA-RNA interactions with *rpoS* mRNA (Lease *et al.*, 1998; Lease and Belfort, 2000). A stem-loop of DsrA binds to the 5' untranslated leader sequence of *rpoS* mRNA just before the translation initiation site, resulting in increased RpoS protein levels (Lease *et al.*, 1998; Majdalani *et al.*, 1998).

We propose the following model to explain *rpoS* antisense RNA repression of *rpoS* mRNA translation. The ratio of introduced antisense *rpoS* RNA to *rpoS* mRNA generated from the chromosome is very high. Thus, antisense RNA is sufficient to bind to the sense *rpoS* RNA strand and produce a double-strand RNA molecule. The double-stranded RNA molecule is degraded by ribonucleases (Inouye, 1988). The 5' - untranslated region of the *rpoS* mRNA is self-complementary, forming a secondary structure (Majdalani *et al.*, 1998; Majdalani *et al.*, 2001; Cuning *et al.*, 1998) which is inaccessible to ribosomes, and therefore is not translated into RpoS protein (Figure 10). Induced expression of *rpoS* antisense RNA inhibits the expression of *rpoS* and RpoS-

dependent genes such as *katE* and *glgS* by binding *rpoS* mRNA and inhibiting translation, and thereby ablating synthesis of RNA polymerase sigma factor required for RpoS-dependent gene transcription.

Inhibition of bacteriophage replication by antisense expression in host cells requires optimization during a phage infection in the cell (Walker and Klaenhammer, 2000). By reducing the expression of RpoS using controllable antisense RNA during bacterial infection, expression of a number of RpoS-dependent genes will be influenced. Control of expression of RpoS-dependent genes by RNA antisense may offer a good model for the regulation of RpoS translation and provide a new tool to study bacterial infection processes. One limitation is that the high-copy-number plasmid pET21 may have an effect on *E. coli* cell growth and the expression of *rpoS*. Future work includes insertion of the *rpoS* antisense RNA construct in a low-copy-number plasmid or as a single copy in the host chromosome.

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Table 1. Bacterial strains used and constructed in this study

<i>E. coli</i> strain	Genotype/Description	Source/Comment
DH5 α	<i>supE44 ΔlacU169(ϕ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Stratagene
MC4100	<i>(argF-lac)205 araD139 flbB5301 relA1 rpsL150 thi ptsF25</i>	(Schellhorn and Stones, 1992)
MC4100DE3	As MC4100 but expresses T7 RNA polymerase	This study
MC4100DE3Y	As MC4100DE3 but <i>osmY-lacZ</i>	This study
HS1600	As MC4100, but <i>rpoS13::Tn10</i>	Laboratory collection
HS1600DE3	As HS1600 but expresses T7 RNA polymerase	This study
BL21DE3	<i>F⁻ompT hsdS_B(r_B⁻m_B⁻) gal dcm</i> (DE3)	Novagen
MC4100DE3pET21	As MC4100DE3Y, but pET21	This study
MC4100DE3YpET21	As MC4100DE3Y, but pET21	This study
MC4100DE3pSOPRL	Contains <i>rpoS</i> gene in pET21, but fragment in opposite orientation to <i>T7lac</i> promoter	This study
MC4100DE3YpSOPRL	As MC4100DE3Y, but pSOPRL	This study
MC4100DE3pSOPR	Contains <i>rpoS</i> antisense in pET21	This study
MC4100DE3YpSOPR	As MC4100DE3Y, but pSOPR	This study
BL21DE3pSOPR	Contains <i>rpoS</i> antisense in pET21	This study

Table 2. Plasmids used and constructed in this study

Plasmid	Size (bp)	Description and resistance(s)	Source
pGEM-T Easy	3001	Amp ^R , blue/white colour selection, single 3'-T overhangs to allow efficient ligation with PCR products	Promega
pET21	5369	Amp ^R , transcriptional plasmid, lacks the RBS and start codon	Novagen
pET22b	5493	Amp ^R , translational plasmid, pelB for protein export/folding plus His. Tag [®] for His. Bind resin purification	Novagen
pGC2	4298	1278 bp <i>rpoS</i> fragment cloned in the opposite orientation with respect to <i>T7lac</i> promoter in pGEM-T Easy vector	This study
pGC2a	4042	pGEM-T Easy containing 1022 bp of <i>rpoS</i> sequence from MC4100 DNA	This study
pSOPR	6411	pET21 containing 1042 <i>EcoRI</i> fragment from pGC2a, <i>T7lac</i> promoter in orientation with respect to <i>rpoS</i> antisense	This study
pSOPRL	6667	<i>T7lac</i> promoter in opposite orientation with respect to <i>rpoS</i>	This study
pC225a	6535	pET22b with 1042 <i>EcoRI</i> fragment from pGC2a, <i>T7lac</i> promoter in orientation with respect to <i>rpoS</i> antisense	This study
pGC226	6791	pET22b containing 1298 bp <i>EcoRI</i> fragment from pGC2, <i>T7lac</i> promoter in orientation with respect to <i>rpoS</i> antisense	This study

Figure 1. Schematic representation of the *rpoS* gene in the *E. coli* chromosome (top) and in antisense expression plasmids pSOPR and pSOPRL. Promoter positions are indicated (*P1*, *P2* and *pT7lac*). The *rpoS* gene RBS, and start (ATG) and stop codons (TAA) are also shown.

Figure 1.

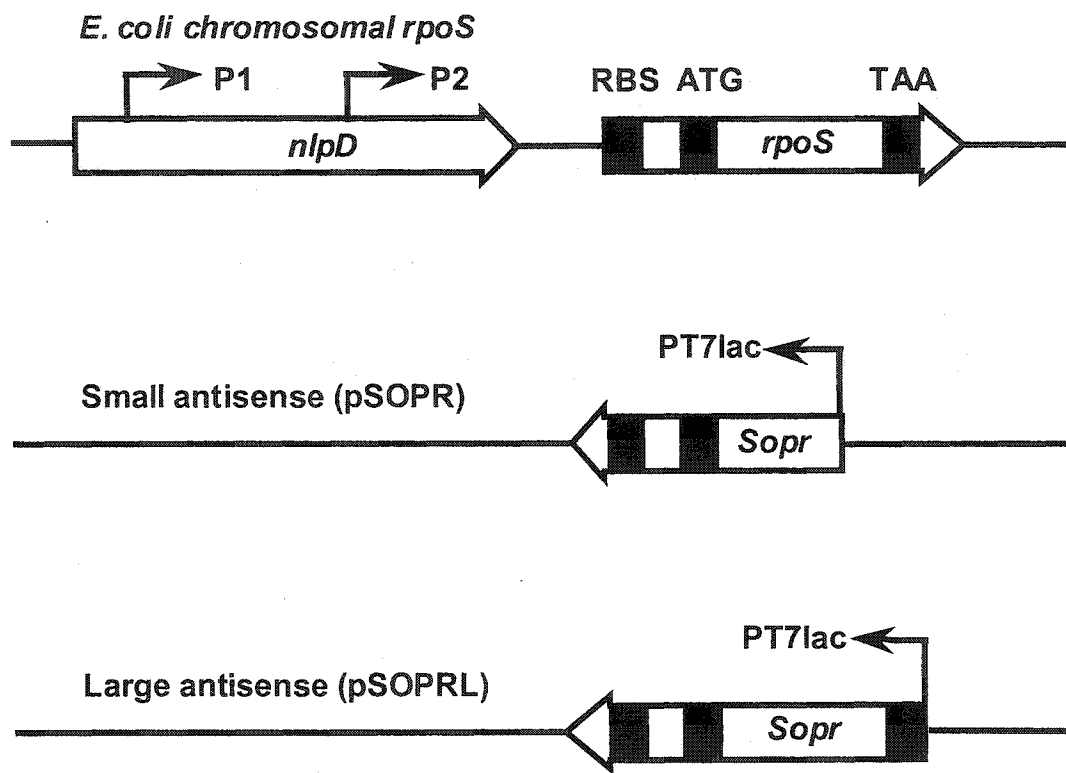


Figure 2. Antisense RNA plasmid pSOPR. The unique restriction endonuclease recognition sites are shown. Abbreviation: P_{T7lac} , $T7lac$ promoter, *ori*, plasmid origin of DNA replication, *rpoSa*, *rpoS* antisense fragment, *Ap*, ampicillin resistance gene.

Figure 2.

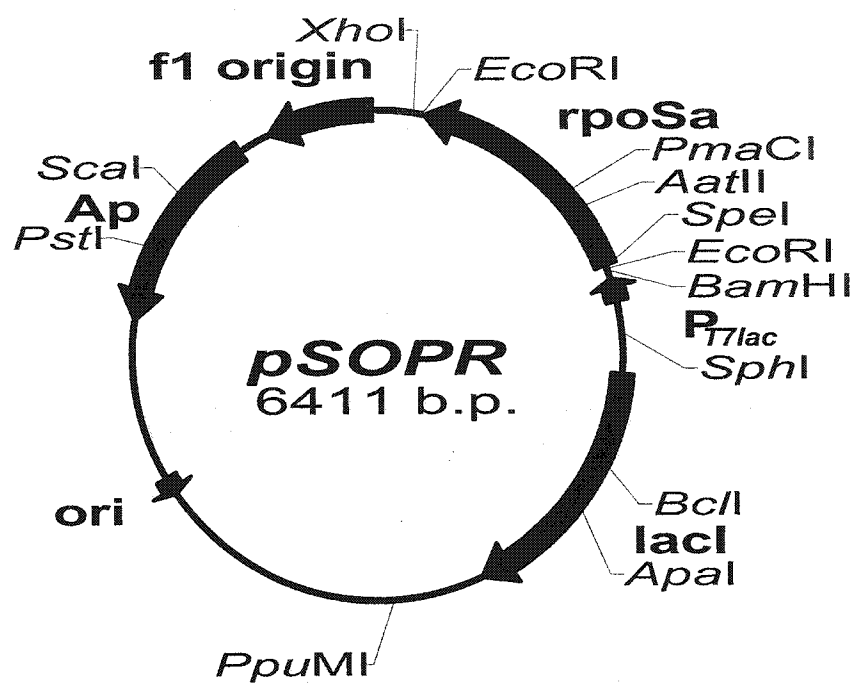


Figure 3. Northern blot analysis of *rpoS* antisense RNA induced with IPTG in MC4100DE3 series in exponential (A) and stationary (B) phases showing the effect of IPTG on *rpoS* antisense RNA expression. Total RNA was isolated from *rpoS* wild type strain MC4100DE3 and its transformants containing control plasmid pET21 and antisense *rpoS* plasmid pSOPR. RNA was isolated from cultures grown in the absence (lanes 1, 7, 13) or in the presence of 0.2 mM, 0.3 mM, 0.5 mM, 0.75 mM, and 1 mM IPTG (lanes 2-6, 8-12, 14-18). As a positive control, BL21DE3pSOPR was induced with 0.75 mM IPTG (lane 19). The lower panels in A and B show that relative amounts of RNA were applied to formaldehyde-agarose gels and then transferred to Hybond-N⁺ membranes. The expression of antisense RNA was demonstrated with an *rpoS*-specific α -³²P-dCTP-labeled double-stranded DNA probes.

Figure 3.

