Identification and genetic mapping of *perR*, a novel stationary phase gene that mediates oxidative stress protection in *Escherichia coli*

by

Jeanna Brandy Lee Strutinsky

A thesis
submitted to the Faculty of Graduate Studies
in partial fulfillment of the requirements for the degree of
Master of Science

Department of Microbiology
University of Manitoba
Winnipeg, Manitoba
© September, 2000

National Library of Canada
Acquisitions and Bibliographic Services
395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada
Acquisitions et services bibliographiques
395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-53229-1

Canada

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES
*****
COPYRIGHT PERMISSION PAGE

Identification and genetic mapping of *perR*, a novel stationary phase gene that mediates oxidative stress protection in *Escherichia coli*

BY

Jeanna Brandy Lee Strutinsky

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of Master of Science

JEANNA BRANDY LEE STRUTINSKY © 2000

Permission has been granted to the Library of The University of Manitoba to lend or sell copies of this thesis/practicum, to the National Library of Canada to microfilm this thesis/practicum and to lend or sell copies of the film, and to Dissertations Abstracts International to publish an abstract of this thesis/practicum.

The author reserves other publication rights, and neither this thesis/practicum nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

1.0 ABSTRACT

In bacteria, stationary phase is a time of intricate cellular metabolic and physiological changes necessary for the maintanence of essential cell functions and resistance to environmental stresses, such as oxidative stress. This study investigated *perR*; a locus potentially involved in the hydrogen peroxide resistance of *Escherichia coli* in stationary phase. The strain UM397 containing a mutation in *perR* was created as part of this study and showed 13.6 fold greater sensitivity to H_2O_2 in stationary phase when compared to its parent strain MG1655. This suggested that PerR is involved in oxidative stress resistance in stationary phase. Further, the predicted sequence of PerR is similar to LysR-type transcriptional regulators, suggesting a regulatory role for the protein.

ACKNOWLEDGEMENTS

To begin, I would like to thank my advisor, Dr. Peter Loewen, for the opportunities provided to me and valuable lessons learned while in his laboratory. As well, I would like to thank my committee members, Drs. Mike Mulvey and Ross McGowan for their timely availability and thoughtful inspection of my thesis.

I would also like to acknowledge the members of the Loewen Lab, both past and present for their support and camaraderie. Specifically, I would like to thank Jacek Switala, for his patience in teaching me numerous techniques and assistance in the final phases of the project. I also extend a great deal of gratitude to my lab mates: my 'big brother' Dr. Alex Hillar, for his guidance and encouragement in scientific and all other matters; Bei Hu, for her bright smile and otherwise cheery disposition and George 'Pure Evil' Golding for his eternal optimism and undisputable efficiency in the co-looting of a Christmas tree.

My heartfelt appreciation is also extended to the Department of Microbiology, faculty, staff and students alike. Specifically, I acknowledge Dr. Linda Cameron, Dr. Glen Klassen and Michelle Wall for their friendship and 'non-science' chats. To the other members of the self-titled fabulous four -- Tamiko Hisanaga, Paul 'Mr. Greedy' Humenchuk and Fiona Punter, I will always cherish the memories we have created, both one-on-one and as a group. To my fellow graduate students -- Dr. Lateef Adewoye, Dr. Doug Barker, James Bedard, Dwayne Elias, Ayush Kumara, Tricia Magsino,

Andrew Schurko and Dr. Denny Wong -- it was truly a pleasure sharing this experience with you. To my close friend and confidante, Lorelee Tschetter -- Lori, here's to early morning phone calls, ill-fated Christmas baking and laughing fits in never-ending UMSU meetings!

I would like to thank my current employer for Dr. James Johnston for his endless understanding as it pertains to this and other endeavors. As well, I would like to thank Dr. Priti Baijal, Elizabeth Henson, James Paul, and Christine Vande Velde for helping me count the 'number of sleeps'.

To my parents, I am eternally grateful for your unconditional love, understanding, and support. The person that I am is largely a reflection of your mentorship. To my brother Vern, thanks for helping me to laugh when I don't think that I can (Notice that you got your own sentence?). And finally to my touchstone, Ian McIntyre, whose own unfaltering motivation and love of learning continues to be my constant source of inspiration. This document is largely the result of your unwavering faith in my abilities and me.

TABLE OF CONTENTS

1.0 Abstract i

Acknowledgements ii

Table of Contents iv

List of Figures vi

List of Tables vii

List of Abbreviations viii

2.0 Literature Review 1

2.1 Oxygen 2
2.1.1 Introduction 2
2.1.2 Oxygen Toxicity 2
2.1.3 Effects of Reactive Oxygen Species 5
2.2 Cellular Defenses 7
2.2.1 Predamage Protection 7
2.2.2 Postdamage Repair 14
2.2.3 Oxidative Stress Regulons 16
2.3 Stationary Phase 18
2.3.1 Introduction 18
2.3.2 Regulation of σ^s-Dependent Genes in Stationary Phase 19
2.3.3 Regulation of σ^s in Stationary Phase 23
2.3.4 Oxidative Stress in Stationary Phase 30
2.4 Purpose 32

3.0 Materials and Methods 33

3.1 *E. coli* Strains, Plasmids and Bacteriophage 34
3.2 Media, Growth Conditions and Storage of Cultures 37
3.3 Hydrogen Peroxide Assay 38
3.4 Transposon Mutagenesis 39
3.4.1 Transposon Carrying Phage Lysate 39
3.4.2 Transposon Mutagenesis and Collection of Mutagenized Strain 39
3.5 Transductional Mapping 40
3.5.1 P1 Lysate Preparation 40
3.5.2 P1 Transduction 41
3.6 DNA Isolation 42
3.6.1 Plasmid DNA 42
3.6.2 Genomic DNA 42

Table of Contents (continued)

3.6.3 Single-stranded DNA 43
3.7 Agarose Gel Electrophoresis 44
3.8 Transformation 44
3.9 Restriction Endonuclease Digestion 45
3.10 Southern Hybridization 45
3.10.1 Southern Blotting 45
3.10.2 Hybridization 46
3.10.3 Colourmetric Detection 47
3.11 Extraction of DNA from Agarose Gel 47
3.12 Ligation Reactions 47
3.13 Primer Extraction and Phosphorylation 48
3.14 Site-Directed Mutagenesis 48
3.15 Homologous Recombination 50
3.16 Polymerase Chain Reaction 50
3.17 DNA Sequencing 51
3.18 Protein Sequence Analysis 52

4.0 Results 53

4.1 Genetic Mapping of Mini-Tn*10* insertions in UM367 and UM369 54
4.2 Attempted Transposon Mutagenesis of *perR* 56
4.3 Site-Directed Transposon Mutagenesis of *perR* 60
4.4 Homologous Recombination into *E. coli* Chromosome 64
4.5 Altered H_2O_2 Resistance of *perR*::Tn10 65
4.6 *perR* Sequence Analysis 68

5.0 Discussion 74

5.1 Summary 75
5.2 Future Perspectives 78

6.0 References 80

LIST OF FIGURES

Figure 2.1 The univalent pathway of oxygen reduction 3

Figure 2.2 Predamage protection enzymes found in *Escherichia coli* 10

Figure 2.3 The role of glutathione in decreasing oxidative stress.............. 13

Figure 4.1 Genetic map based on linkage analysis from the three-factor transductional crosses. 57

Figure 4.2 Diagrammatic representation of the sequential experiments performed in the attempted creation of a *perR* knockout via random transposition.. 59

Figure 4.3 Creation of pPerR::Tn*10*. 62

Figure 4.4 Confirmation of transposon insertion in *perR* via DNA sequencing.. 66

Figure 4.5 Comparison of hydrogen peroxide survival curves of stationary phase cells 67

Figure 4.6 The *perR* DNA sequence with predicted amino acid sequence 69

Figure 4.7 Sequence comparison of PerR with other LysR-type proteins. 71

LIST OF TABLES

Table 2.1 Modulators and their regulation of RpoS 26

Table 3.1 Bacterial strains, plasmids and bacteriophage used in this work 35

Table 3.2 Single-stranded oligonucleotides used in this study 49

Table 4.1 Mapping of the *perR* locus relative to adjacent genes by three factor transductional crosses 55

ABBREVIATIONS

°	degrees
Δ	deletion
%	percentage
2°	secondary
σ^{70}	sigma factor – housekeeping
σ^{s}	sigma factor – stationary phase
$^{1}\Delta g\ O_2$	singlet oxygen (paired, parallel electrons)
$^{1}\Sigma g\ O_2$	singlet oxygen (unpaired, antiparallel electrons)
A	adenosine
AP	alkaline phosphatase
AP	apurinic/apyrimidinic
Ap^{r}	ampicillin resistant
ADP	adenosine diphosphate
ATP	adenosine triphosphate
C	Celcius/cytosine
cAMP	cyclic adenosine monophosphate
Cm^{r}	chloramphenicol resistant
conc	concentrated
Da	Dalton(s)
dH_2O	distilled water
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP(s)	deoxynucleoside triphosphate(s)
dUTP	deoxyuridine triphosphate
e-	electron(s)
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetraacetic acid
et al.	et alia
g	gram(s)
G	guanosine
glc	glucose
GSH	glutatione
$GS^{\bullet}$	glutathione radical
GSSG	glutathione dimer
H_2O_2	hydrogen peroxide
h/hr	hour(s)
H^{+}	proton
H_2O	water
HPI	hydroperoxidase I
HPII	hydroperoxidase II
HPLC	high pressure liquid chromotography
HSL	homoserine lactone
kda/kDa	kilodalton(s)
Km^{r}	kamamycin resistant

L/l	litre(s)
LB	Luria Betani
LTTR	LysR-type transcriptional regulator
μg	microgram(s)
M	molarity/molar
mA	milliamp(s)
min	minute(s)
mini-Tn*10*	mini-transposon*10*
ml	millilitre(s)
mM	millimolar
MNNG	N – methyl – N' nitro N – nitroguanidine
mRNA	messenger RNA
ng	nanogram(s)
NAD(P)H	nicotinamide dinucleotide (phosphate)
O_2	oxygen
O_2^-	superoxide anion radical
$OH^•$	hydroxyl radical
ORF	open reading frame
PCR	polymerase chain reaction
pers. comm.	personal communication
PEG	polyethylene glycol
poly(P)	polyphosphate
pm	picomole(s)
ppGpp	guanosine tetraphosphate
RBS	ribosome binding site
RNA	ribonucleic acid
S	sedimentary unit
s/sec	second(s)
SOD	superoxide dismutase
sup	suppressor
T	thymidine
TAE	Tris – acetate – EDTA buffer
TCA	tricarboxylic acid
TE	Tris – EDTA buffer
Ts	temperatrue sensitive
U	units
UDP	uridine diphosphate
vir	virulent
v/v	volume/volume
w/v	weight / volume

LITERATURE REVIEW

2.0 LITERATURE REVIEW

2.1 Oxygen

If an organism is going to reap the rewards of an evolutionary occurrence, so must it endure the disadvantages. An example of this is inherent in the appearance of oxygen and its involvement with aerobic organisms. Initially, the Earth was without significant amounts of oxygen. Then, due to the evolution of oxygen-producing photosynthetic organisms, oxygen was made readily available to those organisms able to utilize it. Aerobic organisms evolved, depending on oxygen for the efficient production of energy via oxidative phosphorylation (Halliwell and Gutteridge, 1989). Subsequently, these same organisms acquired a tolerance to the toxic side effects produced as a consequence of aerobic respiration (Demple, 1991). The following review examines aerobic bacterial responses to oxygen toxicity.

2.1.1 Introduction

2.1.2 Oxygen Toxicity

Oxygen is toxic, indirectly, through the production of reactive oxygen species. Reactive oxygen species are formed by the sequential reduction of oxygen to yield superoxide (anion) radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\bullet$) (Figure 2.1), or by exciting the molecule to yield singlet oxygen ($^1\Delta g\ O_2$) (Fridovich, 1977). Since oxygen has two unpaired electrons of parallel spin in separate antibonding orbitals, reduction by an electron pair of antiparallel spin is unlikely. This phenomenon, known as spin

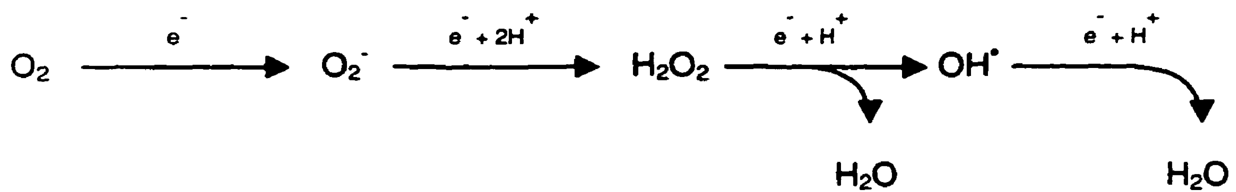


Figure 2.1 The univalent pathway of oxygen reduction. Sequential reduction yields superoxide anion radical, hydrogen peroxide, hydroxyl radical and water (Fridovich, 1978).

restriction, is responsible for the unreactivity of oxygen and the need for univalent reduction (Fridovich, 1977).

The univalent reduction of oxygen or the univalent oxidation of hydrogen peroxide (Fridovich, 1976) forms the superoxide radical. One source of O_2^- in aerobic cells is aerobic respiration itself, in which coupling the oxidation of NADH + H^+ by O_2 and the generation of ATP from ADP occurs through the proton gradient established across the plasma membrane. The electron transport chain is not completely efficient and electron carrier molecules (e.g. coenzyme Q) can 'leak' electrons to O_2 for the subsequent univalent reduction to O_2^- (Halliwell and Gutteridge, 1989). Sources of O_2^- also include: autoxidation of enzymes such as NADH dehydrogenase, succinate dehydrogenase, D-lactate dehydrogenase and glutathione reductase (Imlay and Fridovich, 1991); nonenzymatic autoxidation of ubiquinols, catechols, thiols and flavins; redox-cycling agents such as quinones and paraquat, and catalytic transition metals (Farr and Kogoma, 1991). Autoxidation by enzymes or other cellular components results only in electron donation whereas reduction by redox-cycling agents and transition metals can occur repeatedly after these molecules are cycled back to their reduced forms (Fridovich, 1978).

Hydrogen peroxide can be formed via the divalent reduction of O_2 by oxidases or the univalent reduction of O_2 followed by a simultaneous oxidation and reduction process, or dismutation, of the O_2^- molecule (Fridovich, 1976). The latter is considered here. Since O_2^- is unstable

compared to O_2 and H_2O_2, it will dismutate to form these compounds (Fridovich, 1977). This dismutation can be spontaneous, mediated by redox-cycling agents and transition metals or generated by superoxide dismutases (SODs, see below; Fridovich, 1978).

Extra electrons in the antibonding orbitals of hydrogen peroxide will result in decreased strength of the O-O bond and subsequent decomposition to hydroxyl radicals (Halliwell and Gutteridge, 1989). The interaction of H_2O_2 and reduced iron, also known as the Fenton Reaction, readily yields hydroxyl radicals. Since this reaction is made possible by O_2^--generated H_2O_2 and O_2-reduced iron, O_2^-generating systems are indirectly responsible for the production of $OH^•$ (Fridovich, 1977).

Singlet oxygen exists, in theory, in two forms: with electrons unpaired and antiparallel ($^1\Sigma g\ O_2$), and with electrons paired ($^1\Delta g\ O_2$). However, the $^1\Sigma g\ O_2$ state degrades rapidly to the $^1\Delta g\ O_2$ state such that only $^1\Delta g\ O_2$ is considered in biological systems (Halliwell and Gutteridge, 1989). Singlet oxygen ($^1\Delta g\ O_2$) is most often formed when absorption of a photon of light results in the spin inversion of one of the unpaired electrons in O_2 (Fridovich, 1977).

2.1.3 The Effects of Reactive Oxygen Species

Reactive oxygen species are formed in aerobic bacteria intracellularly, as discussed above. Additionally, pathogenic bacteria may encounter reactive oxygen species exogenously via phagocytosis associated with inflammatory response (Klebanoff, 1988). Regardless of the source,

interaction of these oxygen derivatives with biological macromolecules is harmful, and potentially lethal, to cells.

The bacterial plasma membrane affords a hydrophobic environment, in which endogenously-formed superoxide ion is most reactive (Halliwell and Gutteridge, 1986). O_2^- can oxidize the thiol groups of proteins and other molecules such as glutathione, proteins with metallic domains (e.g., cytochrome c) and many other biological molecules. It is a more powerful reducing agent in its protonated form ($HO_2^{\bullet}$) although this may not exist in great quantities *in vivo* (Halliwell and Gutteridge, 1989). Also, O_2^- facilitates redox cycling by returning transition metals to their most reduced form (Brunori and Rotilio, 1984). O_2^- located outside the bacteria is seemingly less dangerous since it cannot cross the membrane; however, it may give rise to other reactive oxygen species that can. This secondary generation of other oxygen species is perhaps the most threatening situation in and outside the cell.