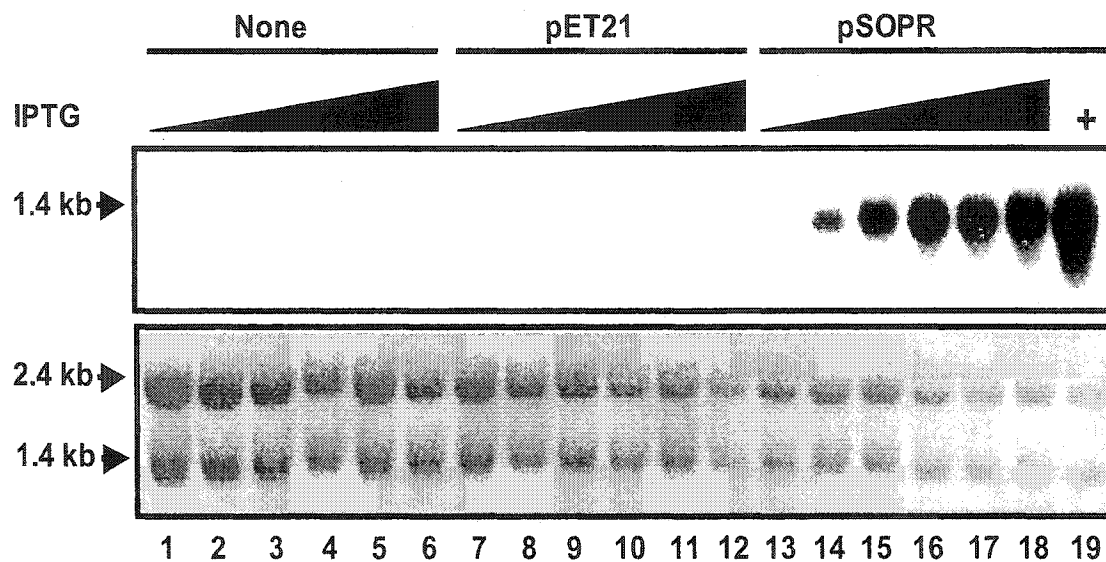
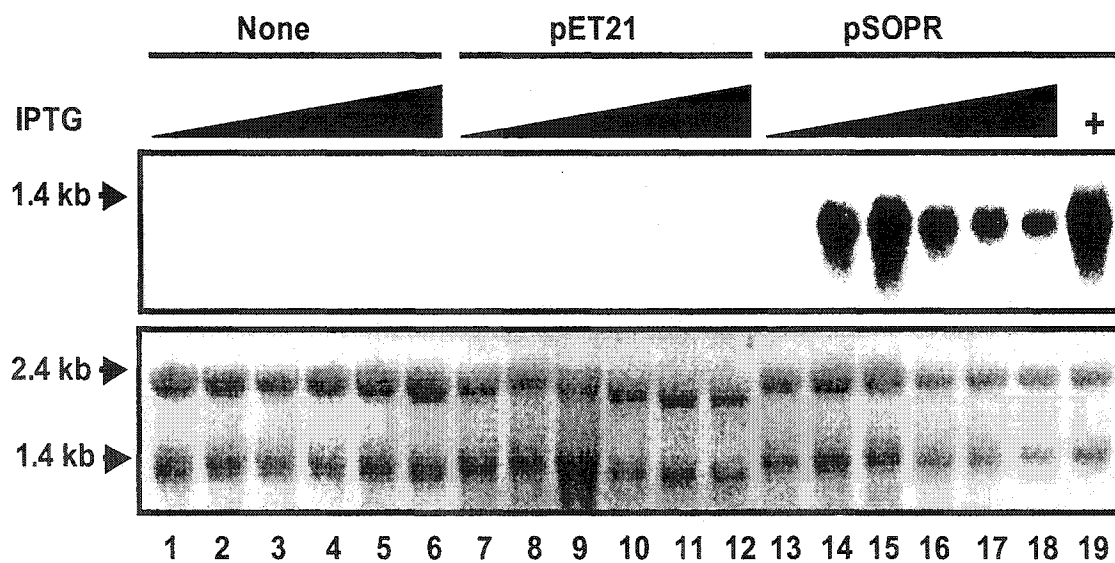
(A) Exponential phase**(B) Stationary phase**

Figure 4. Northern blot analysis of short and large fragments of *rpoS* antisense RNAs induced by IPTG in BL21DE3 and MC4100DE3 in (A) exponential phase and (B) stationary phase. Total RNA was isolated from *rpoS* wild type strains BL21DE3 and MC4100DE3 and their transformants containing control plasmid pET21 and antisense *rpoS* plasmids pSOPRL and pSOPR. RNA was isolated from culture grown in the absence (-) (lanes 1-4, 9-12) or in the presence (+) of IPTG (lanes 5-8, 13-16). The lower panels in A and B show that equal amounts of RNA were applied to formaldehyde-agarose gels and then transferred to Hybond-N⁺ membranes. The expression of antisense RNA was demonstrated with an *rpoS*-specific α -³²P-dCTP-labeled double-stranded DNA probes.

Figure 4.

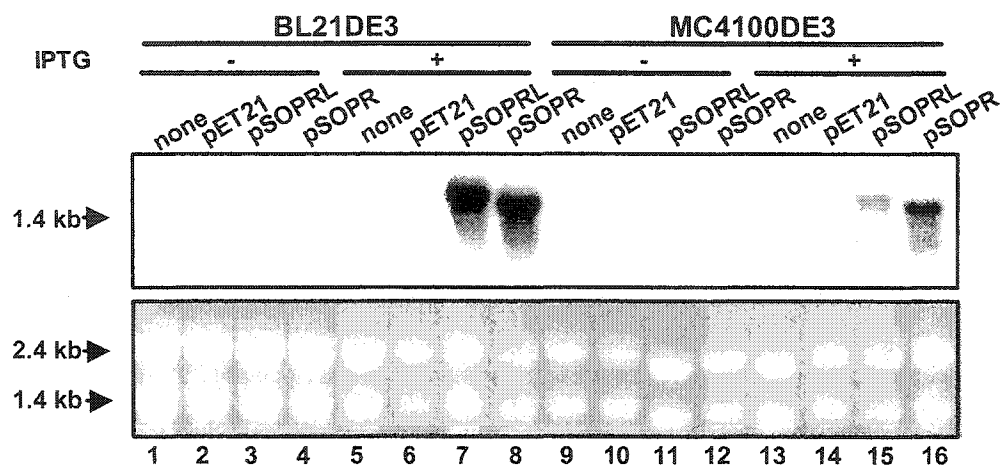
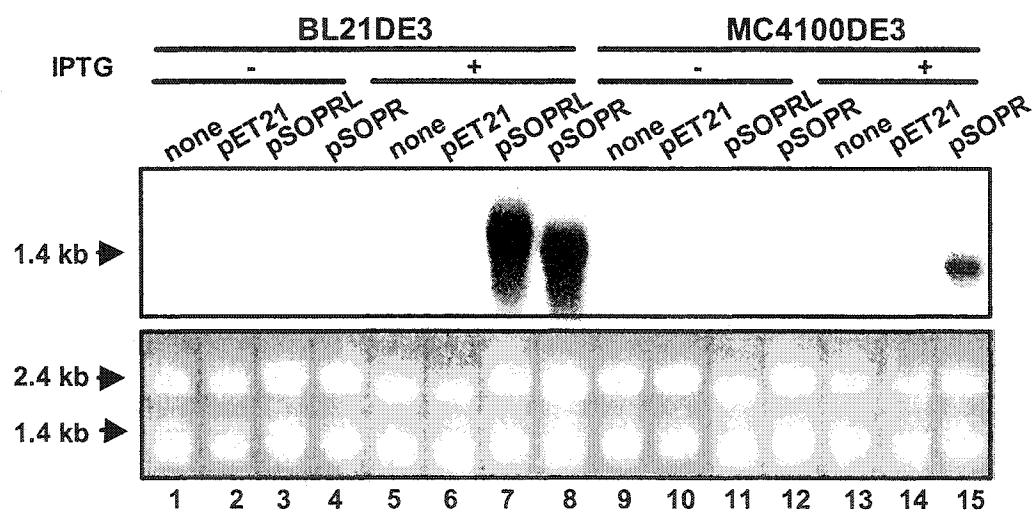
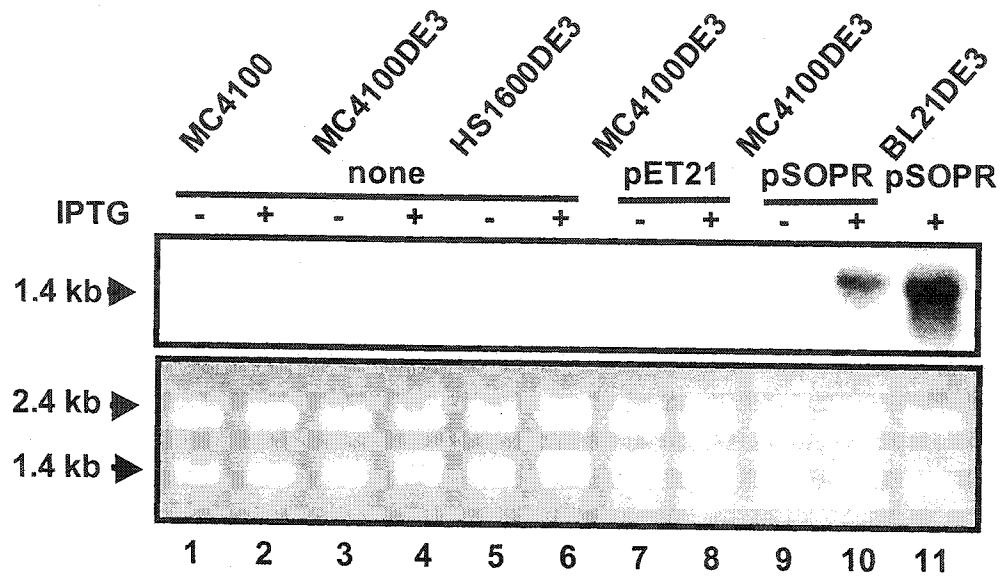
(A) Exponential phase**(B) Stationary phase**

Figure 5. Northern blot analysis of *rpoS* antisense RNA induced by 0.3 mM IPTG in MC4100DE3 series in exponential and stationary phases showing the optimization of induction of *rpoS* antisense RNA expression of short and large fragments of *rpoS* antisense RNA.

(A) Exponential phase

Figure 5.



(B) Stationary Phase

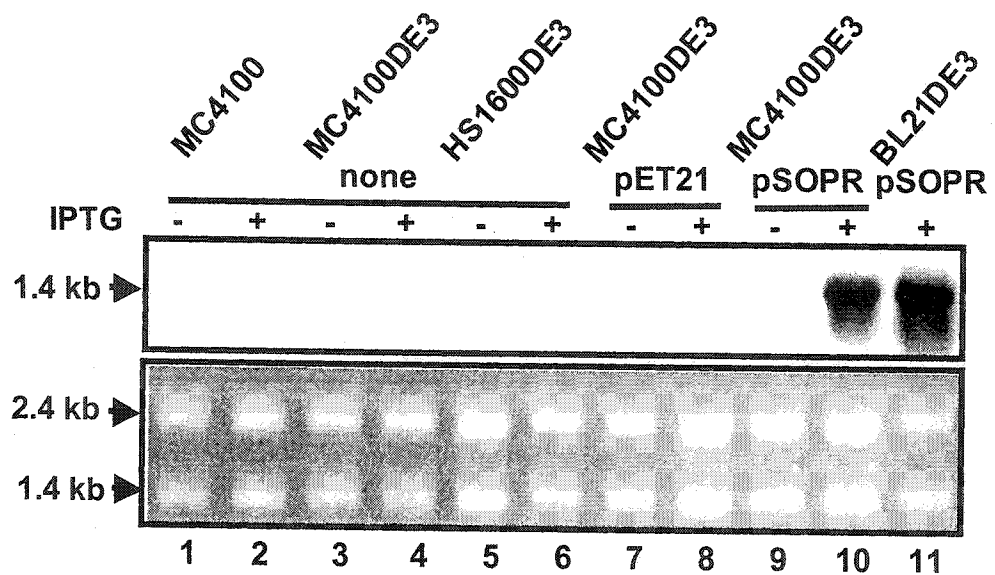


Figure 6. Western blot analysis of RpoS levels in *E. coli* MC4100 derivatives using anti- σ^S -antiserum. Protein was extracted from cultures grown to exponential phase (OD_{600} 0.25) and stationary phase (OD_{600} 1.5). The PVDF membrane was stained with ECL staining solution and exposed to Kodak X-OMAT film (Eastman Kodak Company, Rochester, NY).

Figure 6.

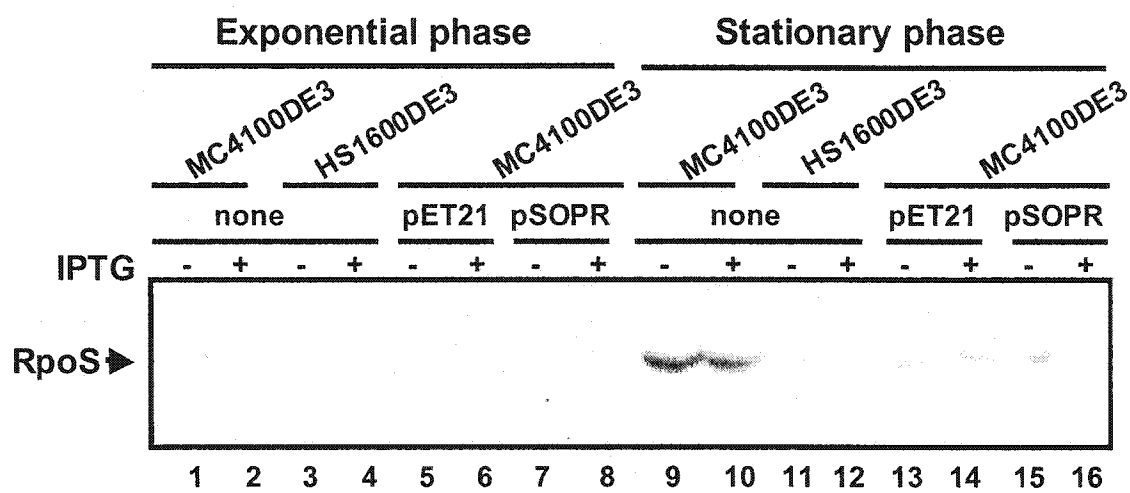


Figure 7. Catalase specific activity of *rpoS* wild type strain MC4100DE3 containing control plasmid pET21 and antisense construct pSOPR grown in the absence and presence of 0.3 mM IPTG in LB media. Induced *rpoS* antisense RNA downregulated expression of RpoS-dependent catalase.

Figure 7.

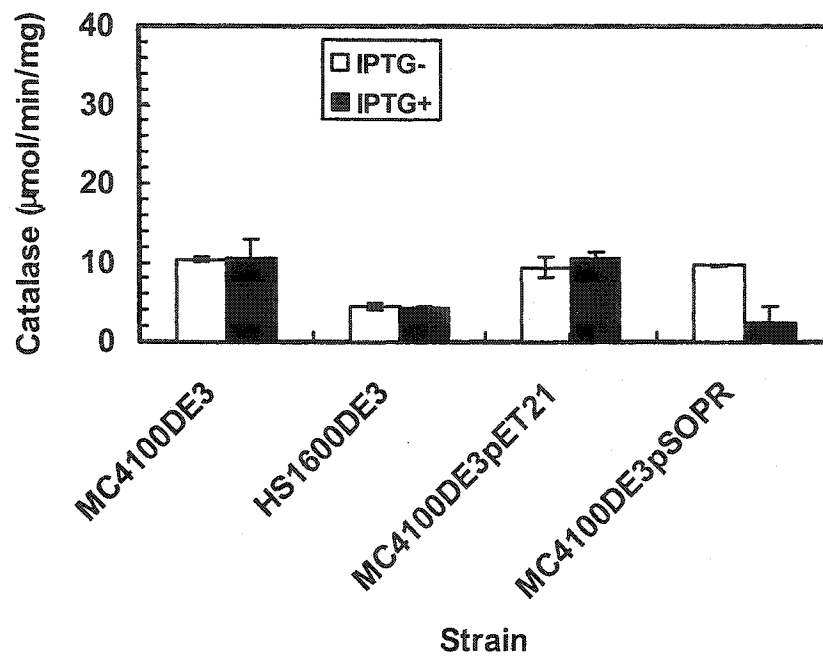
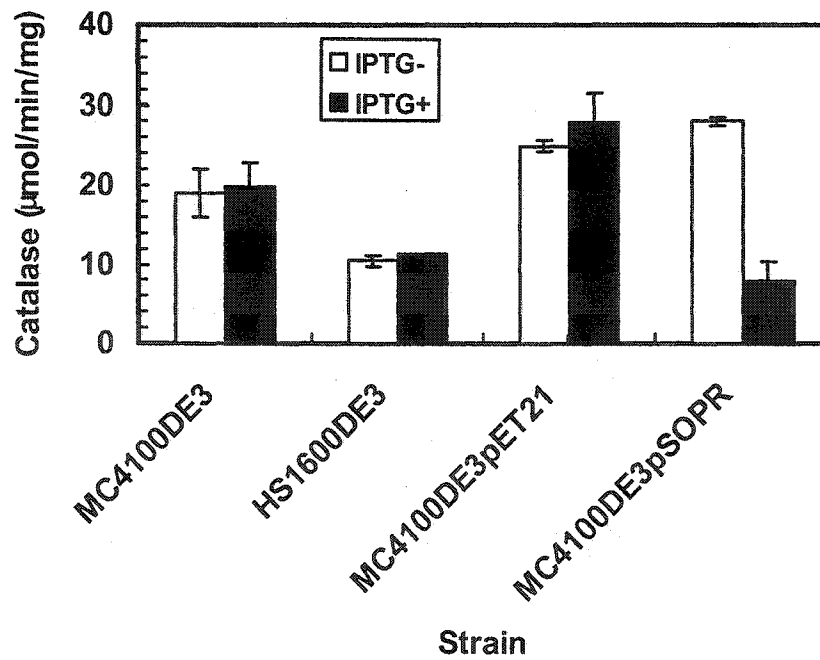
(A) Exponential phase**(B) Stationary phase**

Figure 8. Catalase activity in polyacrylamide gels containing protein extracted from *rpoS* wild type strain MC4100DE3 carrying control plasmid pET21 and antisense construct pSOPR grown in the absence and presence of 0.3 mM IPTG in LB media. Induced *rpoS* antisense RNA downregulated RpoS-dependent *katE* expression (compare lanes 7 and 8 in panel B).

Figure 8.

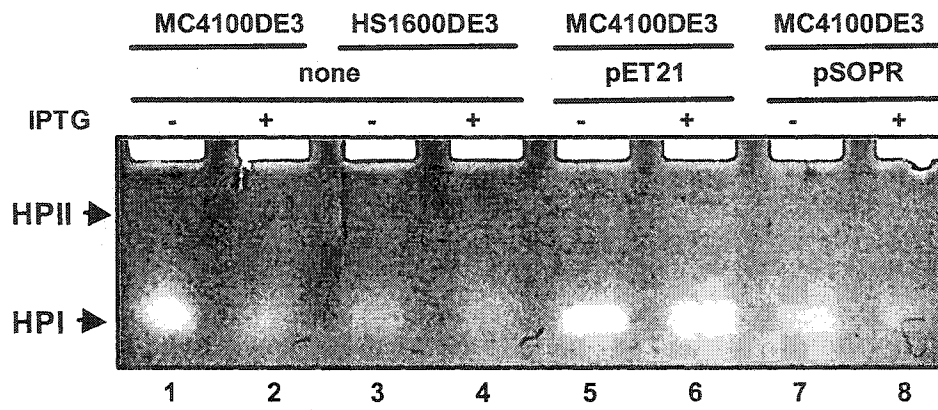
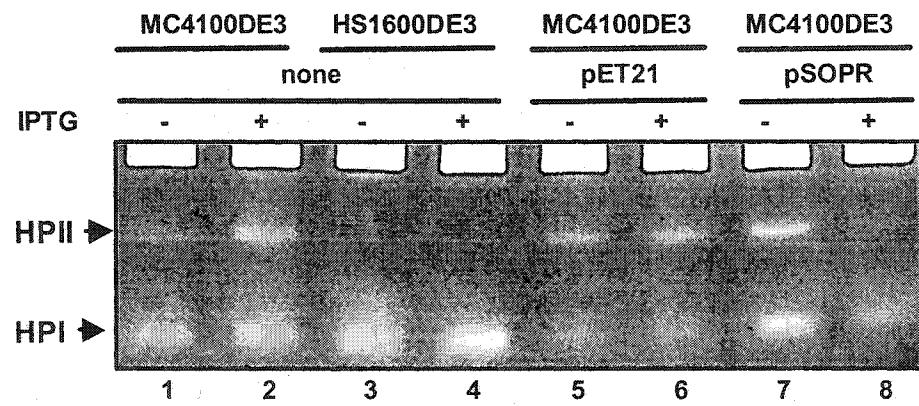
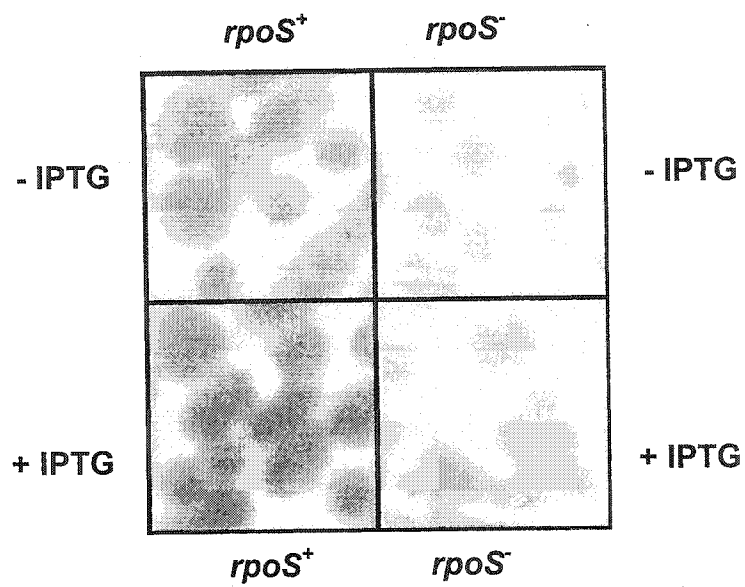
(A) Exponential phase**(B) Stationary phase**

Figure 9. Glycogen accumulation of strains during growth on Kornberg medium plates. The strains were grown 1-2 days at 37°C and intracellular glycogen was stained with iodine vapors.

Figure 9.