Unlike O_2^-, hydrogen peroxide can cross the plasma membrane (Halliwell and Gutteridge, 1986) where it will act as a weak oxidizing agent, attacking thiol groups (Farr and Kogoma, 1991; Sanner and Fihl, 1963) and methionyl residues (Stauffer and Etson, 1969) of proteins. Furthermore, H_2O_2 has been reported to disrupt DNA structure by liberating each of the purine and pyrimidine bases (Rhaese and Freese, 1968) and causing single stranded breaks (Ananthaswamy and Eisenstark, 1977). This damage may be the indirect result of the reduction of H_2O_2 to $OH^{\bullet}$. Much like O_2^-, H_2O_2

presents the most damage when it reacts to form more reactive oxygen species.

The most reactive of all reactive oxygen species is the hydroxyl radical (reviewed in Halliwell and Gutteridge, 1989). It can cross membranes and will react with sugars, amino acids, phospholipids, nucleotides and organic acids, most often producing a different free radical which may be more reactive than $OH^{\bullet}$ itself. It will react via one of three reactions; hydrogen abstraction, addition, or electron transfer. $OH^{\bullet}$ is also capable of initiating lipid peroxidation, or the oxidative deterioration of polyunsaturated lipids (Fridovich, 1977). Lipid peroxidation not only affects lipids, but also the structure of the membrane, membrane proteins and in the case of bacteria, potentially any DNA that may be close to or attached to the cell wall. Singlet oxygen ($^{1}\Delta g\ O_2$) also reacts rapidly at carbon-carbon double bonds and may initiate lipid peroxidation (Fridovich, 1977).

2.2 Cellular Defenses

2.2.1 Predamage Protection

Numerous predamage protection mechanisms exist against reactive oxygen species acting passively or actively to prevent oxidative damage before it occurs. One passive predamage mechanism is any enzyme that exhibits multivalent reduction of oxygen including divalent reduction by a flavin-containing oxidase (Fridovich, 1977) or tetravalent reduction by cytochrome oxidase (Antonini and Brunori, 1970). Another is the inherent protection of DNA by DNA-binding proteins such as the DNA-binding protein

in stationary phase or Dps in eubacteria (Almirón *et al.*, 1992). More active predamage protection methods include the scavenging of oxygen reactive species by enzymes such as superoxide dismutases, catalases and others.

Superoxide dismutase (SOD) catalyses the dismutation of O_2^- to H_2O_2 (Figure 2.2a) via a mechanism whereby a metal at the active site is alternately reduced and reoxidized (Figure 2.2b) by successive encounters with O_2^- (Fridovich, 1978). In *Escherichia coli*, four different SODs exist: maganosuperoxide dismutase (MnSOD), a homodimer approximately 40 Kda with a Mn^{3+} ion at the active site (Keele *et al.*, 1970); ferrisuperoxide dismutase (FeSOD), a homodimer approximately 39 KDa with a Fe^{3+} ion at the active site (Yost and Fridovich, 1973); a magano-ferrisuperoxide dismutase (Mn/FeSOD) consisting of one subunit of each enzyme (Halliwell and Gutteridge, 1989) and a 17 Kda monomeric copper- and zinc- containing SOD (Benov and Fridovich, 1996; Battistoni and Rotilio, 1995; Battistoni, *et al.*, 1996; Benov *et al.*, 1997). Though it was initially reported that MnSOD was located in the cytoplasm and FeSOD in the periplasm (Gregory *et al.*, 1973), it has since been found that both are present in the cytoplasm (Britton and Fridovich, 1977). Alternately, CuZnSOD is localized solely in the periplasm (Benov *et al.*, 1995) and may function to protect the cell from exogenous O_2^- produced by phagocytes (Imlay and Imlay, 1996). MnSOD is encoded by *sodA* and is induced by O_2^- whereas FeSOD is encoded by *sodB* and is produced constitutively (Touati, 1988; Hassan, 1988). CuZnSOD is encoded by *sodC* and is induced in stationary phase (Benov *et al.*, 1996;

Imlay and Imlay, 1996, Gort *et al.*, 1999). The comparatively late discovery of CuZnSOD in *E. coli* paired with functional studies suggests that CuZnSOD is most important in the oxidative stress defense of *sodA sodB* mutants (Benov and Fridovich, 1996).

Although SOD produces H_2O_2, a substance toxic to the cell, catalases and hydroperoxidases work to remove H_2O_2 before it can damage cellular components (Yonei *et al.*, 1987). Catalase catalyses the reaction of two H_2O_2 molecules to one oxygen and two water molecules (Figure 2.2c) using a mechanism in which H_2O_2 is used as an electron source. Peroxidase catalyses a similar reaction in which H_2O_2 is reduced to water via the oxidation of some substrate (Figure 2.2d). Some catalases can work as either a catalase or a peroxidase depending on the substrate concentration. In such situations, the enzyme is usually peroxidatic when the H_2O_2 concentration is low and catalatic when the concentration is high (Fridovich, 1977).

Hydroperoxidases I and II are both formed in *E. coli*. HPI is a bifunctional enzyme that exists as a homotetramer with a molecular weight of 337 000 Da and two protoheme IX (heme b) groups (Claiborne and Fridovich, 1979). HPI is encoded by *katG* at 89.2 minutes on the chromosome (Loewen *et al.*, 1985b). Sequencing predicted a subunit 726 amino acids in length and indicated no homology with other known catalases (Triggs-Raine *et al.*, 1988). Although initially thought to exist in the periplasm, more recently, spheroplast fractionation and immunogold labeling *in situ* performed by Hillar *et al.* (1999) indicate that HPI is found throughout the cytoplasm. HPI can be

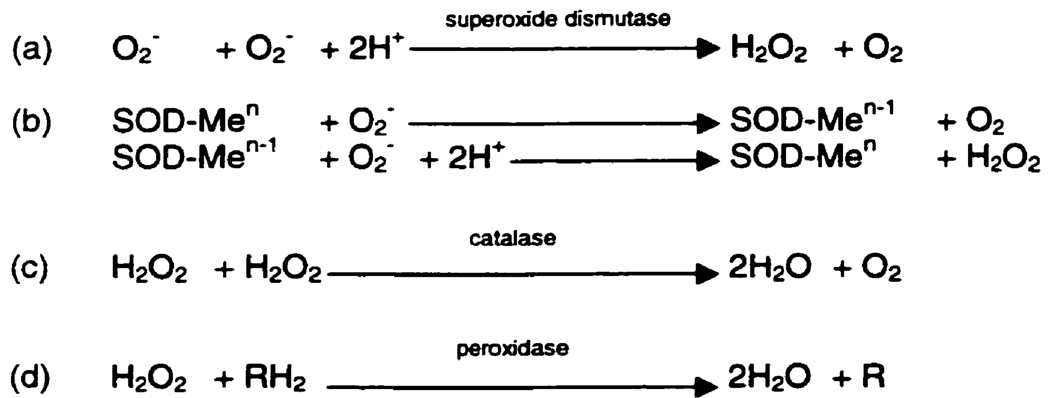


Figure 2.2 Predamage protection enzymes found in *Escherichia coli.*

(a) Superoxide dismutase (SOD) catalyses the conversion of the superoxide radical to hydrogen peroxide and oxygen via a 2-step mechanism (b) in which the metal (Me) at the active site is alternately reduced and reoxidized by successive interactions with a superoxide radical. (c) Catalase catalyses the conversion of two hydrogen peroxide molecules to one oxygen and two water molecules using H_2O_2 as an electron source. (d) Peroxidase catalyses a similar reaction in which hydrogen peroxide is reduced to water using a different substrate (R) as an electron source (Modified from Fridovich, 1978 and Halliwell and Gutteridge, 1989).

induced by H_2O_2 and ascorbate (Richter and Loewen, 1982; Loewen *et al.*, 1985a) in log phase (Loewen *et al.*, 1985a).

HPII is a monofunctional enzyme initially thought to exist as a homotetramer of 312 000 Da with an uncharacterized heme group (Claiborne *et al.*, 1979). By employing different methods, Loewen and Switala (1986) characterized HPII as a homohexamer with a subunit molecular weight of approximately 90 KDa and one heme per subunit although crystallography revealed that it was a homotetramer like all other catalases (Bravo *et al.*, 1995). Heme d was found to be the principal heme bound to HPII (Chiu *et al.*, 1989; Loewen *et al.*, 1993a). HPII is encoded by *katE* at 37.8 minutes on the chromosome (Loewen, 1984). Cloning (Mulvey *et al.*, 1988) and sequencing (von Ossowski *et al.*, 1991) confirmed the molecular weight of HPII and indicated significant sequence similarity to shorter catalase sequences. HPII is categorized as an atypical monofunctional catalase because it differs from eukaroytic monofunctional catalases (Loewen, 1997). It is induced in stationary phase and by TCA cycle intermediates in midlog phase (Loewen *et al.*, 1985a; 1993a).

Another predamage protective enzyme is alkyl hydroperoxide reductase (Ahp) which is encoded by the *ahp* operon (Storz *et al.*, 1989). It provides additional defense to the cell by reducing various organic hydroperoxides including cumene hydroperoxide and t-butyl hydroperoxide (Jacobson *et al.*, 1989).

Additionally, glutathione (GSH) is a proposed protection molecule in

bacteria and a known antioxidant in mammals (Meister and Anderson, 1983). Its synthesis is carried out in two steps using γ-glutamylcysteine and glutathione synthetases encoded by *gshAB*. Once formed, GSH is thought to protect the cell by reacting with H_2O_2, O_2^-, and $HO_2^•$ to form stable glutathione radicals ($GS^•$) via oxidation of the molecule's thiol group. These radicals then dimerize to form GSSG, which is harmless to the cell. Glutathione reductase (encoded by *gor*) then transfers electrons from NADPH to GSSG to reform GSH (Figure 2.3).

In early work, *E. coli* N-methyl-N'-nitro-N-nitroguanidine (MNNG) induced mutants deficient in glutathione synthesis and showing sensitivity to oxygen radicals produced by X-irradiation (Apontoweil and Berends, 1975), suggested that GSH was in fact an antioxidant in bacteria. However, subsequent experiments using *gshA* knockout mutants, and thus GSH deficient cells, indicated that GSH provides no protection from oxidative stress suggesting that MNNG may have caused a second mutation affecting another protective mechanism (Greenberg and Demple, 1986). Furthermore, *gor*$^-$ mutants when compared with wild type cells in oxidative stress experiments behave similarly, suggesting that glutathione reductase has no antioxidant properties. However, the possibility of up-regulation of another stress enzyme or the GSH biosynthesis genes was not eliminated (Becker-Hapak and Eisenstark, 1995). While GSH as a bacterial antioxidant remains an intriguing hypothesis, to date, no component of the GSH mechanism has been shown to play a protective role against oxidative stress in bacteria.

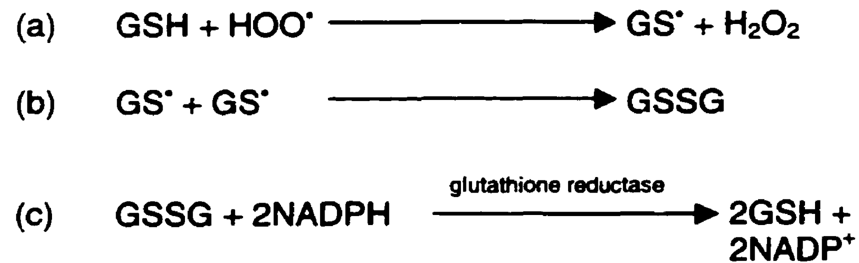


Figure 2.3 The role of glutathione in decreasing oxidative stress. (a) Glutathione (GSH) reacts with a reactive oxygen species (here $HO_2^{\bullet}$) to form a glutathione radical ($GS^{\bullet}$). (b) Two glutathione radicals dimerize to form GSSG. (c) Glutathione reductase reforms glutathione using NADPH as an electron source. Modified from Meister and Anderson (1983).

2.2.2 Postdamage Repair

Damage to biological macromolecules will result if cellular protection mechanisms fail to provide sufficient defense against reactive oxygen species. As mentioned previously, reactive oxygen species damage many cellular components, including DNA, proteins and lipids. Alternatives to cellular death after oxidative damage, are repair and tolerance.

The lowered survival of *polA*⁻ and *recA*⁻ mutants exposed to H_2O_2 indicated that damage occurs to components dependent on DNA polymerase I and RecA protein for repair (Ananthaswamy and Eisenstark, 1977). Further studies also indicated that DNA polymerase I and not DNA polymerase III mediates repair of H_2O_2 damaged DNA (Hagensee and Moses, 1986). DNA polymerase I synthesis is required in excision, postreplication, and recombination repair processes. The RecA protein is responsible for the transfer of undamaged DNA from a sister helix to a damaged region without template in recombination repair. In addition, RecA can initiate SOS response via degradation of LexA repressor molecules. Since *recA*⁻ mutants are subject to increased killing when exposed to H_2O_2 and few protective enzymes are found in mutants' cell free extracts, it has been suggested that RecA is more important than catalase and superoxide dismutase in protecting against H_2O_2 damage (Carlsson and Carpenter, 1980). However, more recent studies show that DNA repair and protective enzymes have distinct roles in mediating protection from oxidative stress and indicate that catalase deficient mutants have lower survival rates than *recA*

mutants using different experimental methods (Loewen, 1984; Yonei *et al.*, 1987).

Endonuclease III, exonuclease III and endonuclease IV, encoded by *nth*, *xthA*, and *nfo* respectively, are involved in specialized excision repair. In this repair process, unnatural bases are excised from DNA leaving an apurinic/apyrimidinic (AP) site which is then nicked by an AP endonuclease allowing DNA polymerase to fill in the gap and DNA ligase to join the DNA (Halliwell and Gutteridge, 1989). Endonuclease III liberates the damaged bases (Breimer and Lindahl, 1984) and, in addition to exonuclease III and endonuclease IV, acts as an AP endonuclease (Weiss, 1976; Ljungquist, 1977). *nth*$^-$, *xthA*$^-$, and *nfo*$^-$ mutants all show increased killing when exposed to oxygen reactive species (Cunningham and Weiss, 1985; Demple *et al.*, 1983; Cunningham *et al.*, 1986).

Although much focus in the literature is on DNA repair, repair at the protein level also occurs. Extensive damage occurs to protein primary structure by $OH^{\bullet}$ and singlet oxygen resulting in alterations to secondary and tertiary structures that renders proteins inactive (Davies and Delsignore, 1987). However, evidence of enzymes capable of reversing amino acid damage, and hence reactivating proteins, exists. An example is methionine sulphoxide reductase which restores the damage done to methionine residues by $OH^{\bullet}$ and singlet oxygen allowing for protein reactivation (Halliwell and Gutteridge, 1989). When restorative enzymes do not exist for a particular lesion or when protein damage is too great, damaged

proteins are degraded by proteolytic enzymes at an accelerated rate (Halliwell and Gutteridge, 1989; Davies *et al.*, 1987).

2.2.3 Oxidative Stress Regulons

The genetic response to a given stimulus can result in a complex regulatory hierarchy comprised of stimulons, regulons, operons and individual genes with their respective regulatory regions. A set of genes that is involved in responding to an external stimulus is referred to as a stimulon. Similarly, a regulon is a group of genes and operons under the control of a single regulatory factor, which can be a variety of molecules from proteins to nucleotides (Neidhart, 1987).

Multiple regulated responses to oxidative stress exist in *E. coli.* In fact, two separate stimulons exist for different oxidative challenges (Greenberg and Demple, 1989). Both O_2^- and H_2O_2 induce independent responses which elicit the production of over thirty proteins each (Walkup and Kogoma, 1989; Demple and Halbrook, 1983; Christman *et al.*, 1985). Within the superoxide and hydrogen peroxide stimulons there are two oxidative stress regulons: SoxRS (Greenberg *et al.*, 1990; Tsaneva and Weiss, 1990) and OxyR (Christman *et al.*, 1985), respectively. A third regulon specific to stationary phase, controlled by RpoS, also confers protection from reactive oxygen species.

The superoxide-response SoxRS regulon induces diverse effects on the cell. It is located at 92.2 minutes on the *E. coli* chromosome and up-regulates at least nine proteins including aconitase A (*acnA*), endonuclease

IV (*nfo*), fumarase C (*fumC*), glucose-6-phosphate dehydrogenase (*zwf*), MnSOD (*sodA*), NADPH-ferredoxin reductase (*fpr*), 30S ribosomal subunit protein S6C, and antisense mRNA *micF* (Greenberg *et al.*, 1990; Tsaneva and Weiss, 1990; Liochev and Fridovich, 1992b; Chou *et al.*, 1993; Gruer and Guest, 1994; Liochev *et al.*, 1994). Furthermore, it down-regulates three proteins including outermembrane protein F and 30S ribosomal subunit protein S6A (Greenberg *et al.*, 1990). Additionally, eleven superoxide-inducible genes, or *soi* genes (Kogoma *et al.*, 1988; Greenberg *et al.*, 1990; Mito *et al.*, 1993), *inaA*, encoding a protein of unknown function (Rosner and Slonczewski, 1994) and paraquat-inducible gene, *pqi5* (Koh and Roe, 1995) are thought to have unique loci under the regulation of SoxRS.

Cloning and sequencing of the *sox* operon identified two divergently transcribed genes, *soxR* and *soxS* separated by 85 bp with predicted protein masses of 17 and 13 kDa respectively (Amabile-Cuevas and Demple, 1991; Wu and Weiss, 1991). Regulation of this regulon is via a two-stage induction; *soxR*- dependent activation of *soxS* and *soxS*-dependent induction of the other operons/genes (Nunoshiba *et al.*, 1992; Wu and Weiss, 1992). Thus, SoxR acts as a sensor and SoxS as a regulatory protein. Although previously thought of as a superoxide-generated response, some suggest that this regulon responds to a depletion of NAD(P)H, indicative of redox imbalance (Liochev and Fridovich, 1992a; Liochev and Fridovich, 1992b).

Induced by hydrogen peroxide, the OxyR regulon maps to approximately 88 minutes on the chromosome and positively regulates nine

genes at the transcriptional level including those coding for HPI, MnSOD, Gor, Ahp, Dps, and three heat shock proteins (Christman *et al.*, 1985; Morgan *et al.*, 1986; Greenberg and Demple, 1988; Tartaglia *et al.*, 1989; Altuvia *et al.*, 1994). *oxyR* has been cloned, sequenced (Christman *et al.*, 1989, Tao *et al.*, 1989) and shown to be negatively autoregulated (Tao *et al.*, 1991). OxyR remains at a steady state level in the cell and activates the regulon genes when oxidized. Thus, it acts both as a sensor and transducer molecule (Storz *et al.*, 1990).

2.3 Stationary Phase

2.3.1 Introduction

Stationary phase is defined as the time interval in which the number of bacterial cells in a given culture ceases to increase (Kolter *et al.*, 1993) due to the complete deprivation of an essential nutrient (Hengge-Aronis, 1993). Although originally thought to be a period of metabolic inactivity, it is now known that stationary phase is a time of intricate metabolic and physiological changes (Kolter *et al.*, 1993; Loewen and Hengge-Aronis, 1994). Phenotypically, the cells appear smaller and more round, the cytoplasm condenses while the volume of periplasm increases. Storage compounds like glycogen, polyphosphate and trehalose accumulate, DNA condenses, the cell membrane becomes more fluid, and stable RNA and protein turnover occurs (reviewed in Hengge-Aronis, 1996). Although net bulk protein synthesis decreases upon entry into stationary phase, the synthesis of several dozen new proteins involved in maintaining essential cell functions and increasing

resistance to environmental stresses such as oxidative stress, temperature and osmolarity fluctuations is induced (Groat *et al.*, 1986).

The initiation and maintenance of stationary phase in non-spore-forming Gram-negative bacteria such as *Escherichia coli* is controlled by a complex regulatory network centering on σ^s, the starvation phase sigma factor. Originally, the locus coding for σ^s was identified with different roles and names including nur (Tuveson, 1981) and *katF* (Loewen and Triggs, 1984) responsible for protection from diverse stresses. Subsequent studies identified KatF as an alternate sigma factor (Mulvey and Loewen, 1989) which regulated more than 30 genes in stationary phase (McCann *et al.*, 1991). Following the convention for sigma factor nomenclature, this once multi-named locus and protein was consequently re-designated as *rpoS* coding for σ^s (Lange and Hengge-Aronis, 1991b).

2.3.2 Regulation of σ^s-Dependent Genes in Stationary Phase

σ^s, binds to RNA polymerase, to produce the holoenzyme necessary for the transcriptional activation of many genes in stationary phase. A list of known σ^s-regulated genes was compiled in a recent review article (Loewen *et al.*, 1998). Despite extensive study, the identity of a common inducing factor or mechanism for σ^s has remained elusive. The lack of an existing motif that discriminates between the σ^{70} and σ^s promoter sequences is most probably due to the overlapping use of promoters by both σ^{70} and σ^s (Tanaka *et al.*, 1993). A study of several σ^s-dependent promoters has proposed a tentative

σ^s consensus sequence nearly identical to that of σ^{70} (Espinosa-Urgel *et al.*, 1996).

Other elements that may aid in sigma factor determination between promoters include the intrinsic curvature of the σ^s-recognized promoter (Espinosa-Urgel and Tormo, 1993), the differences in DNase protection patterns between the -10 and -20 gene regions (Nguyen and Burgess, 1997) and a σ^s preference for CC over TT in the -35 region (Wise *et al.*, 1996). These factors, along with the proposed involvement of metabolites such as trehalose (Kusano and Ishihama, 1997) which vary in concentration produce a complex regulatory picture. In addition, simple sigma factor competition may have a role in promoter discrimination (Farewell *et al.*, 1998).

A class of stationary phase–inducible promoters called gearbox promoters have been identified. The gene products of such promoters occur in fixed amounts dependent on growth rate. Gene expression increases as growth rate decreases and this behaviour is abolished if changes are made to the –10 promoter sequence CGGCAGT (Aldea *et al.*, 1990). Speculation into the importance of gearbox promoters and their role in σ^s transcription arose when it was discovered that *bolA* and *mcbA*, both containing gearbox promoters, were regulated by different sigma factors (Lange and Hengge-Aronis, 1991a). A suggestion was made that an unidentified gearbox-binding factor may be the common element that facilitates transcription with different sigma factors from gearbox promoters (Bohannon *et al.*, 1991). Most recently however, researchers have restricted the definition of gearbox promoters to

include promoters: (1) with activity inversely proportional to growth rate and yielding constant amounts per cell, (2) that are transcribed by σ^s, and (3) containing the defined –10 promoter sequence and AT-rich UP element at the –35 region (Ballesteros *et al.*, 1998).

Although the RpoS regulon is known to mediate the expression of many stationary phase operons and genes, RpoS is not the sole controlling factor in the regulatory network. Other molecules, some of which control their own regulons, also regulate some σ^s-dependent stationary genes. These additional regulatory factors can regulate a given gene directly or indirectly, positively or negatively and do not necessarily regulate all the genes in its regulon similarly. Some examples follow.

The cAMP-CRP (cAMP receptor protein) complex has been shown to both positively and negatively affect gene expression in stationary phase. Hengge-Aronis and Fischer (1992) showed that *glgS*, coding for the protein for glycogen synthesis stimulation during entry into stationary phase, is positively regulated by cAMP. The *glgSp1* promoter has a Crp binding site and is positively regulated by cAMP. Alternately, in *Salmonella typhimurium*, *crp*$^-$ mutants in logarithmic phase derepress expression of several genes, suggesting that Crp repression during log phase is alleviated by another regulatory molecule in stationary phase (Fang *et al.*, 1996). Similarly, experiments conducted in a *lacZ* fusion containing *osmY* and monitored for expression in a *cya* deletion strain in the presence and absence of cAMP indicated that cyclic AMP has a negative regulatory effect on *osmY/csi-5*

(Weichart *et al.*, 1993).

Lrp, the leucine responsive protein, binds with some sequence specificity to DNA and changes its structure via bending; thus affecting transcription and assuming a regulatory function (Newman *et al.*, 1992). It is a negative regulator of σ^s-dependent genes such as *aidB*, a gene homologous to the isovaleryl-coenzyme A dehydrogenase in humans, preventing transcription by blocking the promoter region. This repression is alleviated by a rise in leucine concentrations that increase when leucine is not being used to build proteins in stationary phase (Landini *et al.*, 1996).

The histone-like protein H-NS is known to modify DNA structure by compaction and alterations in superhelicity, and thus its role as a regulator of gene function is inherent (Barth *et al.*, 1995). The curlin subunit gene *csgA* is activated in *hns*$^-$ mutants indicating that H-NS affects *csgA* negatively. Additionally, an rpoS$^-$ mutant able to express curli when H-NS is inactivated suggests that H-NS repression can be alleviated by *rpoS* (Olsén *et al.*, 1993).

IHF is a histone-like, DNA-binding protein with some sequence specificity comprised of two nonidentical subunits IHFα and IHFβ encoded by *himA* and *hip/himD* respectively (Friedman, 1988). *himA*, a σ^s-dependent gene is controlled by negative autoregulation (Aviv *et al.*, 1994). Also, IHF binds upstream of the *dps* promoter, altering DNA structure and positively affecting Dps expression. It is proposed that holoenzymes do not bind maximally to the *dps* promoter region making IHF or another regulatory molecule essential for maximal expression (Altuvia *et al.*, 1994).

Fis, a nucleoid protein known to regulate recombination, replication, transcription and also modulates some genes regulated by σ^s, such as *aldB*, *glnQ*, *mglA*, *sdhA*, and *xylF* (Xu and Johnson, 1995). As the expression of transcriptional fusions in *fis*$^+$ and *fis*$^-$ backgrounds indicates, Fis always modulates gene expression negatively. A clear elucidation of the mechanism by which this protein regulates is confused by its regulation of genes both positively and negatively controlled by σ^s (Loewen *et al.*, 1998)

The combination of regulatory factors acting to regulate stationary phase genes adds to the complexity of the regulatory network, but is advantageous to the organism since it can modulate such elaborate gene regulation with so few regulatory molecules (Hengge-Aronis, 1996; Loewen and Hengge-Aronis, 1994). Such a system was discovered with respect to the *osmY* locus, which is a σ^s-dependent locus negatively regulated by cAMP-CRP, Lrp and IHF (Lange *et al.*, 1993). Gene expression is repressed by the concerted effort of these global regulators until its repressive activity is relieved and the gene is induced.

2.3.3 Regulation of σ^s in Stationary Phase

rpoS is transcribed in the same direction as the adjacent gene *nlpD* (Ichikawa *et al.*, 1994). *nlpD* has two promoters which produce polycistronic mRNA including *rpoS* transcripts which affect basal level σ^s expression in exponential phase (Lange and Hengge-Aronis 1994a). *rpoS* transcription occurs from four different promoters (Takayanagi *et al.*, 1994) but the *rpoSp1*

promoter located within *nlpD* controls most transcription in stationary phase (Lange *et al.*, 1995).

Experiments using quantitative Western immunoblot analysis indicated that while σ^{70} maintained fairly constant protein levels throughout the transition from exponential to stationary phase, the initially undetectable σ^{s} increased to at least 30% of the σ^{70} level in stationary phase (Jishage and Ishihama, 1995). This increase in σ^{s} protein levels is undoubtedly a consequence of σ^{s} up-regulation on a number of levels including transcriptional control, translational control, and σ^{s} stabilization. Additionally, two potential signal molecules without clearly defined roles in *rpoS* regulation, homoserine lactone (Huisman and Kolter, 1994) and UDP-glucose (Böhringer *et al.*, 1995), are thought to aid in facilitating σ^{s} induction.

σ^{s} was originally thought to be exclusively regulated at the transcriptional level as the expression of transcriptional *rpoS::lacZ* fusions increased during growth on poor media and at the beginning of stationary phase (Mulvey *et al.*, 1990). However, subsequent studies have indicated that most regulation occurs post-transcriptionally. For example, some studies show that protein fusions have higher induction patterns than operon fusions indicating that regulation occurs at a translational level (Loewen *et al.*, 1993b; McCann *et al.*, 1993; Lange and Hengge-Aronis, 1994b). Alternatively, recent studies using more direct measurements of *rpoS* gene expression suggest that both transitional and translational fusion data results are artifactual

(Zgurskaya *et al.*, 1997) and rising σ^s protein levels are primarily due to increased protein stability (Lange and Hengge-Aronis, 1994b; Zgurskaya *et al.*, 1997). A summary of *rpoS* regulation in provided in Table 2.1.

Although identification of the primary level of σ^s regulatory control is seemingly unresolved, data indicates that some control occurs transcriptionally and is modulated by various molecules. Guanosine tetraphosphate (ppGpp) deficient strains are also deficient in σ^s implying that ppGpp is a positive regulator of σ^s transcription (Gentry *et al.*, 1993) which is further supported by transcriptional fusion data (Lange *et al.*, 1995). In fact, control of some σ^s-dependent genes originally attributed to direct regulation by ppGpp may actually be affected by ppGpp indirectly via *rpoS* as is the case with *cfa*, the cyclopropane fatty acid synthase gene (Eichel *et al.*, 1999).

Although known to be a regulator of *rpoS*, contrary results do not allow a depiction of how cAMP-CRP modulates its transcription. Some transcriptional fusion data suggest that *rpoS* is positively regulated by cAMP-CRP since expression is reduced in a *cya* mutant (McCann *et al.*, 1993). Conversely, σ^s protein levels that increase in a *cya* mutant strain and decrease upon addition of exogenous cAMP suggest that cAMP-CRP is a negative regulator of *rpoS* (Lange and Hengge-Aronis, 1994b).

Another molecule, inorganic polyphosphate or poly(P), is thought to positively regulate *rpoS* transcription. A correlation between poly(P) and *rpoS* expression was first identified when *ppk*, or poly(P) kinase, mutants were observed to lose viability upon entry into stationary phase (Rao and

Table 2.1 Modulators and their regulation of RpoS

Level of Regulation	Modulator	Effect on RpoS	Mode of Action
Unknown[a]	HSL	+ve[b] correlation	signal molecule
	UDP-glc	+ve correlation	signal molecule
Transcriptional	ppGpp	positive	direct
	cAMP-CRP	unresolved[c]	n/a[d]
	poly(P)	positive	direct
Translational	2°[e] structure	negative	direct
	H-NS	negative	direct, indirect via LeuO
	DsrA RNA	positive	direct, indirect via H-NS
	LeuO	positive	indirect via DsrA RNA
	Hfq	positive	direct
	OxyS RNA	negative	indirect via Hfq
Protein stability	ClpPX	negative	direct
	RssB/SprE	negative	indirect via ClpPX
	DnaK	positive	direct
	LrhA	negative	indirect via RssB

[a] The data only indicate a positive correlation between HSL and UDP-glc levels with *rpoS*. Thus, the level of regulation (if any) is unknown.
[b] +ve; positive
[c] Conflicting data identify cAMP-CRP as both a positive and negative regulator (see text for details), and hence is reported here as 'unresolved'.
[d] Since the effect cAMP-CRP has on *rpoS* is unresolved, its mode of action is yet to be determined. n/a; not applicable
[e] 2°; secondary

Kornberg, 1996). Subsequent studies in which overexpression of poly(P)ase mimics the state of a *ppk* mutant also show that reduced poly(P) levels have a negative effect on *rpoS* which can be overcome by extra copies of *ppk* (Shiba *et al.*, 1997).

Many factors also modulate σ^s expression at the translational level. The most successful being the predicted secondary *rpoS* mRNA structure which makes the ribosome-binding site (RBS) and initiation codon unavailable to ribosomes resulting in translation inhibition (Lange and Hengge-Aronis, 1994b). Other modulators include well-established global regulators such as H-NS and newly described anti-sense binding RNA structures.

H-NS levels are known to increase in stationary phase (Dersch *et al.*, 1993) and regulate *rpoS* and other stationary phase genes negatively in both a *rpoS*-dependent and -independent manner (Barth *et al.*, 1995). Increased expression of *rpoS* translational fusions in a *hns*$^-$ background first suggested that this regulation was at the post-transcriptional level (Barth *et al.*, 1995). Enhanced translation of σ^s as shown by Western blotting and increased protein half-life in similar mutants support this claim (Yamashino *et al.*, 1995). This is the foundation of a complex cascade that regulates *rpoS* at the translational level.

DsrA RNA, a small 85 nucleotide RNA molecule, is shown to interrupt the silencing effect of H-NS at various promoters (Sledjeski and Gottesman, 1995), including *rpoS* (Sledjeski *et al.*, 1996). σ^s levels decrease in a *dsrA*

mutant identifying DsrA RNA as an indirect positive regulator via H-NS. Additionally, the LysR-like regulator, LeuO, also affects *rpoS* translation. *leuO*⁻ mutants increase the expression from *rpoS* translational fusions, unless they are present in a *dsrA* null mutant background (Klauck *et al.*, 1997). This suggests that LeuO is indirectly a negative regulator of *rpoS* facilitated by the repression of *dsrA*. Further, in a *hns*⁻ mutant, *leuO* is derepressed, suggesting that H-NS is a negative regulator of LeuO (Klauck *et al.*, 1997).