(A)



(B)

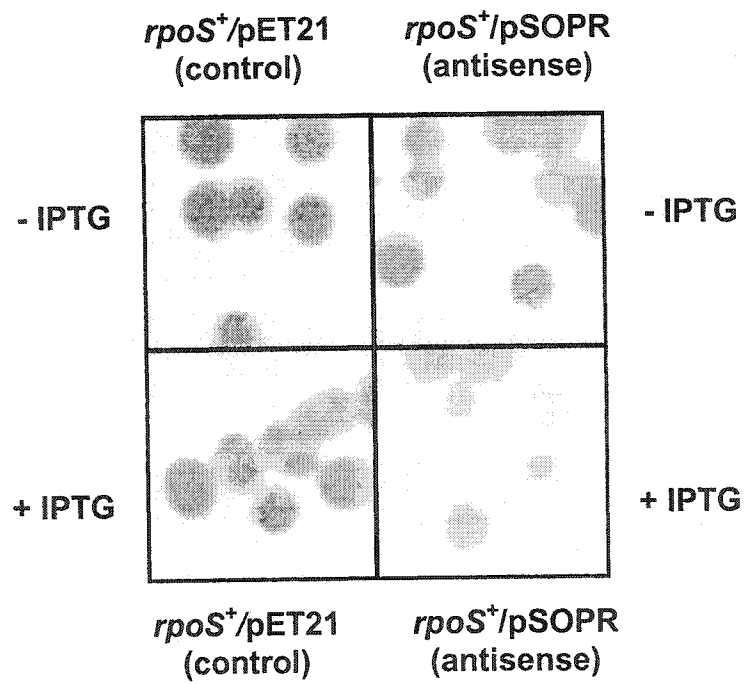
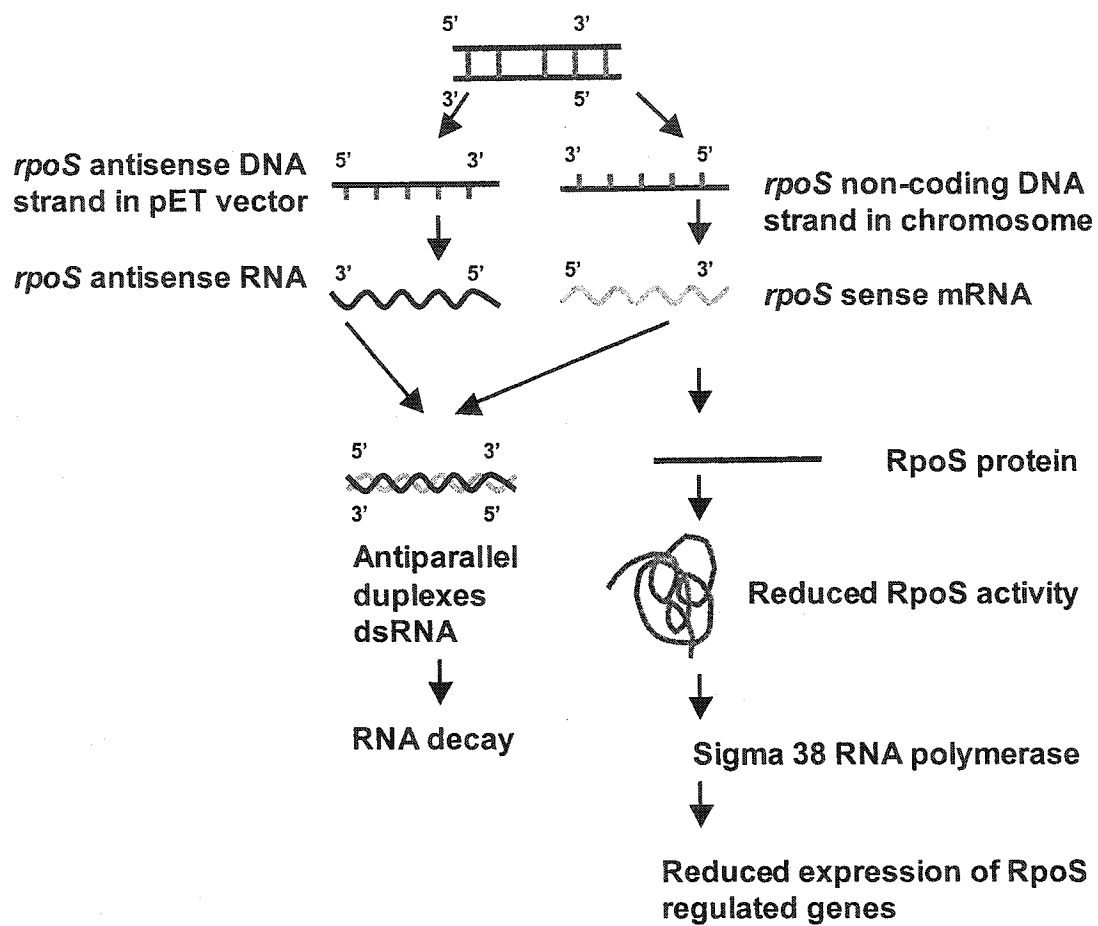


Figure 10. The proposed mechanism of *rpoS* antisense RNA inhibition of *rpoS* expression. Induced expression of *rpoS* antisense RNA can inhibit the expression of *rpoS* and downstream RpoS-dependent genes through (A) the binding and sequestration of *rpoS* mRNA, thereby inhibiting the *rpoS* mRNA translation, and (B) the reduction of RNA polymerase sigma factor and thereby the prevention of RpoS-dependent gene translation.

Figure 10.



CHAPTER 5:

**CONTROLLED INDUCTION OF THE RPOS REGULON IN
ESCHERICHIA COLI USING AN RPOS-EXPRESSING PLASMID**

Preface

Chapter five, entitled “Controlled induction of the *rpoS* regulon in *Escherichia coli* using an *rpoS*-expressing plasmid”, was written as a manuscript that has been formatted to comply with the thesis regulations. All the work presented in this chapter has been carried out by the author.

**Controlled induction of the RpoS Regulon in *Escherichia coli*
using an RpoS-expressing plasmid**

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Running Title: Induction of the RpoS Regulon in *E. coli*

Key words: RpoS, regulon, gene expression, *Escherichia coli*

Abstract

RpoS, an alternative sigma factor produced by many gram-negative bacteria, primarily controls genes that are expressed in stationary phase in response to nutrient deprivation. To test whether induction of RpoS in exponential phase, when RpoS is not normally expressed, results in increased expression of RpoS and RpoS-dependent gene expression, we constructed a plasmid carrying the *rpoS* gene under the control of an IPTG inducible-*T7lac* promoter. Western analysis revealed that both RpoS mRNA and protein were expressed in response to the inducer IPTG. Assays of RpoS-dependent function (measured as catalase activity) and expression of glycogen accumulation, an RpoS-dependent phenotype, confirmed that induction of RpoS in exponential phase is sufficient for expression of the RpoS regulon. Controlled expression of RpoS and RpoS-dependent genes by plasmid-encoded *rpoS* gene may offer a useful tool to establish requirements of expression of the RpoS regulon.

Introduction

RpoS is an alternative sigma factor of bacterial RNA polymerase (Tanaka *et al.*, 1993; Jishage *et al.*, 1996). In response to environmental stress and nutrient starvation, RpoS mediates the increase in expression of many genes during transition to stationary phase (Hengge-Aronis, 1996). Many stationary-phase-inducible proteins that are regulated by RpoS have been identified, including the *katE* encoded catalase HPII (Loewen *et al.*, 1985; Schellhorn and Hassan, 1988), glycogen synthetase (Liu *et al.*, 1995; Romeo and Preiss, 1989), acid phosphatase (Touati *et al.*, 1986; Touati and Danchin, 1987b; Touati and Danchin, 1987a), and the morphogene BofA (Hengge-Aronis *et al.*, 1993; Lange and Hengge-Aronis, 1991a; Santos *et al.*, 1999). RpoS is thus responsible for induction of many genes under starvation and stress conditions. RpoS plays a role in synthesis of storage polymers, for example, glycogen accumulation in *E. coli* is stimulated by RpoS during entry into stationary phase (Hengge-Aronis and Fischer, 1992; Liu *et al.*, 1995).

Expression of RpoS-regulated genes is controlled during both exponential and stationary phases. While it is clear that increased stability of RpoS in stationary phase is an important factor in control of expression of the RpoS regulon (Loewen *et al.*, 1998), transcription of *rpoS* is tightly controlled and is a prerequisite for expression of the regulon (Lange and Hengge-Aronis, 1994).

In this study, an *rpoS* gene expression system was constructed using a transcriptional vector pET21 under the control of the *T7lac* promoter. We were primarily interested in assessing if non-physiological induction of the regulator would result in expression of the regulon. The expression of *rpoS* gene was evaluated to

examine whether plasmid-encoded *rpoS* gene could affect the level of *rpoS* mRNA and RpoS-dependent catalase HPII levels. The results indicate the expression of the RpoS regulon in *E. coli* can be modulated by plasmid-encoded *rpoS* gene expression in early exponential and stationary phases.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1 and plasmids used are listed in Table 2.

Liquid and solid media were prepared as described by Miller (1992). Glycogen accumulation was tested on Kornberg Medium agar plates to allow maximal glycogen synthesis (Hengge-Aronis and Fischer, 1992; Romeo and Preiss, 1989). All strains were routinely grown in Luria Bertani (LB) broth (Miller, 1992) containing the appropriate antibiotics at 37°C at 200 rpm. M9 minimal media (Miller, 1992) were also used as indicated. Growth was monitored turbidometrically by measuring optical density at 600 nm (OD₆₀₀) using a Kontron Uvikon 930 spectrophotometer (Kontron AG, Munich, Germany).

Construction of λ DE3 lysogens

To integrate the T7 RNA polymerase gene into the *E. coli* chromosome, the protocol supplied by Novagen Inc., Madison, WI, was used. Single colonies of *E. coli* strains were used to inoculate 10 ml LB broth containing 0.2% maltose, 10 mM MgSO₄ and grown overnight at 37°C with shaking (200 rpm). The overnight culture was diluted and grown to an OD₆₀₀ of 0.5. Cells were mixed with 10⁸ PFU of phage λ DE3, 10⁸ PFU of helper phage λ B10 and 10⁸ PFU of selection phage λ B482. The resulting mixture was incubated at 37°C for 20 min with shaking (200 rpm) to allow the phage to adsorb to the host. Aliquot of mixtures was spread onto LB agar containing selective antibiotics. As a control, cells were mixed with 10⁸ PFU of selection phage and treated as above to check for viability of λ DE3 lysogen.

To verify λ DE3 lysogenization, a tester phage (T74107) that produces large plaques on permissive (lysogenic) strains and small plaques on non-permissive (non-lysogenic) strains was used according to the manufacturer's instructions.

DNA isolation and transformation of *E. coli*

Small scale preparations of *E. coli* plasmid DNA were prepared by alkaline lysis (Sambrook et al., 1989) while larger amounts were isolated using a Midi kit (Qiagen Inc., Mississauga, ON). Chromosomal DNA isolation, endonuclease restriction, ligation, transformation and agarose gel electrophoresis were performed using standard techniques (Sambrook et al., 1989). Restriction enzymes, T4 DNA ligase and shrimp alkaline phosphatase were obtained from Invitrogen (Mississauga, ON) and used according to the manufacturer's instructions.