DsrA RNA exists as a three-stem loop secondary structure (Sledjeski and Gottesman, 1995) with different regions of the structure modulating different factors in this cascade (Majdalani *et al.*, 1998; Lease *et al.*, 1998). The anti-H-NS effect of DsrA RNA is independent of stem-loop 1, yet dependent on the central stem loop for RNA-RNA base-pairing of *hns* mRNA and prevention of H-NS silencing. Stem loop 1 is necessary for anti-sense binding of the 5' end of the *rpoS* leader mRNA, prohibiting the secondary structure conformation that inhibits translation of *rpoS* indicating that DsrA RNA can control *rpoS* directly.

A separate cascade includes Hfq and OxyS RNA. Hfq, a RNA-binding protein, is required for the translation of *rpoS* and is known to regulate stationary phase genes independently and via σ^s (Muffler *et al.*, 1996b). OxyS RNA, a small 109 nucleotide RNA molecule, is a negative regulator of *rpoS* (Altuvia *et al.*, 1997) and is controlled by OxyR (Gonzalez-Flecha and Demple, 1999). Also existing as a three-stem loop structure, mutational analysis shows it is actually a 27 base pair inter-stem loop region between

the second and third stem loops that facilitates repression of *rpoS* (Altuvia *et al.*, 1998). Subsequent studies have shown that OxyS RNA achieves inhibition by binding Hfq. Thus, repression of *rpoS* translation by OxyS RNA is due to the alterations of Hfq activity (Zhang *et al.*, 1998).

As σ^S stability is observed to increase greatly in stationary phase, it has been suggested that increased protein stability is another regulation level for σ^S (Lange and Hengge-Aronis, 1994b). Most regulators at the post-translational level have a role in the genetic pathway centred around the protease ClpPX. σ^S is found to be more stable in the absence of ClpPX as indicated by the measurement of σ^S levels by Western blotting with monoclonal σ^S antibodies in different genetic backgrounds.

Another molecule referred to as RssB or SprE (Muffler *et al.*, 1996a; Pratt and Silhavy, 1996) contributes to the σ^S degradation pathway in a protease dependent manner (Pratt and Silhavy, 1996) while acting σ^S-specifically (Zhou and Gottesman, 1998). It has been proposed that RssB interacts with RpoS to form a complex that affects its activity or stability.

Interestingly, it was observed that the middle portion of σ^S, specifically amino acid residues 173-188 from the N-terminus end, makes the protein susceptible to proteolytic attack either directly or indirectly by ClpPX (Schweder *et al.*, 1996). This was confirmed with hybrid protein studies indicating that an element located between nucleotides 397 and 742 is required for σ^S degradation (Muffler *et al.*, 1996c) and is needed for RssB

activity (Muffler *et al.*, 1996a).

Surprisingly, the increase in σ^s stability is not due to the decrease of ClpPX in stationary phase, as ClpPX levels actually increase in stationary phase (Schweder *et al.*, 1996). This would suggest that a chaperone protein might protect σ^s from degradation by ClpPX. DnaK, a known heat shock protein chaperone, is suggested to play a positive role in σ^s regulation since dnaK$^-$ mutants suffer a decrease in σ^s levels and exhibit phenotypes similar to rpoS$^-$ mutants (Muffler *et al.*, 1997; Rockabrand *et al.*, 1998). The decrease in protein synthesis inherent in stationary phase may decrease the availability of other chaperone substrates for DnaK and increase the frequency of it protecting σ^s (Muffler *et al.*, 1997).

Another regulator, found to exist upstream of all others so far in this pathway is LrhA, yet another LysR homolog (Gibson and Silhavy, 1999). LrhA does not regulate synthesis of any other modulator in this pathway, but is thought to modulate SprE either directly or indirectly via another unknown mechanism.

2.3.4 Oxidative Stress in Stationary Phase

Stationary phase cells are observed to have heightened resistance against many stressors, not the least of which is oxidative stress. This is dependent on the *rpoS* induction of defense mechanisms, such as catalase, which protect from the effects of reactive oxygen species. As discussed earlier, hydroperoxidase II is encoded by *katE* (Loewen, 1984) and affected by a second locus originally referred to as *katF* (Loewen and Triggs, 1984).

Subsequent studies indicated that *katE* is in fact regulated by the KatF protein (Mulvey *et al.*, 1990; Schellhorn and Stones, 1992) now realized to be the stationary phase sigma factor σ^s (Lange and Hengge-Aronis, 1991b). Thus, HPII is turned on by σ^s in stationary phase. Other genes regulated by σ^s and thought to confer oxidative protection in stationary phase include *xthA* (Sak *et al.*, 1989), *dps* (Almirón *et al.*, 1992), *gor* (Becker-Hapak and Eisenstark, 1995) and sodC (Gort *et al.*, 1999).

It was initially proven that both catalases are regulated independently (Loewen *et al.*, 1985a; reviewed in Schellhorn, 1994) and thought that no regulatory overlap between stationary phase protection and induced oxidative stress responses produced by regulons SoxRS and OxyR existed (Farr and Kogoma, 1991). In agreement was the observation that *katE* is not affected by *oxyR* (Schellhorn and Hassan, 1988). However, evidence suggests that HPI, encoded by *katG* (Loewen *et al.*, 1985b), is regulated by OxyR (Christman *et al.*, 1985) and also controlled by *rpoS* (reviewed in Eisenstark *et al.*, 1996). Mukhopadhyay and Schellhorn (1994) showed that growth phase induction of *katG* is not dependent on OxyR induction, but rather relies on σ^s. Furthermore, decreases in HPI protein levels and expression levels of *katG::lacZ* fusions occurred in *rpoS⁻* backgrounds, and *rpoS⁻ΔoxyR* double mutants had lower HPI levels than *ΔoxyR* strains with the levels being restored by a *rpoS*-carrying plasmid (Ivanova *et al.*, 1994). More recent data has shown that the RpoS regulon is activated in $\Delta oxyR^{sup}$ strains and is

responsible for the suppressor phenotype further confirms the overlap between the inducible exponential phase OxyR regulon and constitutive stationary phase RpoS regulon (Ivanova *et al.*, 1997). Other genes exist that confer antioxidant properties and are regulated by both OxyR and σ^s, such as *dps* (Altuvia *et al.*, 1994). Furthermore, a recent study showing that OxyR controls OxyS RNA (Gonzalez-Flecha and Demple, 1999), a known negative regulator of *rpoS* (Altuvia *et al.*, 1997), suggests an integrated control mechanism for these two regulons.

2.4 Purpose

All organisms requiring oxygen for respiration must also tolerate the consequent side effects. As most aerobic organisms have evolved similar protection mechanisms, the study of oxidative stress response of any one species may provide insight into others. This study examines the response to H_2O_2 in stationary phase of *E. coli.* This is of particular interest as naturally occurring bacteria are commonly found in stationary phase, not logarithmic phase as seen in most laboratory settings (Kolter *et al.*, 1993). Continuing the growing inquiry into bacterial stationary phase genetics, this work identifies a new locus, *perR*, which is involved in hydrogen peroxide resistance in *Escherichia coli* in stationary phase.

MATERIALS AND METHODS

3.0 MATERIALS AND METHODS

3.1 *E. coli* Strains, Plasmids and Bacteriophage

All bacterial strains, plasmids and bacteriophage used in this work are listed in Table 3.1. *E. coli* A594, A594::Tn*10*, H1230, MP180, UM367 and UM369 were used primarily for genetic mapping studies of *perR*. Specifically, strain A594 is a suppressorless strain frequently chosen to facilitate transposon jumping. Strains MV1161 and MV2640 were used for the same genetic mapping studies and as examples of strains that were hydrogen peroxide sensitive and resistant in stationary phase respectively (Volkert *et al.*, 1994). Strain MG1655 was also used in the genetic mapping studies and was the strain chosen for the homologous recombination event of the knocked-out *perR* which lead to the creation of strain MG1655-JS. Strain CJ236 was used for production of the single-stranded DNA needed for site-directed mutagenesis, JM109 was used for DNA sequencing and NM522 was used for cloning and plasmid propagation. All plasmids listed were used in the creation of *perR* mutagenized with the mini-transposon Tn*10*. The suicide vector, pK03, which aided in the homologous recombination event in *E. coli* MG1655 is explained more fully in section 3.15. P1(vir) was used to create the P1 lysates used in the transductional crosses for the genetic mapping studies. Bacteriophage λNK1316 contains the mini-transposon Tn*10* used for transposon mutagenesis. Bacteriophage R408 is the helper phage used for infection of *E. coli* CJ236 for single-stranded DNA production.

Table 3.1 Bacterial strains, plasmids and bacteriophage used in this work.

Name	Genotype/Phenotype	Reference/Source
A. Bacterial Strains		
A594	F⁻ *lac-3350 galK2 galT22* λ⁻ *rpsL179* IN *(rrnD-rrnE)1*	Campbell, 1965
A594::Tn*10*	As A594, Km^r	this work
CJ236	*dut1 ung1 thi1 rel*A1/pCJ105/*~cam*^r F'	Kunkel *et al.*, 1987
H1230	*thr-20 leu-32 fhuA48 proA35 argF58 relA1 spoT1 arl60*	CGSC[a]
JM109	*recA1 supE44 endA1 hsdA1 hsdR17 gyrA96 relA1 thi* Δ*(lac-proAB)*	Yanisch-Perron *et al.*, 1985
MG1655	LAM-1, *rph-1*	Guyer *et al.*, 1981
MP180	*thi-1* HfrH	Pearson, 1972
MV1161	*thr-1 ara-14* Δ*(gpt-proA)62 lacY1 tsx-33 supE44 galK2 hisG4 rfbD1 mgl-51 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1 rfa-550*	Volkert and Nguyen, 1984
MV2640	As MV1161 Δ*(argF-lacZ)205 (U169) (also* Δ*katC) pro*⁺	Volkert *et al.*, 1994
NM522	*recA*⁺ *(supE thi* Δ*(lac-proAB) hsd5* {F' *proAB lacI*^Q *lacZ* Δ*M15})*	Mead *et al.*, 1985
NK5012	*thr*⁻ *leu*⁻ *thi*⁺ *sulI*⁺ *T1R T5R phi80R*	Kleckner *et al.*, 1978
UM367	As MV1161 *perR2*	Peter Loewen, pers. comm.[b]

Table 3.1 (continued)

Name	Genotype/Phenotype	Reference/Source
A. Bacterial Strains (continued)		
UM369	As MV1161 *perA3*	Peter Loewen, pers. comm.
MG1655-JS	As MG1655 *perR::Tn10*	this work
B. Plasmids		
pSK+/pSK-	Ap^r	Stratagene Cloning Systems
p129-1.8SK+	Ap^r	Peter Loewen, pers. comm
pPerR*	Ap^r	this work
pPerR**	Ap^r	this work
pNK2857	Ap^r Km^r	Kleckner *et al.*, 1991
pPerR::Tn*10*	Ap^r, Km^r	this work
pK03	Cm^r, Ts, SacB	Link *et al.*, 1997
C. Bacteriophage		
P1(A594::Tn*10*)		this work
P1(UM367)		this work
P1(UM369)		this work
λNK1316		Kleckner *et al.*,1991; Bender *et al.*, 1992
R408 (helper phage)		Stratagene Cloning Systems

[a] *E. coli* Genetic Stock Centre
[b] personal communication

3.2 Media, Growth Conditions and Storage of Cultures

Cultures were grown in one of two media, either LB medium or M9 minimal medium supplemented with glucose (glucose minimal medium). LB Medium was made with 10 g of prepared LB powder (Difco) per 500 ml of distilled water. Solid medium is prepared with the addition of 7.5 g Bacto-agar (Difco)/500 ml of media. Antibiotics such as ampicillin, chloramphenicol, and kanamycin were added after autoclaving in final concentrations of 200 μg/ml, 20 μg/ml, and 50 μg/ml, respectively. Additionally, 5% (v/v) sucrose was added for the negative selection of the suicide vector pK03.

M9 Minimal Medium (Miller, 1972) was made by combining 1 ml 5.0 M NaCl, 42 ml 0.5 M Na_2HPO_4, 5 ml 2.0 M NH_4Cl, 11 ml 1.0 M KH_2PO_4 and bringing the volume up to 500 ml before autoclaving. Solid medium is prepared with the addition of 7.5 g of Bacto-agar/500 ml media. After autoclaving the medium is supplemented with 0.5 ml 1.0 M $MgSO_4$, 0.5 ml 1 mg/ml Vitamin B1, 0.5 ml Trace Elements (2.5 g $FeSO_4 \cdot 7H_2O$, 2.9 g H_3BO_4, 1.2 g $CoSO_4 \cdot 7H_2O$, 0.1 g $CuSO_4 \cdot 5H_2O$, 0.09 g $MnCl_2 \cdot 4H_2O$, 2.5 g $Na_2MoO_4 \cdot 2H_2O$, 2.1 g $ZnSO_4 \cdot 7H_2O$, 5.0 ml H_2SO_4 (conc) in 1.0 L distilled H_2O) and 5.0 ml 30% (w/v) glucose. For amino acid auxotrophic selection, 2.0 ml of each necessary D-amino acid solution (2% (w/v)) was also added per 500 ml of media.

In the event that cultures were pour-plated, R top (1.25 g tryptone, 1.0 g NaCl, 1.0 g agar, 0.125 g yeast extract in 125 ml dH_20, after autoclaving add 0.25 ml sterile 1 M $CaCl_2$ and 0.42 ml sterile 30% glucose) and F top

(0.8 g agar, 0.8 g NaCl, in 100 ml dH_2O) agars were used atop of LB and Glucose Minimal Media respectively.

Cultures were grown at 28°C or 37°C with aeration depending on the characteristics of the strain. Cultures were stored in 8% DMSO at –60°C. Bacteriophage stocks were stored in LB culture supernatant at 4°C with a small amount of chloroform to prevent bacterial contamination.

3.3 Hydrogen Peroxide Assay

A 5 ml culture of the strain of interest was grown for 16 to 24 hours to ensure that the culture was in stationary phase. The cells from a 100 μl aliquot of the culture were collected by centrifugation and resuspended in 900 μl of 1 x E salts (to 670 ml of distilled H_20 add sequentially 10 g $MgSO_4{\cdot}7H_2O$, 100 g citric acid, 500 g K_2HPO_4, 175 g $NaNH_4$ $HPO_4{\cdot}4H_2O$, adjust volume to 1 L and pH to 7.8). A 10 μl aliquot of this suspension was serially diluted in SM buffer (10 ml 5.0 M NaCl, 0.5 ml 1.0 M $MgSO_4$, 10 ml 1.0 M Tris-HCl pH 7.6, 0.05 g gelatin, adjust volume to 500 ml) and plated on LB plates (with kanamycin if containing the mini-Tn*10*) for a time point at zero (0). Hydrogen peroxide was diluted in 1 x E salts to the appropriate molarity (45-135 mM) and 100 μl was added to the cell suspension. The cells were incubated at room temperature (25°C) for 30 minutes. To establish a survival curve, samples were taken at time points 5, 10, 20 and 30 minutes whereas to solely determine resistance/sensitivity a sample was taken at 30 minutes alone. The cells are then serially diluted in SM buffer plated on LB plates (with kanamycin if necessary) and incubated at 37°C overnight. *E. coli* strains

MV1161 and MV2640 were run in parallel to other samples as an example of how hydrogen peroxide sensitive and resistant cells respond respectively. Percentage survival was determined by taking a ratio of bacterial titre at a given time point to bacterial titre at time point zero and multiplying by 100%.

3.4 Transposon Mutagenesis

3.4.1 Transposon carrying phage lysate creation

A subculture of *E. coli* strain NK5012 was grown to midlog phase in LB broth. Aliquots of 0.1 ml were mixed with 1 ml aliquots of λNK1316 serially diluted in SM buffer and allowed to incubate for 20 minutes at room temperature to facilitate phage adsorption. The mixtures were pour-plated with R top agar onto LB plates and incubated at 42°C overnight. Between 5 and 10 isolated plaques were picked into 0.5 ml of SM buffer and 3 drops of chloroform and incubated at room temperature for 2-3 hours before collection by centrifugation. The lysate was used to infect a midlog culture of NK5012, incubated at room temperature for 20 minutes and pour-plated with R top agar onto LB plates. After ensuring confluent lysis, the top agar was collected and combined with 2.0 ml of SM and 5 drops of chloroform and was vortexed for 30 seconds. The lysate was collected by centrifugation and stored at 4°C. Lysates were titred as single plaques were isolated above for calculating multiplicity of infection (moi).

3.4.2 Transposon Mutagenesis and Collection of Mutagenized Strain

Transposon mutagenesis was performed as described by Way *et al.*, (1984). A subculture of the strain to be mutagenized was grown to midlog

phase in LB broth supplemented with 50 µl 10% w/v maltose. The cells were collected by centrifugation and resuspended in one-tenth the original volume of the culture. Bacteriophages harbouring the appropriate transposon were added ensuring a moi of 0.3. This infection mixture was first incubated at room temperature for 30 minutes followed by a subsequent incubation at 37°C for 90 minutes. A mixture without phage was used as a negative control. A 0.1 ml aliquot was spread-plated onto LB plates containing the appropriate antibiotic for transposon selection and 1.25 mM sodium pyrophosphate to minimize problems with unabsorbed phage. The plates were incubated at 42°C overnight as a block to lysogeny.

The collection of bacteria containing transposons was done by resuspending the colonies in approximately 2 ml of LB broth and kanamycin. The cells were collected with a Pasteur pipet and added to 20 ml of the same media. The cells were allowed to replicate for 3 hours at 37°C with aeration to improve yield.

3.5 P1 Transductional Mapping

P1 lysate preparation and transductional mapping procedures were based on the protocols as described by Triggs-Raine (1987).

3.5.1 P1 lysate preparation

A 5 ml subculture of the donor strain was grown to midlog phase in LB broth. The cells were collected by centrifugation and resuspended in 2.5 ml SM buffer, 100 µl of 1.0 M $MgSO_4$ and 50 µl 1.0 M $CaCl_2$. A 0.1 ml aliquot of the resuspended cells was combined with 20 µl of P1(vir) and incubated at

37°C for 30 minutes. The phage absorbed bacteria were then pour-plated with molten R-top agar onto LB plates and incubated at 37°C overnight. After ensuring confluent lysis, the top agar was collected, combined with 2.0 ml LB broth and 0.5 ml chloroform and vortexed for one minute. After an hour incubation at room temperature, the supernatant was collected by centrifugation to provide a P1 lysate.

3.5.2 P1 Transduction

A 5 ml subculture of the recipient strain was grown to midlog phase in LB broth. The cells were collected by centrifugation and resuspended in 2.5 ml SM buffer, 100 μl 1.0 M $MgSO_4$ and 50 μl 1.0 M $CaCl_2$. A 2.0 ml aliquot of the resuspended recipient cells was combined with 20 μl of the P1 lysate. A control containing no P1 lysate was prepared simultaneously. After a 45 minute incubation at 37°C, 0.2 ml 1.0 M sodium citrate was added. The transduction mixture was the pour-plated with molten R top agar or F top onto the appropriate selective medium and incubated at 37°C overnight. Colonies from these plates were picked onto the same media to form master plates and onto other media to score for particular genetic markers. Specifically, Km^r, Per^r and *proA*$^-$ (gamma-glutamyl phosphate reductase) selected for the mini-Tn*10*, H_2O_2 resistance and proline auxotrophy. The genetic linkage data were used to calculate map distance (Wu, 1966). The map distance between markers (d) in minutes is equal to the cubed root of cotransduction frequency (x) of any two markers subtracted from one, multiplied by 2 or $d = 2 (1- x^{-3})$. Additionally, a rough estimate of distance measured in base pairs is

determined by the relationship one minute equals 47, 000 bp.

3.6 DNA Isolation

3.6.1 Plasmid DNA

Plasmids were prepared as described in Sambrook *et al.* (1989). A 5 ml culture harbouring the necessary plasmid was grown overnight with the appropriate antibiotic for plasmid selection. The procedure was carried out on ice unless otherwise specified. Cells were harvested from a 1.5 ml volume of culture by centrifugation and resuspended in 200 µl of Tris-EDTA-glucose (25 mM Tris-HCL, 10 mM EDTA, 50 mM glucose, pH 8.0). The cells are lysed by a 5 minute incubation with 400 µl of 0.2 M NaOH + 0.1 % SDS (prepared fresh) and the genomic DNA precipitated by a 10 minute incubation with 300 µl of 6.2 M NH_4OAc (pH 5.9). The supernatant containing plasmid DNA was collected by two consecutive centrifugations for 15 and 10 minutes, respectively. The plasmid DNA was precipitated by a 10 minute centrifugation. The plasmid DNA was washed twice with cold 70% ethanol, dried by vacuum and resuspended in 50 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). For low yielding plasmids (i.e. pK03) this procedure was performed using 10 times the amounts described here.

3.6.2 Genomic DNA

To isolate genomic DNA, a procedure modified from Triggs-Raine (1987) was used. A 3.0 ml volume from an overnight culture was used to inoculate 200 ml of LB broth. At mid-logarithmic phase the cells were collected by centrifugation and washed in 50 ml saline-EDTA (0.15 M NaCl,

0.1 M EDTA, pH 8.0). Following a subsequent centrifugation, the cells were resuspended in 4.0 ml saline-EDTA. After 8 mg of lysozyme was added the solution was incubated for 30 min at 37°C and subsequently frozen at –60°C for 30 min. A volume of 4.0 ml of Tris-SDS-NaCl (0.1 M Tris, 1% SDS, 0.1 M NaCl, pH 7.6) was added to the frozen cells which were resuspended on thawing. A phenol – chloroform extraction was performed by adding an equal volume of saturated phenol (buffered with 0.1 M NaCl, 0.1 M Tris-HCl, 1.0 mM EDTA, pH 7.6) and water-saturated chloroform and chilling for 20 minutes at 4°C. The emulsion was separated by centrifugation and the upper layer was combined with twice the volume of cold ethanol. The nucleic acids were precipitated by centrifugation and resuspended in 0.1 ml of 0.1x SSC (15 mM NaCl, 1.5 mM Na citrate). Removal of RNA was done by adding 1 mg of RNaseA and incubating for 30 minutes at 37°C. Then twice the volume of ethanol and one tenth the volume of 4 M NaOAc (pH 6.0) were added and left for 30 minutes at –20°C. The genomic DNA was collected by centrifugation and resuspended in 1 ml of TE buffer.

3.6.3 Single-stranded DNA

Single-stranded DNA rescue was performed as described by Vierira and Messing (1987). A 5 ml culture containing the plasmid of interest was grown in LB broth with the appropriate antibiotic for plasmid selection and a supplement of 50 µl 1 M $MgSO_4$ for enhanced yield. Upon reaching early logarithmic phase, the culture was infected with 10^{10} R408 helper phage particles and allowed to grow at 37°C for 6-8 hours. The lysate was

centrifuged and the supernatant combined with 300 μl of 1.5 M NaCl + 20% PEG for an incubation at room temperature for 15 minutes. The phage particles are collected by centrifugation. The pellet was resuspended in 200 μl of TE buffer and the DNA was extracted by equal amounts of saturated phenol and water-saturated chloroform sequentially. A volume of 150 μl 7.5 M NH_4OAc (pH 7.4) and 600 μl of cold 100% ethanol were added and incubated at –20°C for 20 minutes. The single-stranded DNA was collected by centrifugation, washed twice with 70% ethanol and resuspended in 25 μl of HPLC-grade water just before use.

3.7 Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed as in Sambrook *et al.* (1989). 0.6 – 2 % agarose gels were made with TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and 0.1 μg/ml ethidium bromide. Samples of 10 μl were combined with 3 μl of stop buffer (40% v/v glycerol, 10 mM EDTA pH 8.0, 0.25% bromophenol blue) and added to the wells. The 1 kilobase ladder or 1 kilobase plus ladder (GIBCO-BRL) were used for size standards. The gels were run at 40 mA-constant current on a Bio-Rad Mini Sub Cell horizontal electrophoresis system for 2 – 3 hours, or until the tracking dye reached near bottom of the gel. Bands were visualized using a UV light and captured as a digitized image using Gel Doc 1000 image capture system (BioRad).

3.8 Transformation

E. coli strains were transformed as in Chung *et al.* (1989). The strain of

choice was grown to midlog phase in 5 ml of culture. The cells were harvested by centrifugation and made competent by resuspension in 0.1 M cold $CaCl_2$ and a subsequent incubation on ice for an hour. Between 2-10 µg of plasmid DNA were added to 100 µl aliquots of competent cells. After a 30 minute incubation on ice, the cells were heat shocked at 42°C for 90 seconds. A volume of 1 ml LB medium was added and the cells were incubated at 37°C for one hour without aeration. The cells were then spread-plated or pour-plated with molten (55°C) R top agar onto LB plates with the appropriate antibiotic for plasmid selection.

3.9 Restriction Endonuclease Digestion

All restriction endonucleases and buffers were purchased from GIBCO-BRL. Restriction endonuclease digests of plasmid DNA were performed in a reaction volume of 10 µl including 1 µl RNaseA (1 mg/ml), 1 µl reaction buffer, 1-5 µg DNA and 5-10 units (U) of endonuclease. The reactions were incubated at 37°C for 2-3 hours. Vector DNA used for subsequent cloning had the 5' phosphate groups removed during the last 0.5-1 hour of digestion by adding 25 U of calf intestinal alkaline phosphatase (GIBCO-BRL).

3.10 Southern Hybridization

Southern hybridizations were performed using the DIG system using conditions as supplied by the manufacturer (Boehringer Mannheim).

3.10.1 Southern Blotting

First the gel was submerged in the denaturation solution (0.5 N NaOH,

1.5 M NaCl) and incubated with shaking for 30 minutes at room temperature. The solution was poured off and the gel was rinsed with deionized water. The gel was submerged in the neutralizing solution (0.5 M Tris-HCl, pH 7.5; 3 M NaCl) and incubated with shaking for 2 x 15 minutes at room temperature. The DNA was blotted overnight using capillary transfer and cross linked for 3 minutes with UV light. In assembling the blotting apparatus, the Hybond-N nylon membrane (Amersham) and Whatman 3 MM filter paper were soaked in 10 x SSC (1.5 M NaCl, 150 mM sodium citrate, pH 7.0) to facilitate capillary transfer of the denatured DNA.

3.10.2 Hybridization

The membrane was prehybrized for one hour in 15 ml of the standard hybridization buffer (5 x SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% w/v blocking reagent to maleic buffer (0.1 M maleic acid, 0.15 M NaCl, (pH 7.5)) at 60°C. Fresh standard hybridization buffer containing approximately 5-25 ng/ml of DIG-labelled probe was then added and allowed to hybridize overnight at 60°C. Oligonucleotide probes were labelled with digoxigenin-11-dUTP using the random primed method according to instructions supplied by the manufacturer (Boehringer Mannheim).

To ensure the removal of unbound probe, the membrane underwent a series of washes after hybridization. First the membrane was washed twice in 2 x wash solution (2 x SSC, 0.1% SDS) at room temperature for 5 minutes per wash. Then it was washed twice in 0.1 x wash solution (0.1 SSC, 0.1% SDS) at 40°C for 15 minutes per wash.

3.10.3 Colourmetric Detection

The following procedure was carried out at room temperature. The membrane was equilibrated in washing buffer (0.3% (w/v) Tween 20 to maleic acid buffer) for one minute. Then it was incubated with the blocking buffer (1% w/v blocking reagent to maleic acid buffer) for 30 minutes with shaking.

After pouring the blocking solution off, a 1:5000 dilution of Anti-Digoxigenin-AP in fresh blocking buffer was added to the membrane for 30 minutes with shaking. The antibody solution was removed and the membrane was washed twice with the washing buffer for 15 minutes with shaking to remove the unbound antibody. The membrane equilibrated in the detection buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl) for two minutes before 45 μl nitroblue tetrazoleum (NBT) and 35 μl 5-bromo-4-chloro-3-indoyl phosphate (X-phosphate) were added per 10 ml detection buffer for colour development. The membrane was incubated in the dark until colour developed (minimum 12 hours).

3.11 Extraction of DNA from Agarose Gel

Fragments of interest were excised from the agarose gel using a Gene Clean DNA Extraction Kit (Bio/Can Sci Inc) using instructions as provided by the manufacturer.

3.12 Ligation Reactions

Ligation reactions were performed as in Sambrook *et al.* (1989). A ratio of 0.5:5 of insert to phosphatased vector DNA were contained in a reaction volume of 10 μl including 1 unit of T_4 DNA ligase and 2 μl 5x ligase mix (both

GIBCO-BRL). A reaction mix without insert DNA was used as a control. The reactions were incubated at 15°C overnight.

3.13 Primer Extraction and Phosphorylation

Oligonucleotide primers as listed in Table 3.2 were created with the PCR-Mate DNA synthesizer (Applied Biosystems Inc) according to manufacturer's instructions. The bound oligonucleotides were extracted from the column with concentrated ammonium hydroxide and incubated at 55°C overnight. The primer solution was evaporated (Savant Instruments, Inc), resuspended in HPLC grade water and stored at –20°C. Oligonucleotide primers used in site-directed mutagenesis were phosphorylated at the 5' end with T_4 kinase (GIBCO-BRL) as described by Ausubel *et al.* (1989).

3.14 Site-Directed Mutagenesis

Site-directed mutagenesis was performed as described in Kunkel *et al.* (1987). First the phosphorylated mutagenic primer was annealed to the single-stranded DNA template in 1x SSC at 73°C for 3 minutes and at room temperature for 20 minutes. The complementary strand was synthesized *in vitro* by unmodified T_7 DNA polymerase (New England Biolabs) and used the annealed oligonucleotide as the primer. The ends of the newly synthesized strand were ligated with T_4 DNA ligase (GIBCO-BRL) which is contained in the reaction mixture. Since original templates were degraded, plasmids containing the mutagenic sequence are recovered upon transformation into *E. coli* NM522.

Table 3.2 Single-stranded oligonucleotides used in this study

Name	Use	Sequence
pPerR+Bam*	Site-directed mutagenesis	5' – GCT GAT GG*A TCC AGG CCT TTG – 3'
pPerR-BamHI	Site-directed mutagenesis	5' – TAG AAC TAG TGG T*TA* CCC CGG G– 3'
Pr367A1	Polymerase Chain Reaction	5' – ATC GCC ATA AAG CTA CGG TC – 3'
Pr367E1	Polymerase Chain Reaction	5' – GGT GGT TTG CCC TGC CGT TC – 3'
pTn367B	DNA Sequencing	5' – GCT GAA AGT CGC AGA TCG ATG – 3'

3.15 Homologous Recombination

To reincorporate a mutagenized open reading frame into the *E. coli* genome, the gene replacement vector pKO3 (Link *et al.*, 1997) was used. This plasmid contains a temperature sensitive origin of replication, chloramphenicol resistance as a positive selection and the *sacB* gene as a negative selection for the manipulation of mutagenized open reading frames. Thus, upon subcloning the mutagenized *ORF* into pKO3, the subclone (pKO3-JS) was transformed into the strain of choice and incubated at 28°C on LB containing chloramphenicol and kanamycin overnight allowing for growth of colonies containing pKO3-JS. These colonies were used to inoculate LB broth containing both antibiotics and incubated at 28°C to allow homologous recombination events to occur. The cultures were then serially diluted using SM buffer and plated on LB chloramphenicol and kanamycin at 43°C for selection of plasmid cointegrates. Colonies were pick-plated onto LB kanamycin and sucrose plates (growth), replica plated on LB kanamycin, sucrose and chloramphenicol plates (no growth) and incubated at 28°C to test for the loss of the replacement vector. The genomic DNA of potential positive colonies was then screened for the integrated mutagenized ORF via PCR and confirmed by DNA sequencing.

3.16 Polymerase Chain Reaction

PCR was used to screen for successful homologous recombination events with the mutagenized open reading frame. Approximately 0.2 pmol of genomic DNA was amplified in a reaction volume of 100 µl using 1.5 µg of

each primer (Table 3.2), 2.5 units of *Taq* DNA polymerase (GIBCO-BRL), 10 µl 10 mM MgCl, 0.8 µl 25 mM dNTPs and 10 µl reaction buffer containing 500 mM KCl, 100 mM Tris-HCl, pH 9.0 and 0.1% Triton X-100 (Ausubel *et al.*, 1989). Additionally, 10 µl 20% (v/v) DMSO and 0.5 µl DNA polymerase extender (Stratagene Cloning Systems) were added for PCR enhancement.

The polymerase chain reaction was performed using a Perkin Elmer Cetus DNA Thermal Cycler with denaturation at 93°C for 1 minute, primer annealing at 50°C for 1 minute and primer extension at 72°C for 2 minutes. After 25 cycles, there was a final extension at 72°C for 5 minutes and the reaction was stopped by chilling to 4°C. The reaction mixture was separated by electrophoresis on an agarose gel which was then prepared for Southern blotting to check for the presence of the kanamycin cassette.

3.17 DNA Sequencing

DNA sequencing was used to verify that the *perR* locus had been interrupted with the kanamycin cassette from the mini-Tn*10*. Double-stranded sequencing was performed as described by Sanger *et al.* (1977) and the primers used are listed in Table 3.2.

Approximately 5 µg of plasmid was denatured by a 10 minute incubation at 37°C in 40 µl of freshly prepared 0.4 M NaOH. The denatured plasmid was reprecipitated by the addition of 10 µl 3 M sodium acetate (pH 4.8) and 150 µl cold absolute ethanol. After an incubation at –20°C for 30 minutes, the pellet was collected by centrifugation at 4°C for 15 minutes and

washed twice with ethanol. The pellet was resuspended in 10 μl of sterile deionized water. The annealing and sequencing reactions were performed using a T7 sequencing kit (Pharmacia) to supplier specifications with 5-15 μCi [α -^{35}S] dATP (NEN-duPont).