Polymerase chain reaction and DNA sequencing

PCR was performed with either *Taq* DNA polymerase (Invitrogen, Mississauga, ON) or *Expand* DNA polymerase (Roche Diagnostics, Laval, QC) using *E. coli* MC4100 chromosomal DNA as a template. The 5' primer sequence used was 5'-CTTGCATTTTGA AATTCGTTACA-3' while the 3' primer was 5'-TTAACGACCATTCTCGGTTTTAC-3'. Annealing temperatures were 5°C below the lowest T_m of each primer pair. DNA primers were synthesized by the MOBIX lab, McMaster University. PCR products were purified using QIAquick PCR purification kit (Qiagen Inc., Mississauga, ON) prior to further manipulation. All DNA used in cloning reactions and probe preparation were extracted from agarose gels using the QIAEX II DNA extraction kit (Qiagen, Inc., Mississauga, ON). DNA sequences were analyzed using the program Gene Runner 3.04 (Hastings Software, Inc., Moraga, CA).

Construction of *rpoS*-containing plasmids for *in vivo* experiments

Chromosomal DNA was isolated from *E. coli* MC4100 as outlined (Sambrook *et al.*, 1989). After PCR amplification, the isolated 1278 bp PCR product was purified using QIAquick PCR purification kit (Qiagen Inc., Mississauga, ON). The purified PCR fragments containing 3'-A overhangs was ligated with compatible overhangs in plasmid pGEM®-T Easy vector (Promega Corporation, Madison, WI) overnight and the ligation mixture was transformed into *E. coli* DH5 α . Transformants were selected for ampicillin resistance on LB agar containing X-gal (50 μ g/ml, final concentration). The white colonies containing insertion of PCR product were obtained and plasmids were isolated from several ampicillin resistant white colonies. The resulting plasmids were designated pGC2. The *rpoS* gene was cloned in the reverse orientation with respect to the *T7* promoter of pGC2. The 1298 bp *EcoRI* fragment of pGC2 was then subcloned into high-copy-number transcriptional plasmid pET21 to yield pRPOS (Figure 1). As indicated, the *rpoS* gene is in the same orientation as *T7lac* promoter. The nature of the plasmids was confirmed by enzyme digestion with *EcoRI*, *HincII* and *AccI* and DNA sequencing.

Northern blot analysis

To produce probes for Northern blots, a 1022 bp fragment of DNA was PCR-amplified from *E. coli* MC4100 chromosomal DNA using *Taq* DNA polymerase. The 5' primer sequence used was 5'- CTTGCATTTTGAAATTCGTTACA -3' while the 3' primer was 5'- GTGAGGCCAATTTACGACCTA -3'. PCR product was then purified using QIAquick PCR purification kit (Qiagen Inc. Mississauga, Ontario). The PCR product was labeled using the random primer labeling method with [α -³²P]-dCTP

to generate high specific activity probes ($\sim 10^9$ cpm/ μ g DNA). The probe was purified using ProbeQuant G-50 micro columns (Amersham Biosciences, Inc., Piscataway, NJ).

Cultures were harvested and RNA extracted using an RNeasy Mini Kit (Qiagen Inc. Mississauga, ON). Total RNA concentration was determined by measuring absorbance at 260 nm (Sambrook *et al.*, 1989). RNA was loaded onto an RNA formaldehyde agarose gel and electrophoresed at 50 V for 2 h. Expression of *rpoS* mRNA was determined by Northern blotting. The RNA gel was blotted to Hybond-N⁺ membrane (Amersham Pharmacia Biotech Inc. Piscataway, NJ) using the capillary action method (Sambrook *et al.*, 1989) and fixed by baking at 80°C for 2 h. Prehybridization and hybridization were performed at 60°C with gentle agitation. The membranes were hybridized with ³²P-labeled *rpoS* DNA probes. The resulting blot was exposed to X-ray film (Eastman Kodak Company, Rochester, N.Y.) or a Storage Phosphor Screen (Molecular Dynamics Inc., Sunnyvale, CA) to visualize hybridization. Bands were quantified by image densitometry using ImageQuant 5.2 software (Molecular Dynamics Inc., Sunnyvale, CA).

Preparation of cell extracts

Single colonies were inoculated into 5 ml LB with the appropriate antibiotics in the tubes. The tubes were incubated overnight at 37°C at 200 rpm. The overnight cultures were diluted 1/1,000 into LB broth. After growth to an OD₆₀₀ of 0.25, the cultures were diluted 1/1,000 again and monitored to OD₆₀₀ = 0.01. The cultures were then divided into two flasks, to one flask was added 0.3 mM IPTG, the other flask was used as a control. After growth to an OD₆₀₀ of 0.25, 70 ml cells of exponential phase cells from each flask were collected. After a further induction to an OD₆₀₀ of 1.5, 25 ml

cells of stationary phase cells from each flask were collected. Samples of cultures were washed in 0.05 M phosphate buffer, pH7.0 and sonicated for 20 min of 30 s run and 20 s pause using a Heat Systems sonicator (Misonix Inc., Farmingdale, New York) equipped with a cup horn. Cell debris was removed by centrifugation at 4°C for 15 min at 12,000xg. Total protein concentration was determined using a kit (Bio-Rad Laboratories, Hercules, CA) (Bradford, 1976).

SDS-PAGE and Western blotting analysis

Protein (30 µg) samples were separated on 10% SDS polyacrylamide gels overnight using Vertical Slab Gel Unit, Model SE400 (Hoefer Scientific Instruments, San Francisco, CA) and then transferred to a Hybond-P membrane (Amersham Pharmacia Biotech Inc. Piscataway, NJ). The membranes were stained with Ponceau S to confirm efficient transfer of protein (Amersham Pharmacia Biotech Inc. Piscataway, NJ). Following transfer, the blots were placed into blocking buffer (0.5% albumin bovine Fraction V, 5% skim milk in TBS-T) overnight. The blots were then incubated with blocking buffer containing primary antibody (anti- σ^S antibody, a gift from R. Hengge-Aronis, University of Konstanz) for 2 h at room temperature. Following washing three times with TBS-T (0.1% Tween 20 in TBS, pH 7.6) or blocking buffer, the blots were placed into blocking buffer containing secondary anti-rabbit antibody (Rabbit Immunoglobulin, horseradish peroxidase-linked whole antibody from donkey, 1:10,000 dilution), and shaken for 1 h. The blots were again washed three times and then incubated in 10 ml of the ECL staining solution (1:1 detection reagents mixture, Amersham Pharmacia Biotech Inc. Piscataway, NJ) and exposed to X-ray film (Kodak

X-OMAT AR or BioMax MR film, Eastman Kodak Company, Rochester, New York) for 10 s to 10 min.

Catalase activity assays

Catalase activity in cell extracts was assayed spectrophotometrically as described previously (Schellhorn and Stones, 1992). One ml of hydrogen peroxide (0.5 ml of 30% H₂O₂ freshly diluted in 250 ml 50 mM potassium phosphate buffer, pH7.0) was added, and the decrease in OD₂₄₀ was monitored. The specific activity of catalase was calculated as $(1,000 \times \text{OD}_{240} / \text{time of incubation}) / (43.6 \times \text{mg of protein/ml of reaction mixture})$ (Beers and Sizer, 1952).

Samples were loaded onto 10% non-denaturing polyacrylamide gel for electrophoresis using a Bio-Rad Mini-PROTEAN II system (Bio-Rad Laboratories, Hercules, CA). To detect catalase activity, non-denaturing gels were stained using horseradish peroxidase and diaminobenzidine (Clare *et al.*, 1984). To confirm equal protein loading, parallel gels were stained with Coomassie Blue (Sambrook *et al.*, 1989). Catalase activity on agar plates was qualitatively determined by adding a drop of 30% hydrogen peroxide to a colony. All assays were performed in duplicate.

Glycogen staining

Glycogen accumulation was tested on Kornberg medium agar plates in the presence or absence of 0.3 mM IPTG. The plates were inverted on a 500 ml beaker containing 3.3% I₂, 6.6% KI solution. The colonies were stained with the iodine vapor by heating the solution for 3-5 min. Colonies in which glycogen had accumulated were dark brown, those with little or no glycogen production stained yellow. Staining was reversible within a few min and the plates were thus photographed immediately.

Results

Construction of T7 RNA polymerase-expressing lysogens

To make an expression host for the pET expression system, λ DE3 fragment carrying the T7 RNA polymerase gene was recombined into the chromosome of HS1600 (Novagen Inc., Madison, WI). About 700 potential lysogens carrying T7 RNA polymerase gene were obtained from LB agar plates. Four colonies were tested for λ DE3 lysogenization using a tester phage (T74107) that makes very large plaques on strains carrying T7 RNA polymerase gene in the presence of IPTG, while much smaller plaques are observed in the absence of IPTG. One lysogen strain was selected and designated HS1600DE3. This strain carries the T7 RNA polymerase gene under the control of *lacUV5* promoter (Dubendorff and Studier, 1991; Studier *et al.*, 1990).

Construction of *rpoS* mRNA expression system in a transcriptional plasmid pET21

To test if increased expression of *rpoS* mRNA can induce the expression of *rpoS* at an early stage of cell growth when RpoS levels are normally extremely low (i.e., in early exponential phase) and activate the expression of an RpoS-dependent gene, the *rpoS* gene was cloned into a pET expression vector and used to determine if plasmid-encoded *rpoS* can complement the deficiency in an RpoS null mutant strain in exponential phase and stationary phase. The resulting plasmid was transformed into *E. coli* strains for the *in vivo* expression of mRNA and RpoS protein. Concentrations higher than 0.3 mM IPTG inhibited cell growth (data not shown) due to protein overexpression. As a result, subsequent experiments were performed using 0.3 mM IPTG, a concentration that was found to have little effect on growth.

Expression of inducible *rpoS* in early exponential phase cultures

To evaluate the levels of mRNA expressed from pRPOS, Northern blot analysis was performed and the results are shown in Figure 2. The levels of *rpoS* mRNA expressed from cultures in early exponential phase and stationary phase were high (Figure 2, A and B). In exponential phase (Figure 2, A), *rpoS* mRNA was highly expressed in the *rpoS* sense-expressing strain HS1600DE3pRPOS in the presence of 0.3 mM IPTG (lanes 10). Expression could not be detected in cultures grown in the absence of IPTG (lane 9) indicating that the expression of *rpoS* was tightly controlled. mRNA from *rpoS* strain HS1600DE3 and its transformants containing the control plasmid pET21 was undetectable in either the absence or presence of IPTG (lanes 7-8). In stationary phase (Figure 2, B), *rpoS* mRNA was highly expressed in the presence of IPTG (lanes 10). As shown in Figure 3, normalized to the induced stationary phase level (HS1600DE3pRPOS in the presence of IPTG), *rpoS* mRNA levels were much higher when induced by IPTG (over 40 fold in both exponential (Figure 3, A) and stationary phase (Figure 3, B) as measured by image densitometry). The results demonstrate that *rpoS* mRNA levels in HS1600DE3pRPOS could be effectively controlled by addition of an inducer.

Western blot analysis of plasmid-encoded RpoS protein

To compare RpoS levels in samples taken from overexpressing strain HS1600DE3pRPOS and *rpoS* mutant strain HS1600DE3 in the presence and absence of IPTG, Western blot analysis was performed using polyclonal anti- σ^S antibody. In exponential and stationary phases, RpoS protein was expressed in the *rpoS* strain HS1600DE3 carrying *rpoS*-expressing plasmid when induced by IPTG (Figure 4, lanes

8, and 16) but was not expressed in the absence of IPTG (lanes 7 and 15). As expected, RpoS protein levels were higher in stationary phase (lane 16) than in exponential phase (lane 8). In the *rpoS* wild type strain, RpoS protein was present in stationary phase (lanes 9 and 10) and was absent in exponential phase (lanes 1 and 2).