The sequencing reaction were separated on a 5% (w/v) polyacrylamide gel made with 7 M urea, 0.13 M Tris, 0.13 boric acid and 10 mM EDTA. The gels were run at 18-24 mA constant current in TBE (90 mM Tris, 89 mM boric acid, 2.2 M EDTA) for 1.5 – 5 hours as required. The dried gels were exposed to X-ray film (Kodak X-OMAT AR) which was consequently developed for band visualization.

3.18 Protein Sequence Analysis

Analysis of the predicted amino acid sequence was perfomed using the BLAST computer program (Altschul, *et al.*, 1997) as provided on the National Centre for Biotechnology Information (NCBI) website – http://www.ncbi.nlm.nih.gov/blast/. Specifically, the blastp program and the database containing all non-redundant peptide sequences available from GenBank CDS translations and PDB, SwissProt, PIR and PRF databanks were used. The sequence was filtered for low complexity and gaps were allowed. Default values for alignment view, descriptions and alignments were employed.

RESULTS

4.0 RESULTS

4.1 Genetic Mapping of Mini-Tn*10* insertions in UM367 and UM369

A comparison of the genotypes and sensitivities to hydrogen peroxide in stationary phase of strains MV1161 and MV2640 may suggest a location for a resistance locus, tentatively called *perR*. That is, the strain MV1161 is hydrogen peroxide sensitive and contains a known deletion from *gpt-proA* (5.4-5.6 min), which may continue downstream to encompass more uncharacterized ORFs. Alternatively, strain MV2640 contains this deleted region and is hydrogen peroxide resistant. Therefore, it is suggested that an ORF affecting resistance to H_2O_2 is located downstream of *proA* in a largely uncharacterized region.

Transductional mapping was performed to verify the location of the putative *perR* locus with *E. coli* strains UM367 and UM369 as donors and H1230 as the recipient strain (Table 4.1). Phenotypically, UM367 and UM369 are hydrogen peroxide resistant, *proA*$^+$ strains that contain the kanamycin resistant mini-Tn*10* around 5.7 and 5.4 minutes respectively (P. Loewen, pers. comm.). Conversely, H1230 requires proline supplementation and is sensitive to hydrogen peroxide and kanamycin. Thus, the location of the putatative gene was mapped with respect to the known locations of *proA* and the mini-Tn*10*.

The map distance between markers was calculated as described in Section 3.5.2 and averages from the following crosses were used to generate a genetic map (Figure 4.1). In the first cross of H1230 x P1(UM367), the initial

Table 4.1 Mapping of the *perR* locus relative to adjacent genes by three-factor transductional crosses

Donor	Recipient	Selected Marker	Unselected Markers Class	No (%)
UM367	H1230	Km^r	*proA*$^+$ Per^r	73 (60.8)
(Km^r, *proA*$^+$, Per^r)[a]	(Km^s, *proA*$^-$, Per^s)$^-$		*proA*$^+$ Per^s	7 (5.8)
			proA$^-$ Per^r	33 (27.5)
			proA$^-$ Per^s	7 (5.8)
UM367	H1230	*proA*$^+$	Km^r Per^r	89 (37.1)
(Km^r, *pro*$^+$, Per^r)	(Km^s, *proA*$^-$, Per^s)		Km^r Per^s	78 (32.5)
			Km^s Per^r	36 (15.0)
			Km^s Per^s	37 (15.4)
UM369	H1230	*proA*$^+$	Km^r Per^r	13 (10.8)
(Km^r, *pro*$^+$, Per^r)	(Km^s, *proA*$^-$, Per^s)		Km^r Per^s	98 (81.7)
			Km^s Per^r	2 (1.7)
			Km^s Per^s	7 (5.8)

[a] When possible, genotypes are given as strain characteristics (e.g. $proA^{+/-}$), otherwise characteristics are reported as phenotypes only (e.g. $Km^{r/s}$, $Per^{r/s}$).

selection was made for the mini-Tn*10* allowing sequential scoring of the transductants for proline auxotrophy and H_2O_2 resistance. According to this cross, the distances from the mini-Tn*10* to *proA*$^+$ and mini-Tn*10* to *perR* is 0.23 min or 11, 800 bp and 0.08 min or 3, 800 bp respectively. In another H1230 x P1(UM367) cross, the initial selection was made for the ability to synthesize proline and transductants were scored for kanamycin and H_2O_2 sensitivities. As determined by this cross, the distances from *proA*$^+$ to the mini-Tn*10* and *proA*$^+$ to *perR* are 0.23 min or 10, 800 bp and 0.39 min or 18, 300 bp respectively. The third cross, H1230 x P1(UM369), also selected for proline synthesis and scored for kanamycin and H_2O_2 sensitivities. The distances calculated from *proA*$^+$ to the mini-Tn*10* is 0.05 min or 2, 400 bp and from *proA*$^+$ to *perR* is 1.0 minute or 47 kb. The large distance between *proA*$^+$ and *perR* is a less accurate determination and is not represented as a map in Figure 4.1. These data suggest that there may be two different loci affecting peroxide sensitivity in these strains. However, the clear conclusion is that neither mini-Tn*10* is located in a locus affecting peroxide sensitivity making it necessary to search for or construct a *perR*::Tn*10* mutation.

4.2 Attempted Transposon Mutagenesis of *perR*

Experiments attempting to create a *perR* mutation by random transposition were performed (Figure 4.2a). Strain A594 lacked suppressor genes and contained a *perR*$^+$ locus based on its hydrogen peroxide phenotype, making it a good candidate for transposon jumping with mini-Tn*10* in which transposon insertion would occasionally interrupt the *perR* locus.

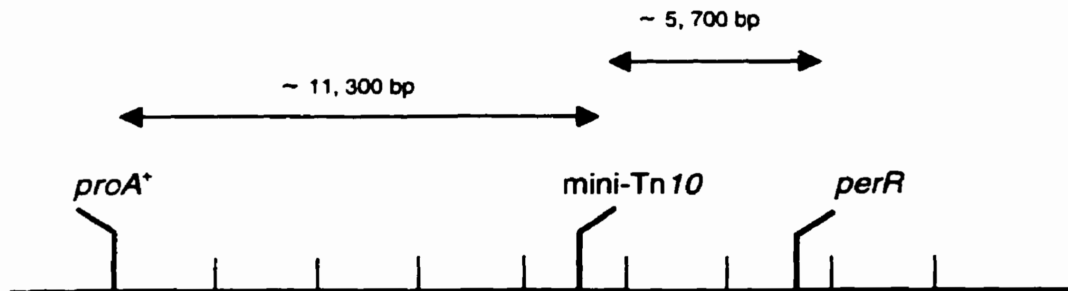


Figure 4.1 Genetic map based on linkage analysis from three-factor transductional crosses. The known location of the *proA*+ locus provides a marker around minute 5.6 or base pair position 260, 727 on the *E. coli* chromosome. Other loci are averaged from approximated distances as discussed in Section 4.2. The thick vertical lines represent loci and the thin vertical lines mark 2, 500 bp.

Because transductional mapping suggested the *perR* locus was near *proA*, the screening of transposon mutants was concentrated in this region of the chromosome. Using P1 lysates from A594 mutagenized colonies (A594::Tn*10*), MV1161 was transduced and proline synthesis was selected to ensure recombination of the deletion and proximity to *proA* while co-selection for kanamycin resistance ensured cotransduction of the mini-Tn*10*. Thus, successful transductants were assumed to have the *gpt-proA* deletion introduced, and all H_2O_2 sensitivity was attributed to an interrupted rather than deleted *perR* locus. Subsequent screening of 120 transductants for the interrupted *perR* locus, yielded 31 potential candidates.

To confirm the identity of the *perR* mutation, subsequent transductions were performed with MV1161 as the recipient strain and P1 lysates generated from those potential candidates conferring kanamycin resistance, *proA*$^+$ and H_2O_2 sensitivity (MV1161::Tn*10*). The object was to demonstrate that 100% of transductants co-selected for *proA*$^+$ and the mini-Tn*10* were also H_2O_2 sensitive. Unfortunately, the best result was 114/120 transductants being H_2O_2 sensitive, and subsequent transductions with the same lysate and other recipient strains, specifically H1230 and MV2640, produced even lower percentages of cotransduction. It is possible that while these insertions did not interrupt the *perR* locus, their obvious proximity may have affected gene function to a lesser extent.

The same procedure was attempted with H1230 as the recipient strain (Figure 4.2b). H1230 is phenotypically comparable to MV1161 but is without

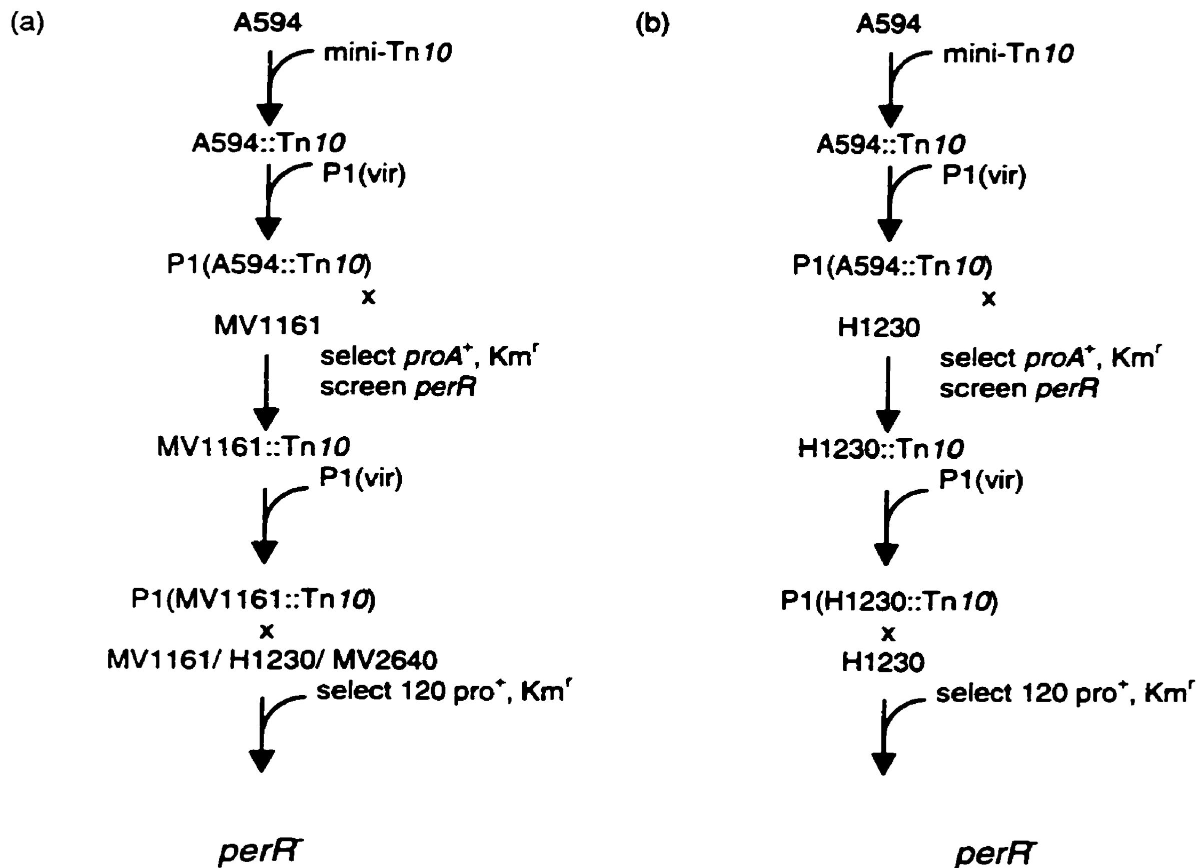


Figure 4.2 Diagrammatic representation of the sequential experiments performed in the attempted creation of a mutation in *perR* via random transposition. In both attempts, strain A594 served as a host for transposon insertion. P1 lysates of the mutagenized A594 were used to infect strains MV1161 (a) or H1230 (b). Subsequent P1 lysates were generated from colonies displaying a phenotype suggestive of a *perR* mutation. The lysates from these potential *perR* mutants were tested via transduction in a variety of strains (MV1161, H1230, MV2640).

the chromosomal deletion, thus, H_2O_2 sensitivity of this strain is thought to be caused by a mutation in *perR*. Nevertheless, transductants from H1230 x P1(H1230::Tn*10*) crosses also failed to produce H_2O_2 sensitive transposon mutants.

4.3 Site-Directed Transposon Mutagenesis of *perR*

Because these other methods failed to identify the precise location of the ORF conferring hydrogen peroxide resistance in stationary phase, a more direct approach was attempted. Three open reading frames, *yafZ*, *ykfA* and *orf307* (*perR*) were selected for mutagenesis based on the rough estimate provided by mapping data and previous findings (P. Loewen, pers. comm.). Specifically, with sequences taken from GenBank, a restriction site was created via site-directed mutagenesis in the chosen ORFs allowing for the subcloning of the mini-Tn*10* kanamycin cassette (Kleckner *et al.*, 1991) into the mutagenized site thereby creating an insertion mutation. The following illustrates the successful insertion mutation of *orf307*.

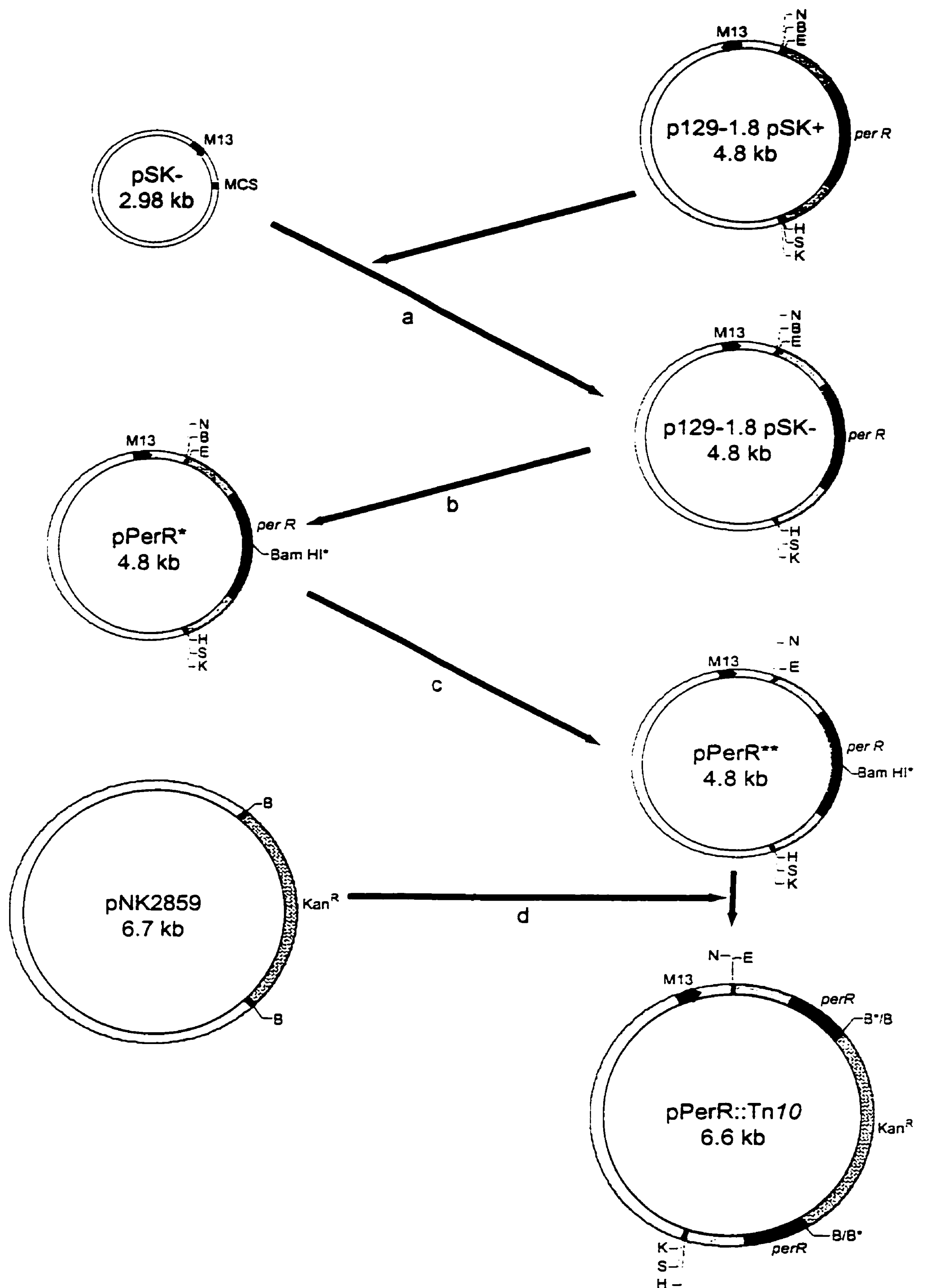
The starting plasmid, p129-1.8 SK+, contains a fragment known to contain *perR*, or *orf307*, from around 5.7 minutes on the *E. coli* chromosome. It was previously created by cloning a *Hind*III – *Eco*RI 1.8 kb genomic fragment from the Kohara collection (Kohara *et al.*, 1987) into Bluescript SK+ (Stratagene Cloning Systems). To ensure the sense strand for *perR* would be rescued as single-stranded DNA in subsequent experiments, this same fragment was subcloned into the multiple cloning site (MCS) of Bluescript SK- to create p129-1.8 SK- (Figure 4.3a).

As discussed above, a *Bam*HI site was created within *perR* using oligonucleotide pPerR+Bam* which binds to nucleotides 268-285 and alters the thymidine at nucleotide 275 to cytosine via site-directed mutagenesis (pPerR*, Figure 4.3b). Confirmation of this mutagenic event via a *Bam*HI restriction digest of pPerR* yielded a restriction profile with 4.0 and 0.8 kb fragments in successful mutants due to the newly introduced *Bam*HI site in *perR* and a pre-existing *Bam*HI site in the MCS of Bluescript SK-.

To facilitate ease of insertion of the mini-Tn*10* kanamycin cassette into *perR*, the pre-existing *Bam*HI site was destroyed using site-directed mutagenesis. This second mutagenic primer, pPerR-BamHI, binds to nucleotides 713-734 of Bluescript SK- and modifies the cytosine and adenosine at nucleotides 720 and 722 to adenosine and thymidine respectively (pPerR**, Figure 4.3c). The desired mutant was confirmed by *Bam*HI restriction digestion that yielded only one fragment of 4.8 kb due to the removal of the MCS *Bam*HI site.

Finally, the 1.8 kb kanamycin cassette with flanking *Bam*HI ends from the mini-Tn*10* carried on pNK2859 was subcloned into the mutagenic *Bam*HI site, thus producing pPerR::Tn*10* (Figure 4.3d). Two separate restriction digests confirmed the transposon mutagenesis. A *Bam*HI restriction digest yielded 1.8 kb and 4.8 kb fragments corresponding to the size of the kanamycin cassette and the remainder of pPerR::Tn*10* respectively, and a combined digestion with *Eco*RI and S*al*I, cutting only in the multiple cloning site on either side of the insert yielded 3.0 and 3.6 kb fragments

Figure 4.3 Creation of pPerR::Tn*10*. (a) Transfer of *Hind*III – *Eco*RI 1.8 kb fragment from p129-1.8 pSK+ into Bluescript SK- to create p129-1.8 pSK-. (b) The construction of a *Bam*HI site within *perR* by site-directed mutagenesis to create pPerR*. (c) Removal of the *Bam*HI site from the MCS by site directed mutatgenesis to create pPerR**. (d) Insertion of the kanamycin cassette from pNK2859 into the *Bam*HI site of pPerR** to create pPerR::Tn*10*. Abbreviations used: B, *Bam*HI; B*, *Bam*HI created; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; N, *Not*I; S, *Sal*I; Kan^R, kanamycin resistance cassette.



pSK-
2.98 kb
M13
MCS
p129-1.8 pSK+
4.8 kb
M13
N
B
E
per R
H
S
K
a
p129-1.8 pSK-
4.8 kb
M13
N
B
E
per R
H
S
K
b
pPerR*
4.8 kb
M13
N
B
E
per R
Bam HI*
H
S
K
c
pPerR**
4.8 kb
M13
N
E
per R
Bam HI*
H
S
K
pNK2859
6.7 kb
B
Kan^R
B
d
pPerR::Tn10
6.6 kb
M13
N
E
perR
B*/B
Kan^R
B/B*
perR
K
S
H

corresponding to the size of the Bluescript vector and the sum of the fragments cloned into it respectively.

4.4 Homologous Recombination into the *E. coli* chromosome

Once the transposom mutagenesis of *perR* on the plasmid was complete, it was necessary to transfer the mutant gene to the chromosome for reliable gene function studies. To achieve this, a 3.6 kb *NotI-SalI* restriction fragment from pPerR::Tn*10* was cloned into matching restriction sites in the 5.7 kb gene replacement vector pKO3 (Link *et al.*, 1997). The successful cloning of the resulting 9.3 kb plasmid, pKO3-JS, was confirmed by a *NotI* restriction digest. pKO3-JS was transformed into MG1655 where the mutagenized *perR* was recombined into the chromosome as per the homologous recombination protocol outlined in Section 3.15.

An attempt was made to characterize the mutation by PCR but similar sized products were obtained from both wild type and mutant DNA. This result is not fully understood, but may be the result of reversions resulting in the loss of the mini-Tn*10* or secondary structure interference, such as hairpin loops caused by inverted repeat regions on the mini-Tn*10* (J. Switala and P. Loewen, pers. comm.).

The presence of the interrupted *perR* locus was verified by Southern blotting. The genomic DNAs from the mutant and wild type strains were digested with *Eco*R1, electrophoresed, blotted onto a nitrocellulose membrane and probed with a DIG-labelled kanamycin cassette from pNK2859. The fragment size without transposon insertion in the parent strain

DNA was 5.8 kb. In the mutant strain DNA a larger fragment was detected confirming transposon insertion close to or in the *perR* locus.

A further confirmation of the insertion was achieved by DNA sequencing. An *Eco*RI fragment of 7.6 kb containing the mutated *perR* gene was cloned from UM397 into Bluescript pSK+ and primer pTn367B, complementary to nucleotides 366-386 of *perR*, only 90 bases from the insertion was used (Figure 4.4).

4.5 Altered H_2O_2 Resistance of *perR*::Tn*10*

Once it had been verified that the mutant *perR* gene had been transferred to the *E. coli* chromosome, it was necessary to test for an altered H_2O_2 resistance phenotype. Four strains assayed simultaneously: i) the H_2O_2-sensitive control, MV1161, ii) the H_2O_2-resistant control, MV2640, iii) the parent strain MG1655, and iv) the mutant strain UM397, all with and without sucrose in the medium. After incubation with 75 mM hydrogen peroxide, UM397 incubated with sucrose exhibited similar sensitivity to MV1161, and was clearly much more sensitive than the resistant controls MV2640 and MG1655 (Figure 4.5). Strains MV2640, MG1655 and UM397 without sucrose in the medium showed 45, 61, and 42 % survival respectively whereas strains MV1161 and UM397 with sucrose-supplemented medium had 0.02 and 4 % survival respectively. The 13.6 fold decrease in survival of the mutant strain, when grown in sucrose-supplemented media for selection against the suicide vector, clearly indicates that the *perR* locus is involved in resistance to H_2O_2 in stationary phase. The unpredicted survival of strain UM397 without sucrose

Figure 4.4 Confirmation of transposon insertion in *perR* via DNA sequencing. The complementary strand of *perR* was sequenced with primer pTn367B. Illustrated here is the sequence beginning at position 313; the arrow indicates the inserted mini-Tn*10* at position 275.

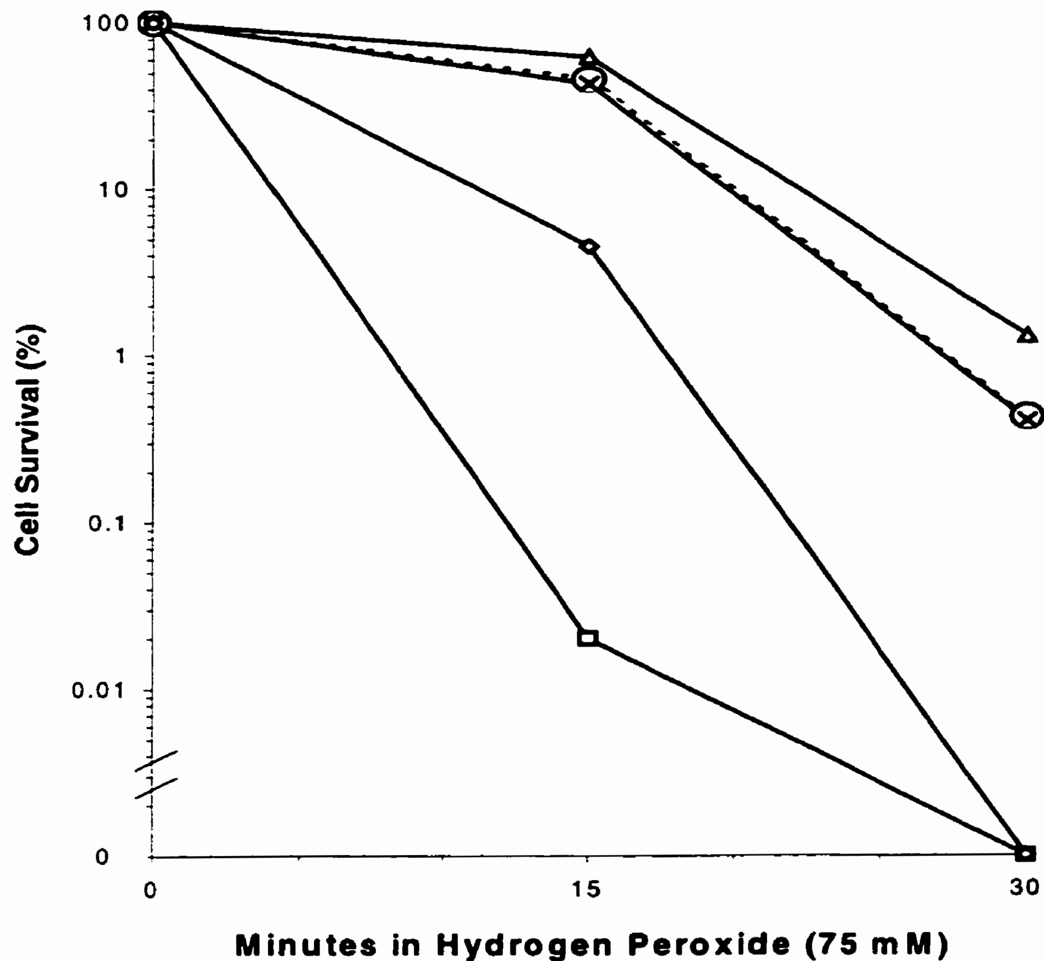


Figure 4.5 Comparison of hydrogen peroxide survival curves of stationary phase cells. Strains studied include H_2O_2 resistant control MV2640 (- - O - -), H_2O_2 sensitive control MV1161 (—□—), parent strain MG1655 (—△—), mutant strain UM397 without sucrose supplement (—x—) and UM397 with sucrose supplement (—◇—). Note strains MV2640 and UM397, both without sucrose in the media, are nearly indiscernible due similar survival profiles.

in the medium can be attributed to the presence of pKO3-JS, which may allow homologous recombination events between the plasmid and chromosome to occur providing a functional *perR* gene.

4.6 *perR* Sequence Analysis

The *perR* sequence as reported in GenBank (Accession Number AE000133.1) and its predicted amino acid sequence are shown in Figure 4.6. The coding region is 881 nucleotides in length and ends with a UGA stop similar survival profiles. codon. A potential Shine-Dalgarno sequence of – AAGGA – is located at position –12. Possible promoter motifs are located approximately 350 bp upstream of the start codon. The -35 region, - TTGACC – , and –10 region, – TTTTTT –, are located at positions –377 and –352 respectively.

The predicted amino acid sequence of PerR is 297 amino acids in length and shows homology to LysR-type transcriptional regulators (LTTRs) as indicated by a BLAST search (Figure 4.7). The highest non-hypothetical matches to PerR are AmpR from *Rhodobacter (Rhodopsuedomonas) capsulatus* (e value, 8×10^{-33}) and PenR from *Burkholderia (Psuedomonas) cepacia* (e value, 4×10^{-30}), both transcriptional activators of β-lactamase or penicillinase operons (Campbell *et al.*, 1989; Trépanier *et al.*, 1997). AmpR has 100 identical and 51 similar residues indicating a 35.3% identity and 53.3% similarity to PerR and PenR has 94 identical and 55 similar residues indicating a 32.5% identity and 51.6% similarity to PerR.

Intraspecifically, PerR shows the most homology to GcvA (e value,

Figure 4.6 The *perR* DNA sequence with predicted amino acid sequence. Nucleotides are shown in regular font and their positions are indicated to the right of the sequence. Positive numbers specify sequence from the start of the open reading frame and negative numbers represent upstream sequence. The first and last codons are indicated above the nucleotide sequence by +1 and &, respectively. A potential ribosomal binding site (RBS) is underlined and located at position −12. Predicted −35 and −10 regions are shaded and located at positions -377 and -352 respectively. Amino acids are shown in bold font and their positions are indicated to the right of the sequence. One letter amino acid abbreviations are used. GenBank accession number for *perR* is AE000133.1.