Increase in RpoS-dependent catalase expression by induction of RpoS in exponential and stationary phases

Catalase activity in *E. coli* is controlled by RpoS (Sak *et al.*, 1989; Schellhorn and Hassan, 1988) and thus catalase can be used to monitor the RpoS status of the cell. Qualitative LB agar plate assays (determined by adding 30% hydrogen peroxide) indicated that catalase activity was much higher in strains transformed with pRPOS in media supplemented with increasing concentrations of IPTG (data not shown). These results are consistent with RpoS expression due to induction of *rpoS* mRNA. Catalase enzyme activities were assayed to quantify the effect of controlled RpoS expression. As shown in Figure 5, A, catalase activity was induced in *rpoS*⁺ expression strain HS1600DE3pRPOS in the presence of 0.3 mM IPTG but not in the absence of IPTG in exponential phase. In the presence of IPTG, increased *rpoS* mRNA levels resulted in a 1.5-fold catalase induction in exponential phase (Figure 5, A) and 4-fold catalase induction in stationary phase (Figure 5, B). These results indicate that increased production of RpoS is sufficient to increase RpoS-dependent gene expression.

Induction of RpoS-dependent HP11 catalase in exponential phase and stationary phase

Since induction of RpoS resulted in a large increase in catalase activity, we wanted to determine which of the two catalases was primarily affected. To test if

induction of *rpoS* mRNA could activate RpoS-dependent catalase HPII in RpoS null mutant strain HS1600DE3 at the protein level, cell extracts were prepared from cultures treated with IPTG. Proteins were separated by non-denaturing PAGE and were stained for HPII and HPI catalases as described in Materials and methods. In the untreated wild type control strain MC4100DE3, HPII levels were low in exponential phase and were induced in stationary phase. As expected, the strain carrying the *rpoS*::Tn10 insertion (HS1600DE3) produced negligible amounts of HPII catalase even in stationary phase. In strain HS1600DE3 transformed with pRPOS, induction of *rpoS* clearly activated RpoS-dependent HPII in both exponential phase (Figure 6 A, lane 8) and stationary phase (Figure 6 B, lane 8). The induced levels of HPII in the *rpoS* deletion strain were much higher than in the control MC4100DE3 strain (compare lane 8 to lane 2 in Figure 6 (A) and Figure 6 (B)). The levels of HPI appeared to be affected by the amount of HPII produced: high levels of HPII are correlated with low levels of HPI. The latter catalase, encoded by *katG*, is primarily controlled by the hydrogen peroxide-activated OxyR regulator (Aslund *et al.*, 1999). In light of this, one possible explanation for this observation is that HPII reduces hydrogen peroxide levels resulting in less OxyR-mediated HPI.

Effect of increased *rpoS* expression on glycogen accumulation

rpoS mutants in *E. coli* do not accumulate glycogen (glycogen negative phenotype) due to the reduced expression of GlgS, a hydrophilic small protein (Lange and Hengge-Aronis, 1991b). Overexpression of *glgS* stimulates glycogen synthesis in early stationary phase (Hengge-Aronis and Fischer, 1992), thus overexpression of *rpoS* in *rpoS* mutant strains could restore a glycogen positive phenotype in these strains.

Glycogen accumulation in *rpoS*⁺ wild type colonies can be visualized by a dark brown stain in the presence of iodine when *rpoS*⁻ colonies are yellow in colour (Figure 7, A). Overexpression of RpoS substantially increased glycogen synthesis resulting in dark brown colonies following iodine staining (glycogen-positive phenotype) (Figure 7, B).

Discussion

The regulation of gene expression by vegetative sigma factors in *Escherichia coli* is correlated with growth phase. More than 200 RpoS-dependent genes are predicted to be transcribed in stationary phase (Schellhorn *et al.*, 1998), while about 1,000 genes are expressed in exponential phase in *E. coli* (Jishage and Ishihama, 1995). In exponential phase, the RpoS protein is highly unstable with a half-life of 1.4 min (Lange and Hengge-Aronis, 1994). The ClpXP protease, a key element controlling RpoS turnover (Schweder *et al.*, 1996), is controlled, primarily during exponential growth, by the RssB response regulator, (Zhou and Gottesman, 1998). As a result, undetectable amounts of RpoS are present during exponential phase (Jishage and Ishihama, 1995; Jishage *et al.*, 1996) and σ^{70} is responsible for transcription regulation during exponential growth. Although some genes are clearly dependent on RpoS in this phase of growth (Schellhorn *et al.*, 1998), the role of RpoS in exponential phase expression has received little attention.

In this work, we examined expression of an RpoS regulon member during exponential phase in *E. coli* by artificially overexpressing RpoS. We placed the *rpoS* gene under the control of a *T7lac* promoter and, by controlled expression of the T7 polymerase (induced by the addition of inducer IPTG), indirectly stimulated the expression of RpoS, a regulator that is normally not expressed during exponential phase. Both *rpoS* messenger RNA and RpoS protein were significantly upregulated. As a consequence, at least one RpoS-regulated gene was efficiently induced during exponential phase. We also found the levels of RpoS were higher in stationary phase following exponential phase induction, indicating that such expression can be persistent

and can be effectively used to amplify expression of the RpoS regulon in non-growing cells.

One potential issue associated with overexpression of RpoS is that such manipulation may alter sigma factor and core polymerase stoichiometry in the cell. Thus increases in RpoS-dependent catalase HPII expression maybe due to sequestration of core polymerase by the overproduced sigma factor. Competition between σ^{70} and RpoS for core RNA polymerase (Farewell *et al.*, 1998) can have a marked effect on gene expression when the concentration of RNA polymerase core enzyme is relatively high (Bedwell and Nomura, 1986). Similarly, this competition between sigma factors in exponential phase cells, has been observed in *Bacillus subtilis* during sporulation: overexpression of σ^A in exponential phase in *B. subtilis* inhibits σ^H -dependent gene expression (Hicks and Grossman, 1996). In *E. coli*, a 2.5-fold overexpression of σ^{70} in stationary phase results in a dramatic decrease in the expression of *rpoS* and RpoS-dependent genes (*uspB*, *bolA* and *fadL*) due to a competition for a limited amount of RNA polymerase core enzyme (Farewell *et al.*, 1998). Introduction of a high-copy-number plasmid pMMkatF2, carrying the *rpoS* gene, increases RpoS levels by 10-fold, and as a consequence, can reduce expression of a σ^{70} -dependent gene (*uspA*), presumably by sigma factor competition for core polymerase (Farewell *et al.*, 1998). Overexpression of RpoS in exponential phase, by analogy, may have a similar effect as overexpression of σ^{70} in stationary phase.

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Table 1. Bacterial strains used and constructed in this study

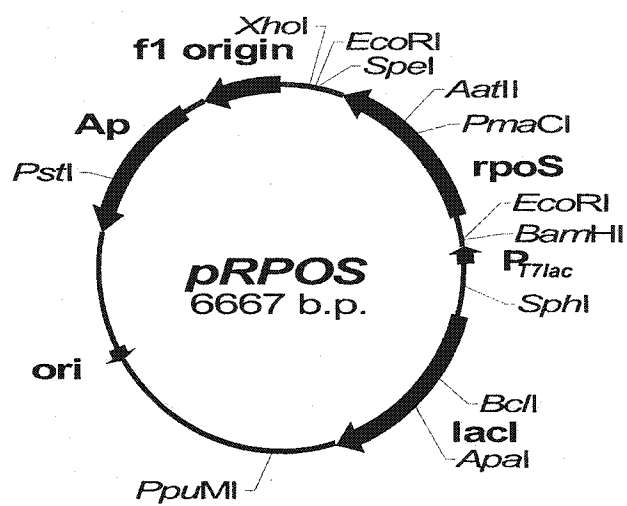
<i>E. coli</i> strain	Genotype	Source or reference
DH5 α	<i>supE44 ΔlacU169(ϕ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Stratagene
MC4100	<i>Δ(argF-lac)205 araD139 flbB5301 relA1 rpsL150 thi ptsF2</i>	(Schellhorn and Stones, 1992)
MC4100DE3	As MC4100, but expresses T7 RNA polymerase	This study
HS1600	As MC4100, but <i>rpoS13::Tn10</i>	(Mukhopadhyay <i>et al.</i> , 2000)
HS1600DE3	As HS1600, but expresses T7 RNA polymerase	This study
HS1600DE3Y	As HS1600DE3, but <i>osmY-lacZ</i>	This study
BL21DE3	F ⁻ <i>ompT hsdS_B(r_B⁻m_B⁻) gal dcm</i> (DE3)	Novagen
HS1600DE3pRPOS	As HS1600DE3, but pRPOS	This study

Table 2. Plasmids used and constructed in this study

<i>E. coli</i> plasmid	Size (bp)	Description and resistance(s)	Source
pGEM-T Easy	3001	Amp ^R , blue/white colour selection single 3'-T overhangs to allow efficient ligation with PCR products	Promega
pET21	5369	Amp ^R , transcription vector lacks the RBS and start codon	Novagen
pGC2	4298	1278 bp fragment as for pGC1 but cloned in the opposite orientation in pGEM-T Easy	This study
pRPOS	6667	1298 <i>EcoRI</i> fragment from pGC1 cloned into pET21, <i>T7lac</i> promoter in same orientation with <i>rpoS</i>	This study

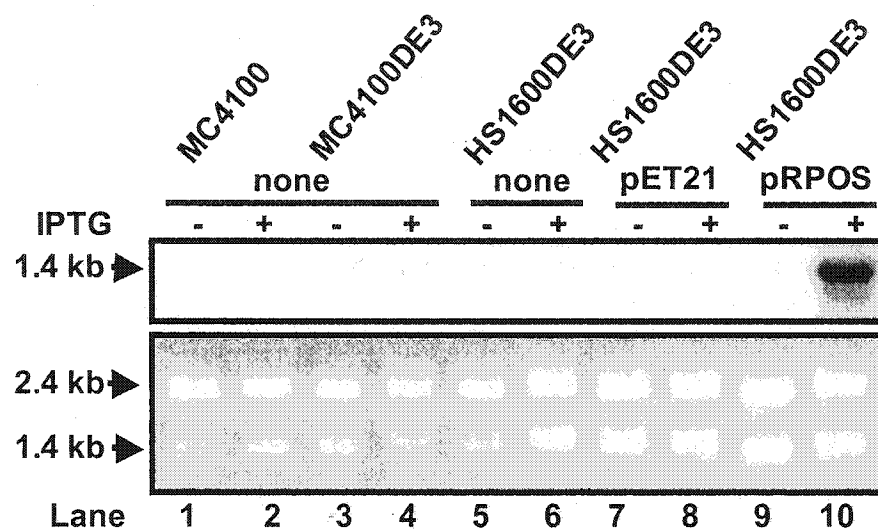
Figure 1. Structure of *rpoS*-encoding plasmid pRPOS. The unique restriction endonuclease recognition sites are indicated. Abbreviation: *P_{T7lac}*, *T7lac* promoter, *ori*, plasmid origin of DNA replication, *rpoS*, *rpoS* gene fragment, *Ap*, ampicillin resistance gene.

Chen and Schellhorn, Figure 1.



Figures 2. Northern analysis of *rpoS* mRNA induced by IPTG. Total RNA was isolated from *rpoS* wild type strain MC4100DE3 and *rpoS* null strain HS1600DE3 and its transformants containing control plasmid pET21 and sense *rpoS* plasmid pRPOS. RNA was isolated from exponential-phase (A) and stationary-phase (B) cultures grown in the absence (lanes 1, 3, 5, 7, 9) and the presence of 0.3 mM IPTG (lanes 2, 4, 6, 8, 10). The lower panels of each figure are loading controls showing 16S and 23S RNA. The *rpoS* mRNA was detected by hybridization with an *rpoS*-specific α -³²P-dCTP labeled double stranded DNA probe (see Materials and methods).

(A) Exponential phase



(B) Stationary phase

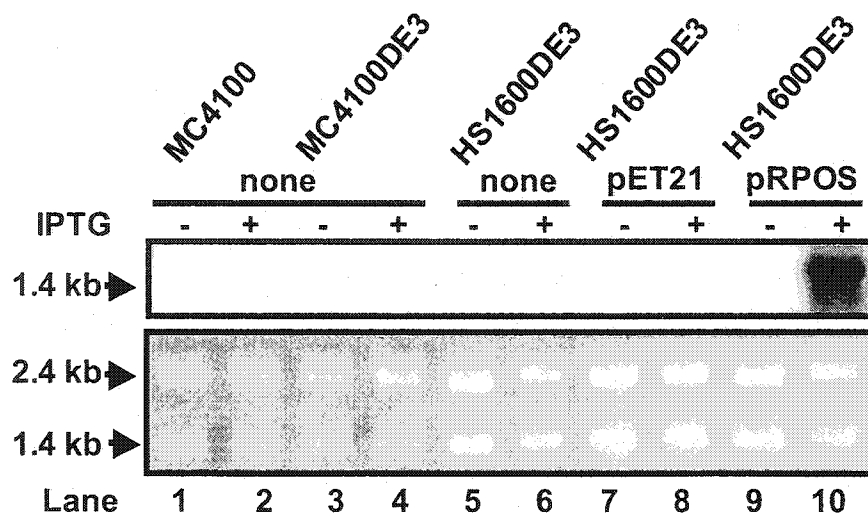
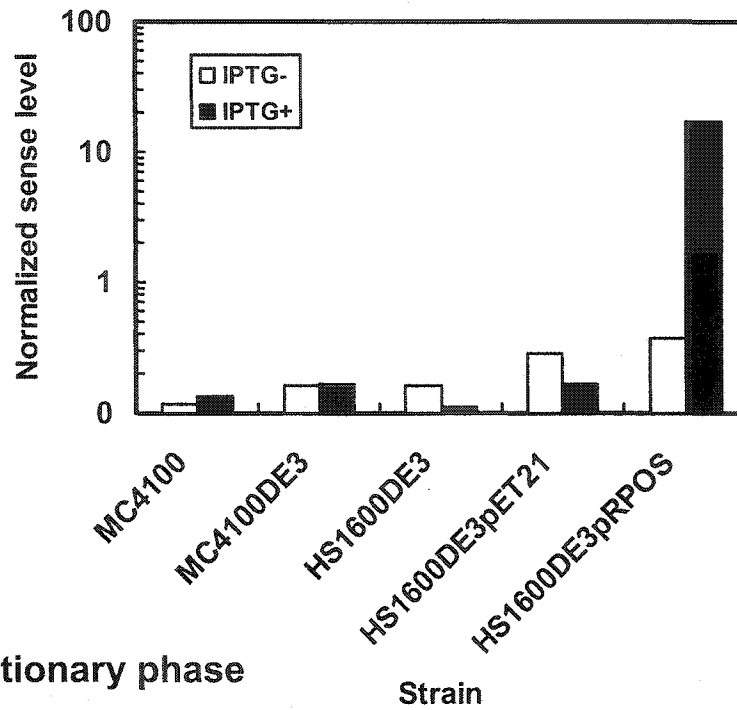


Figure 3. *rpoS* mRNA levels quantified from Northern blot (see Figure 2). All values of *rpoS* mRNA levels during exponential (A) and stationary phase (B) growth were normalized to the value of stationary phase level expressed in HS1600DE3pRPOS in the presence of IPTG.

A. Exponential phase

Chen and Schellhorn, Figure 3.



B. Stationary phase

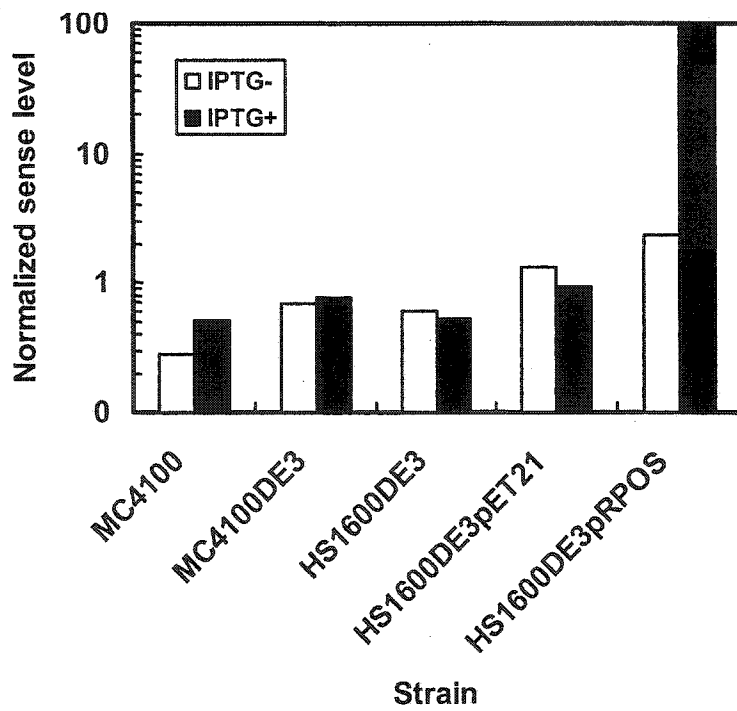


Figure 4. Western blot analysis of RpoS levels using anti- σ^S -antibody. Protein (30 μ g) from cultures of *rpoS* wild type MC4100DE3, null mutation HS1600DE3, HS1600DE3pET21 and HS1600DE3pRpoS strains were sampled during exponential ($OD_{600} = 0.25$) (A) and stationary phase ($OD_{600} = 1.5$) (B) growth. Following transfer, the PVDF membrane was stained with ECL staining solution and exposed to Kodak X-OMAT film.

Chen and Schellhorn, Figure 4.

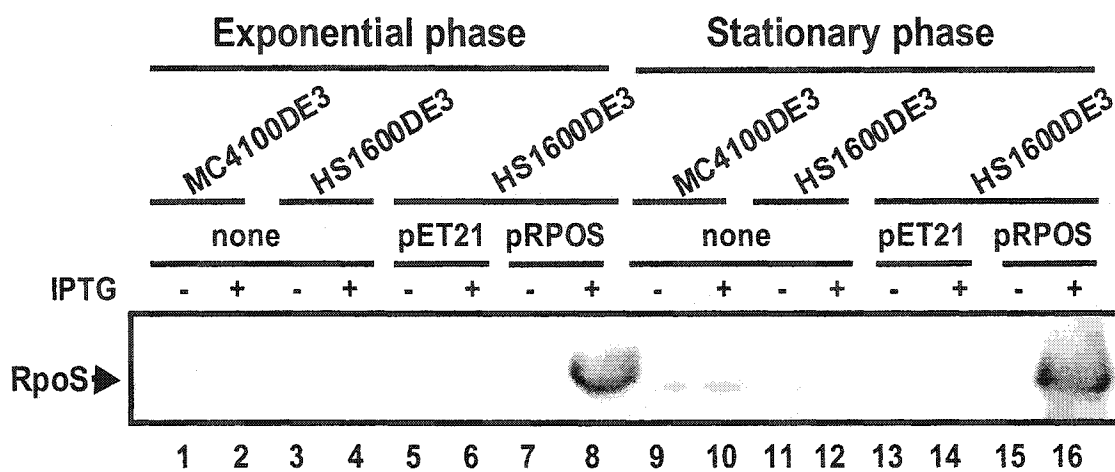


Figure 5. Induced *rpoS* mRNA activates RpoS-dependent catalase expression.

Specific activity of catalase was determined in *rpoS* strain HS1600DE3 containing a control plasmid pET21 and sense construct pRPOS grown with and without 0.3 mM IPTG during exponential phase (A) and stationary phase (B) growth.

Chen and Schellhorn, Figure 5.

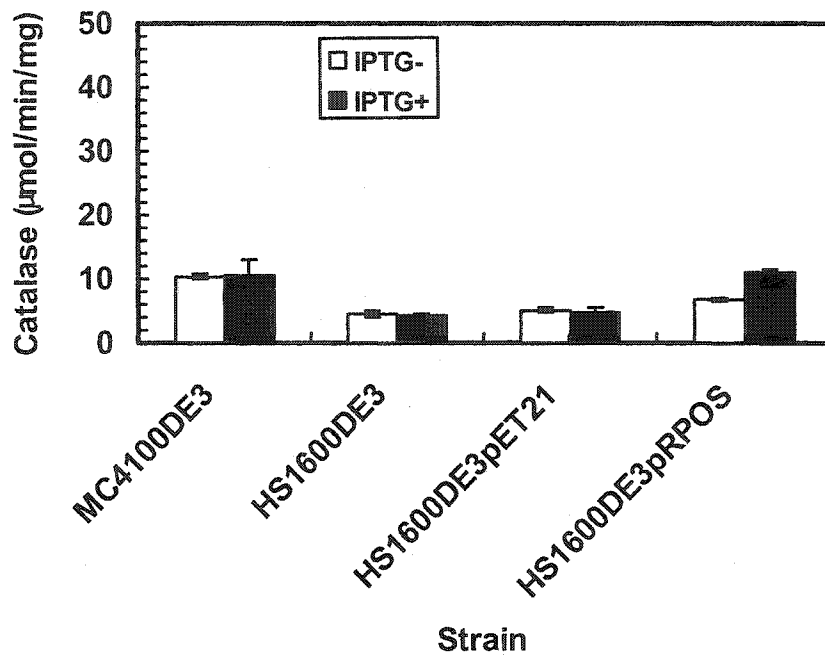
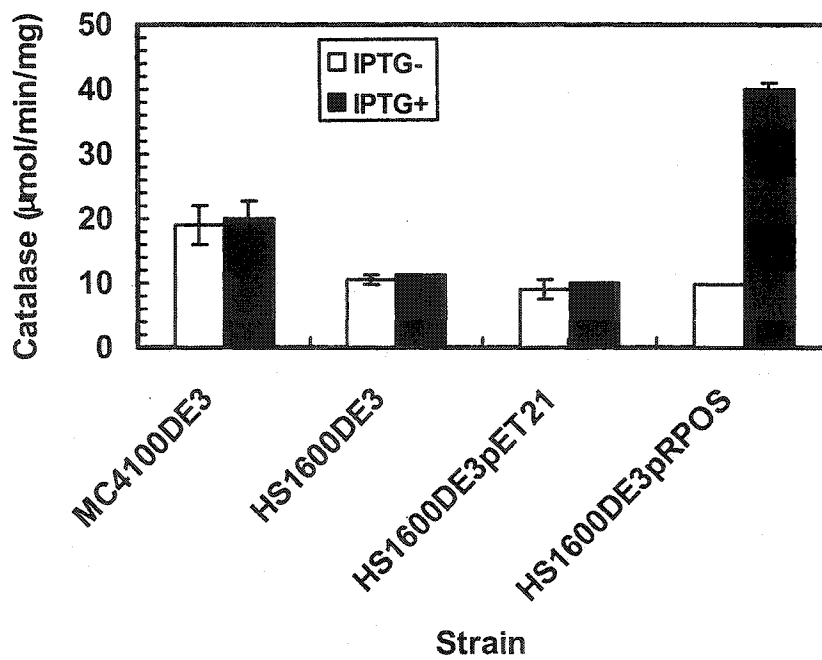
A. Exponential phase**B. Stationary phase**

Figure 6. Induction of *rpoS* activates expression of RpoS-dependent catalase HPII.

Catalase activity in native polyacrylamide gel loaded with protein (10 μ g) was examined in *rpoS* strain HS1600DE3 pET21 and HS1600DE3pRPOS sampled during exponential and stationary phase growth.

Chen and Schellhorn, Figure 6.

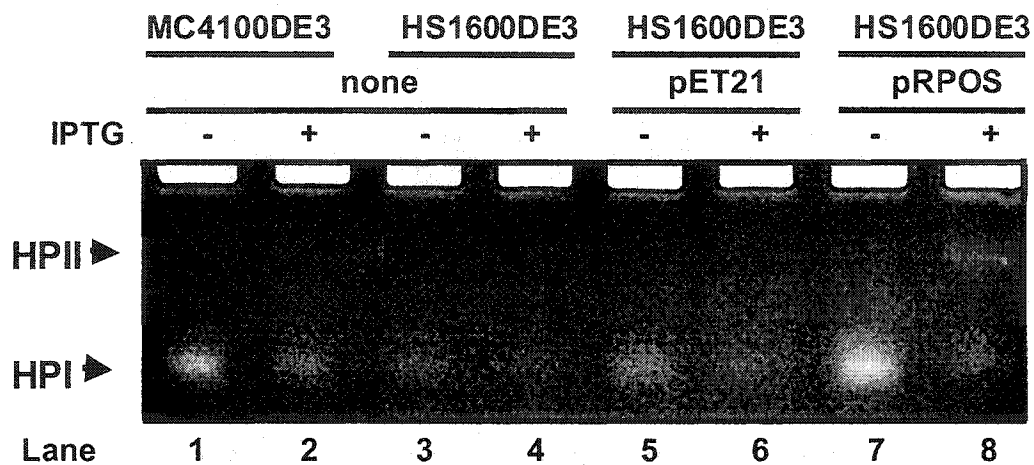
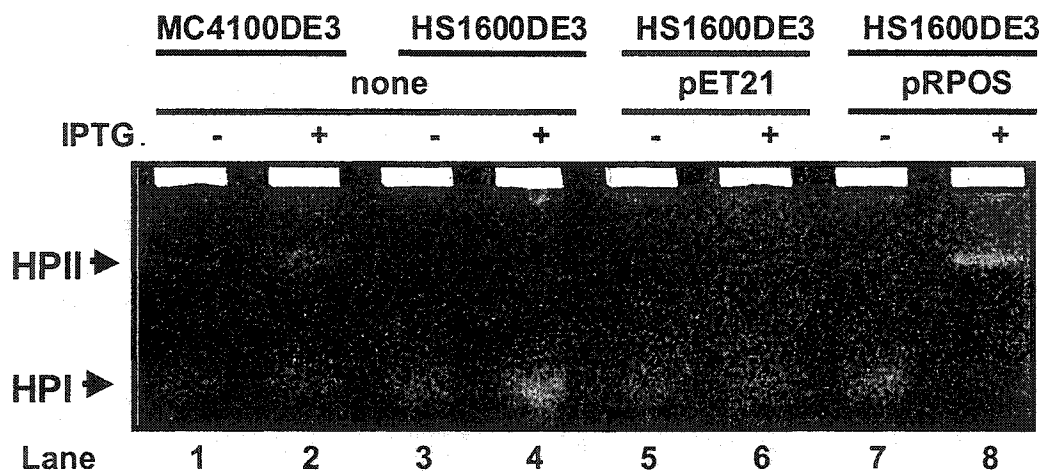
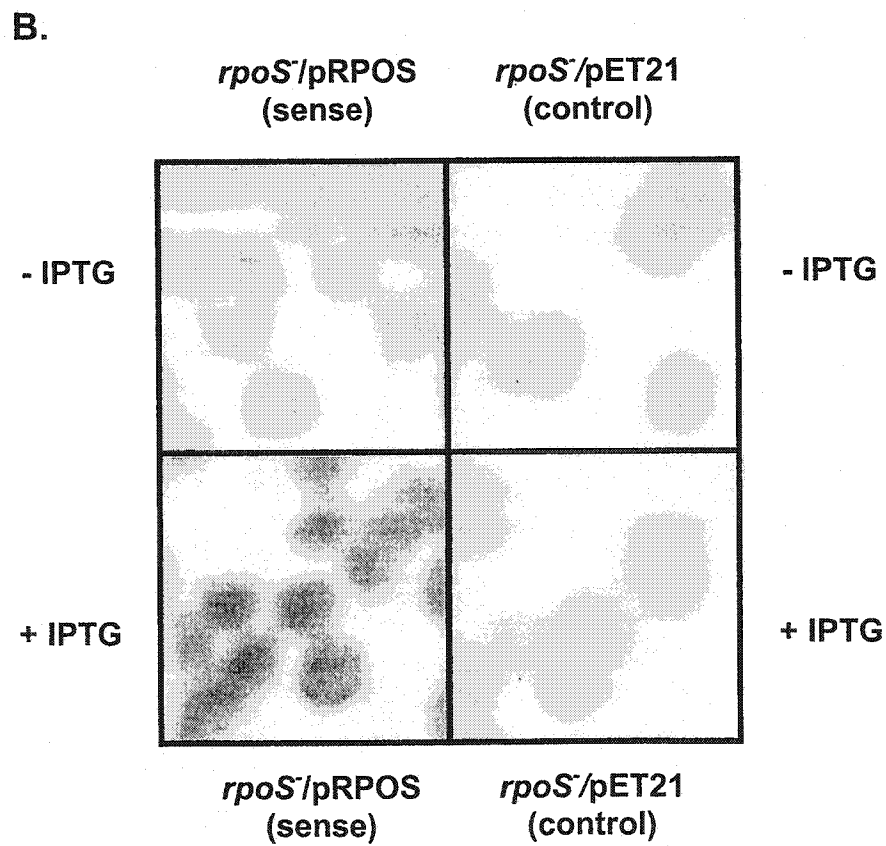
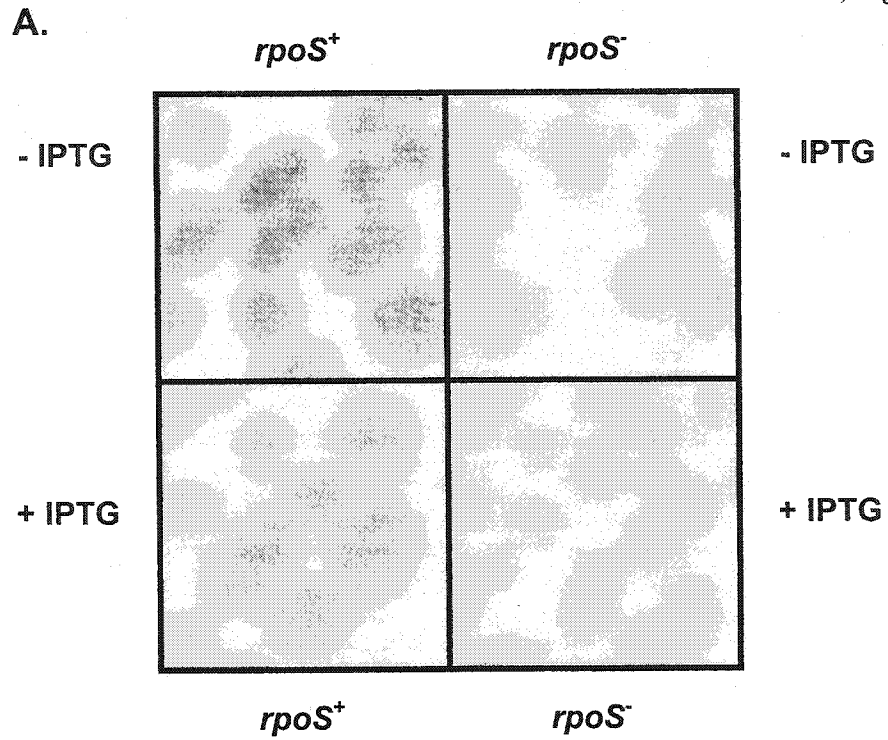
A. Exponential phase**B. Stationary phase**

Figure 7. Glycogen accumulation in RpoS-overexpressing strain

HS1600DE3pRPOS and wild type strain MC4100DE3. The colonies were grown for 2 days at 37°C on Kornberg medium plates and intracellular glycogen was stained with iodine vapor (see Materials and methods).



CHAPTER 6:

SUMMARY AND THESIS REFERENCES

Summary

RpoS and members of the RpoS regulon are induced in response to a variety of stresses during stationary phase (Hengge-Aronis, 1996b). Transition to stationary phase causes a programmed change in gene expression and protein synthesis (Hengge-Aronis, 1996b). Spontaneous *rpoS* mutants arise commonly in different laboratory strains (Visick and Clarke, 1997; Waterman and Small, 1996). In this study, 20 Suc⁺ mutants were isolated and they were found to carry a mutated allele of the *rpoS* gene. This result suggests that *rpoS* mutations can be selected on minimal media containing succinate as the sole source of carbon (see Chapter 3), and helps to explain why *E. coli* strains have acquired mutations in RpoS in certain laboratory conditions. Expression of the *rpoS* regulon can be switched on in *rpoS* mutant transversion revertants under sub-optimal growth conditions.

The attenuation of RpoS has significantly stimulated interest because many RpoS-dependent genes are important for adaptation to the adverse environments in bacteria (Kowarz *et al.*, 1994; Coynault *et al.*, 1992; Coynault *et al.*, 1996). Inducible *rpoS* antisense RNA can inhibit the expression of RpoS in both exponential and stationary phases, and can attenuate expression of the *rpoS* regulon in *E. coli* (see Chapter 4). Catalase and glycogen accumulation and Western blotting analysis revealed that *rpoS* expression was reduced by the expression of the *rpoS* antisense and could be controlled by IPTG during both exponential and stationary phases. The high expression levels of plasmid-encoded antisense *rpoS* reduced the growth rate of cell cultures. To circumvent this problem, the antisense sequence can be inserted into the *E. coli*

chromosome using a modification of a single gene disruption method (Datsenko and Wanner, 2000).

The overexpression of RpoS may offer a useful tool for the study of RpoS-dependent functions (Schellhorn *et al.*, 1998) and a greater understanding of the bacterial environmental adaptation responses. Overexpressed RpoS protein in early exponential phase can activate the expression of the RpoS regulon in exponential phase and stationary phase in *E. coli* (see Chapter 5). Western blotting analysis showed that RpoS levels increased after IPTG-induced expression of *rpoS* from pRPOS in early exponential phase and stationary phase in *E. coli*. RpoS-dependent catalase HPII was positively regulated as determined by catalase activity assays and in native polyacrylamide gels stained for catalase activity. Glycogen accumulation was also increased when RpoS protein levels are high. Induction of plasmid-encoded *rpoS* expression can be used to identify RpoS-dependent function in exponential phase using DNA microarrays.

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