-35

CAACCCCATGTTTTCACATAACTGTTGCG[illegible]AATTGAATCTACAGTA -358

-10

AGCC[illegible]AATATTTCATTTTCCATTTCAATACGTTGTAGCTTTTTCCT -307

GAGCTCACGGATTTCAATTTGTTCCGGGGTAATGGGGGAGGCTTTTGGTGT -256

TTTTGCCCTGCCGTTCATCACGTAATTGTTTCACCCATCGCGTCATTGTGG -205

AAAGGCCGACATCCATAGCGCTGGCTGCATCTGCCACGGTGTAGTTCTGGT -154

CAACGACCAGTTGAGCGGATTCGCGTTTAAACTCTGCGCTGAAATTTCTTT -103

TTTTCATTATGACACCTGTGTTGTTCTGAGGTGAGCATATCACCTCTGTTC -52

RBS

AGGTGGCCAAATTCAGTAAACCACTTCATCCCTAGTGAAAAGGATTCATGA -1

+1

ATGAAGCTCTTAGCAAAAGCACCTCTAAATCTGTTACGCGCCTTTGAAGCG 51
M K L L A K A P L N L L R A F E A 17

GCTGGTCGTACCGGAGCTTTTGCGTTGGCCGCCTCTGAGTTGGAACTGTCA 102
A G R T G A F A L A A S E L E L S 34

CCCAGTGCGATCAGCCATGCCATCCGCAAACTGGAAAACTTGCTTGATGTA 153
P S A I S H A I R K L E N L L D V 51

CGCCTTTTTCAGCGCAGTACGCGAGAAATTACGCTGACGAAAGAAGGTGAA 204
R L F Q R S T R E I T L T K E G E 68

ATACTGCTTGAGCACATACAGCGGGGATTTAACGAATTACAGCAGGGGTTG 255
I L L E H I Q R C F N E L Q Q G L 85

GCATTAGTGACGGCTGATGAATCCAGGCCTTTGCGCCTTCATACTGCACCG 306
A L V T A D E S R P L R L H T A P 102

AGTTTTGCCCATCAATGGCTTTTACCACGTCTTGGTAAGTTCATACGTGAG 357
S F A H Q W L L P R L G K R I R E 119

AACCCAAGCATCGATCTGCGACTTTCAGCCAGCACAGAATATGCACGTTTT 408
N P S I D L R L S A S T E Y A R F 136

GAACAGGATGATTTTGATCTCGATATAGTCTACGGTGAACCCCGCCCATCA 459
E Q D D F D L D I V Y G E P R P S 153

CCCTATGAGAAGATCCCGCTTGCTGTTGAAGAACTTACGCCACTGTGTTCT 510
P Y E K I P L A V E E L T P L C S 170

CCCCAACTGGCTGAGCGGCTAAAGAAACCAGAGGATCTCTATGCGTTGACA 561
P Q L A E R L K K P E D L Y A L T 187

TTGATTCAGTGCGATGTGCAGTTGTACCAGTGGAAAGGATGGTTTGAGGCG 612
L I Q C D V Q L W K G W F E A N K 204

AATAAGATGACGCCACCCAATAATTATGGCCTTCGATTTGACCGTAGCTTT 669
M T P P N N Y G L R F D R S F M A 221

ATGGCGATTGCTGCCGCAGTTGACGGCTTGGGAGTTGTGCTTGAATCAAAA 726
I A A A V D G L G V V L E S K L L 238

CTACTGGCTGAACGTGAAATCGCTAGCGGTAAACTGGTATGCCCACTTGTA 783
A E R E I A S G K L V C P L V N S 255

AACAGTACCAGCGAAATACATTACATCGGTCATTATTTGGTTTTCCCCCAG 834
T S T S E I H Y I G H Y L V F P Q 272

CACCAACACATGCATTCTGCGCTTGATGTGTTCAAAACCTGGCTACTGAAT 885
H Q H M H S A L D V F K T W L L N 289

GAACTCAATCTGGGTAAAATTCGTTGATCATGTTCACATTAATCGGGGCTC 936
E L N L G K I R 297

Figure 4.7 Sequence comparison of PerR with other LysR type proteins. The *E. coli* PerR sequence (middle, bolded) is compared to AmpR from *Rhodobacter (Rhodopsuedomonas) capsulatus* (bottom) and PenR from *Burkholderia (Psuedomonas) cepacia* (top, italicized). One letter amino acid symbols are used, dashes represent gaps generated by the BLAST computer program to optimize homology, and the underlined sequence represents an area of nonspecific homology originally omitted from the Blast-p search to minimize nonspecific matches. A vertical line represents homologous matches whereas other symbols represent conserved residues based on the R-group. Specifically, !, ^, * and o indicate conservation based on hydrophobic, hydrophyllic, small-sized and aromatic R-groups, respectively. Boxed residues 1-66 region shows the most highly conserved region and the highlighted region from residues 23-42 is predicted to contain a helix-turn-helix DNA-binding motif as suggested by Henikoff (1988).

```
B. cepacia      3   KLRPHLPLNALRAFESSARHL[...]RAL      46
                    ||    ||| |||||**!|^![...]| |
E. coli         1  MKLLAKAPLNLLRAFEAAGRTG[...]RKL      45
                          ||| || ||*[ |^|[...]^|
R. capsulatus   7         PLNALRVFEVAMRQG[...]ARL      44

B. cepacia         EERLGCALFTRLPRGLDLTDEGRALLPVLSDAFSRIETVL-QQFD     90
                   |  |   || |  | ! || || ||  !   |^ !^   |
E. coli            ENLLDVRLFQRSTREITLTKEGEILLEHIQRGFNELQQGL-ALVT     89
                   |^||   || |^^^ !  | || !|    !^ ||^ ! ^ |  |
R. capsulatus      EDLLGTALFLRTSQCLIPTDEGRLLFPVLEHGFDAMSRVLDRLGG     89

B. cepacia         GGRLRQVLTLGVVGTFALGWLMPRLKRFRDTHPFVELRLRTHNNV    135
                       |  | |     ^||  ||!||| ^|   ^| !^|||
E. coli            ADESRP-LRLHTAPSFAHQWLLPRLGKFIRENPSIDLRLSASTEY    133
                    ^    |^!     ^||  ||!|||  | ^ ^| ||||!| ^
R. capsulatus      RRDIEV-LKVGVNTTFAMCWLMPRLEAFRQAHPQIDLRISTNNNR    133

B. cepacia         VDLAAEGLDFAIRFGQGNQPATRNERLFDAPLTALCAPEIARRLT    180
                       ^  |  | o|^         |     || ||*|^!| ||
E. coli            ARFEQDDFDLDIVYGEPRPSPYEKIPLAVEELTPLCSPQLAERLK    178
                      ^^  |! | o|    ^ ^^ ||||   ! |||*| || ||
R. capsulatus      VEILREGLDMAIRFGTGGWTGHDAIPLAEAPMAPLCAPGLASRLL    178

B. cepacia         QPADLAHETLLR-SYRTDEWLGWFDAAQLEPWTVNGPVFDSSRLM    224
                   ^| ||   ||!^   ^  ^| |||^| ^! |    |  || | !
E. coli            KPEDLYALTLIQCDVQLYQWKGWFEANKMTPPNNYGLRFDRSFMA    223
                    | ||  !||!^   ^  ^| |||||  !  |    |  || |
R. capsulatus      HPSDLGQVTLLR-SYRSAEWPGWFEAAGVPCPPVTGPVFDSSVAL    222

B. cepacia         VEAAMQGMGIALAPACMFERELQLGLLARPL---DIDVHAGGYWL    266
                   ! ||! |!|! |     ! |||!  |  | ||     ^!|  |^o|
E. coli            IAAAVDGLGVVLESKLLAEREIASGKLVCPLVNSTSEIHYIGHYL    268
                      |  | || |     ! |  || |^|  |   ^ |   |   ||
R. capsulatus      AELATSGAGVALLPISMFESYIAQGRLAQPFGVTVSVGRY---YL    264

B. cepacia         TSLKSKSLTPAMTLFRDWITAE              288
                     ^ ^ !  |! !|^ |!  |
E. coli            VFPQHQHMHSALDVFKTWLLNELNLGKIR       297
                   !|  ^    ||!   |  ||  ^
R. capsulatus      AWPSDRPATSAMSTFSRWLTGQ              286
```

5×10^{-27}), a transcriptional activator of the glycine cleavage enzyme system (Wilson *et al.*, 1993) and DsdC (e value 1×10^{-27}), a transcriptional activator of the D-serine deaminase operon (McFall and Heincz, 1983). These proteins from *E. coli* are over 30% homologous (GcvA, 34.5%; DsdC, 33.4) and more than 50% similar (GcvA, 52.1%;DsdC, 52.1%).

DISCUSSION

5.0 DISCUSSION

5.1 Summary

This study investigated a locus thought to play a role in the H_2O_2 resistance of stationary phase *E. coli*, tentatively called *perR* (P. Loewen, pers. comm.). Preliminary studies had suggested the locus in the vicinity of *proA*, and this was confirmed by three factor transductional crosses relative to *proA* (Figure 4.1). Unfortunately, transductional mapping offers only a crude estimation of map distances and it was not possible to precisely identify the ORF from the data, given the large number of uncharacterized ORFs in the region. Furthermore, attempts to isolate a *perR*::Tn*10* mutant were unsuccessful, possibly a result of inappropriate recipient strains (Section 4.2). A likely candidate for *perR* was identified by surveying the loci around the Tn*10* insertion of UM367 which revealed an ORF with significant similarity to LysR-type regulatory genes. This ORF (*orf307*) was targeted and a mini-Tn*10* cassette was introduced by molecular techniques (Sections 4.3 and 4.4). Sensitivity of the *orf307*::Tn*10* containing strain to H_2O_2 confirmed the role of the locus in modulating peroxide resistance and *orf307* was named *perR*.

The predicted amino acid sequence of PerR is very similar to the sequences of other LysR-type transcriptional regulators, suggesting a regulatory function for the *perR* gene product. LysR-type transcriptional regulators are approximately 300 amino acids in length and share areas of high conservation, predominately the helix-turn-helix motif close to the amino end between residues 23 – 42 (Henikoff, 1988; Schell, 1993). They are

involved in the regulation of a wide variety of systems. Thus, it is conceivable that PerR may regulate genes that confer hydrogen peroxide resistance on the cell in stationary phase rather than influence H_2O_2 resistance directly.

Other LTTRs are observed to share characteristics such as coinduction of and divergent transcription from target genes. Although, no evidence for these traits exists to date for PerR, it is interesting to predict the findings of such future studies. Given that wild type cells survive a H_2O_2 challenge, and PerR-deficient cells do not, it is easy to speculate that hydrogen peroxide is the PerR coinducer molecule. Alternatively, H_2O_2 may oxidize PerR for activation, as is observed with another LTTR, the exponential-phase oxidative stress mediator, OxyR (Storz *et al.*, 1990).

LTTR regulatory regions are frequently similar to and often shared with their divergently transcribed target gene as for AmpR and PenR (Campbell *et al.*, 1989; Trépanier *et al.*, 1997). However, this does not seem to be the case for PerR as an examination of the region as reported in Genbank indicates that insertion sequence IS30 is directly upstream. This is not a rigid criterion, as other LTTRs, such as OxyR, are not linked to their target genes (Schell, 1993). Nevertheless, the *perR* regulatory region does differ from the regulatory regions of other LTTR in having over 350 bp between the AUG codon of the ORF and the transcriptional start site.

The following two conclusions are the main points of this study:

1. The *perR*-knockout strain UM397 has a 13.6 fold decrease in H_2O_2 resistance in stationary phase when compared to the parent strain

suggesting that PerR is involved in oxidative stress resistance in stationary phase.

2. The predicted sequence of the PerR protein is highly conserved among LysR-type transcriptional regulators suggesting a regulatory role for the protein.

As previously mentioned, multiple oxidative stress regulons exist in *E. coli*, namely SoxRS and OxyR. From the conclusions of this study, one can speculate that PerR may be implicated as a stationary phase regulon that responds to oxidative stress. Although this study does not provide evidence that PerR does not protect against H_2O_2 challenge in logarithmic phase, previous findings that neither strain MV1161 nor MV2640 survive when exposed to H_2O_2 in mid-logarithmic phase (Volkert *et al.*, 1994) suggests that PerR does not have a role in oxidative stress protection in growth phase.

While RpoS is not the sole-controlling factor, most stationary phase operons and genes are controlled by some combination of RpoS and additional regulatory factors, such as cAMP-CRP, Lrp or H-NS. Thus, it is suggested that *perR* is likely regulated by σ^s and other factors. Furthermore, it is hypothesized that genes previously shown to be regulated separately by σ^s such as *dps* (Altuvia *et al.*, 1992), *sodC* (Gort *et al.*, 1999), *katE* (Loewen *et al.*, 1985a), *xthA* (Sak *et al.*, 1989) and *gor* (Becker-Hapak and Eisenstark, 1995) may be indirectly regulated by σ^s via the PerR operon. This is suggestive of PerR activation of both pre- and post-damage mechanisms for oxidative stress, but does not exclude the activation or repression of other

related and unrelated genes.

As discussed in Section 2.3.4, little is understood about the interaction or independence of oxidative stress defenses, especially at the regulon level. Recently however, OxyS RNA, a negative regulator of *rpoS* (Altuvia *et al.*, 1997), was demonstrated to be controlled by the OxyR regulon (Gonzalez-Flecha and Demple, 1999), suggesting a mechanism whereby the cell selects a growth phase dependent oxidative stress regulon. That is, if OxyR is induced by protein oxidation in exponential phase (Storz *et al.*, 1990) transcription of the OxyR regulon genes ensues, including the OxyS RNA molecule known to negatively regulate *rpoS* via Hfq (Zhang *et al.*, 1998). Consequently, many σ^s-dependent genes, potentially including the PerR regulon, will not be transcribed. Presumably, some similar mechanism will work to effectively inactivate the OxyR regulon when PerR is induced.

5.2 Future Perspectives

The characterization of *perR*, based on phenotype and sequence analyses, mediates oxidative stress in stationary phase, provides a springboard for a plethora of future initiatives. Purification of the overexpressed PerR generated by a protein fusion to an inducible promoter could aid in characterization of the gene product. Confirmation of the suggested regulatory function for PerR could be examined by comparing the differential display profiles of wild-type and mutant *perR* strains MG1655 and UM397. Alternatively, any individual gene suspected to be regulated by PerR could be fused to a *lacZ* promoter and observed for differences in β-

galactosidase expression in *perR* wild-type and mutant backgrounds. Once the loci of such PerR-regulated genes are known, elucidation of probable PerR binding and potential PerR consensus sequences via gel mobility shift and DNAse I protection assays should be performed. Additionally, modification of target gene promoter regions by site-directed mutagenesis could offer information about the necessity of potential consensus sequences.

Still unanswered are the questions pertaining to the regulation of *perR*. As many LysR-type transcriptional regulators are autoregulated, this should be investigated by generating a *perR*::*lacZ* fusion and monitoring β-galactosidase expression in *perR* wild-type and mutant backgrounds. As well, the induction of PerR by protein oxidation should be explored as was done with OxyR (Storz *et al.*, 1990); this potentiality should be explored via the same method in PerR. Lastly, an attempt to understand the role of *perR* in a global sense should also be undertaken. SpvR, another LTTR from *S. typhimurium* required for spleenic invasion in stationary phase cells is RpoS-regulated (Fang *et al.*, 1992; Norel *et al.*, 1993; Kowarz *et al.*, 1994). Since, *perR* may be induced in stationary phase, the potential role of RpoS should be examined by comparing β-galactosidase expression of the *perR*::*lacZ* mutants in *rpoS* wild-type and mutant backgrounds. If expression is found to be RpoS-mediated, investigations into other potential regulators in the RpoS regulatory network could be examined similarly. Eventually, an investigation of the proposed mechanism whereby the cell selects a growth phase dependent oxidative stress regulon will be required.

REFERENCES

6.0 REFERENCES

Aldea, M., Garrido, T., Pla, J., and M. Vicente. 1990. Division genes in *Escherichia coli* are expressed coordinately to cell septum requirements by gearbox promoters. *EMBO J.* **9**:3787-3794.

Almirón, M., Link, A. J., Furlong, D., and R. Kolter. 1992. A novel DNA binding protein with regulatory and protective roles in starved *Escherichia coli. Genes Dev.* **6**:2646-2654.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, A., Miller, W., and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nuc. Acids. Res.* **25**:3389-3402.

Altuvia, S., Almirón, M., Huisman, G., Kolter, R., and G. Storz. 1994. The *dps* promoter is activated by OxyR during growth and by IHF and σ^s in stationary phase. *Mol. Microbiol.* **13**:265-272.

Altuvia, S., Weinstein-Fisher, D., Zhang, A., Postow, L., and G. Storz. 1997. A small, stable RNA induced by oxidative stress: role as a pleiotropic regulator and antimutator. *Cell* **90**:43-53.

Altuvia, S., Zhang, A., Argaman, L., Tiwari, A. and G. Storz. 1998. The *Escherichia coli* OxyS regulatory RNA represses *fhlA* translation by blocking ribosome binding. *EMBO J.* **17**:6069-6075.

Amabile-Cuevas, C. F., and B. Demple. 1991. Molecular characterization of the *soxRS* genes of *Escherichia coli*: two genes control a superoxide stress regulon. *Nuc. Acids Res.* **19**:4479-4484.

Anathaswamy, H. N., and A. Eisenstark. 1977. Repair of hydrogen peroxide-induced single-strand breaks in *Escherichia coli* deoxyribonucleic acid. *J. Bacteriol.* **130**:187-191.

Antonini, E., and M. Brunori. 1970. Catalytic mechanism of cytochrome oxidase. *Nature* **228**:936-937.

Apontoweil, P., and W. Berends. 1975. Isolation and initial characterization of glutathione-deficient mutants of *Escherichia coli* K12. *Biochim. Biophys. Acta.* **399**:10-12.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Siedman, J. G, Smith, J. A., and K. Struhl. 1989. Current Protocols in Molecular Biology, Green Publishing Assoc., Inc., and John Wiley and Sons, Inc. New York, N. Y.

Aviv, M., Giladi, H., Schreiber, G., Oppenheim, A. B., and G. Giaser. 1994. Expression of the genes coding for the Escherichia coli integration host factor are controlled by growth phase, *rpoS*, ppGpp and by autoregulation. *Mol. Microbiol.* **14:**1021-1031.

Ballestros, M., Kusano, S., Ishihama, S., and M. Vicente. 1998. The *ftsQ1p* gearbox promoter of *Escherichia coli* is a major σ^s-dependent promoter in the *ddlB-ftsA* region. *Mol. Microbio.* **30:**419-30.

Barth, M., Marshcall, C., Muffler, A., Fischer, D., and R. Hengge-Aronis. 1995. Role for the histone-like protein H-NS in growth phase dependent and osmotic regulation of σ^s and many σ^s-dependent genes in *Escherichia coli. J. Bacteriol.* **177:**3455-3464.

Battistoni, A. and G. Rotilio. 1995. Isolation of an active heat-stable monomeric form of Cu,Zn superoxide dismutase from the periplasmic space of *Escherichia coli. FEBS Lett.* **374:**199-202.

Battistoni, A., Folcarelli, S., Rotilio, G., Capasso, C., Pesce, A., Bolognesi, M., and A. Desideri. 1996. Crystallization and preliminary X-ray analysis of the monomeric Cu, Zn superoxide dismutase from *Escherichia coli. Prot. Sci.* **5:**2125-2127.

Becker-Hapak, M. and A. Eisenstark. 1995. Role of *rpoS* in the regulation of glutathione oxidoreductase (*gor*) in *Escherichia coli. FEMS Microbiol.* Lett. **134**:39-44.

Bender, J. and N. Kleckner. 1992. IS10 transposase mutations that specifically alter target site recognition. *EMBO J.* **11:**741-750.

Benov, L., Chang, L. Y., Day, B., and I. Fridovich. 1995. Copper, zinc superoxide dismutase in *Escherichia coli*: Periplasmic localization. *Arch. Biochem. Biophys.* **319:**508-511.

Benov, L., and I. Fridovich. 1996. Functional significance of the Cu,ZnSOD in *Escherichia coli. Arch. Biochem. Biophys. 327:*249-253.

Benov, L. T., Beyer, Jr., W. F., Stevens, R. D., and I. Fridovich. 1996. Purification and characterization of the Cu,Zn SOD from *Escherichia coli. Free Rad. Biol. Med.* **21:**117-121.

Benov, L., Sage, H. and I. Fridovich. 1997. The copper- and zinc-containing superoxide dismutase from *Escherichia coli*: Molecular weight and stability. *Arch. Biochem. Biophys.* **340:**305-310.

Bohannon, D. E., Connell, N., Keener, J., Tormo, A., Espinosa-Urgel, M., Zambrano, M. M., and R. Kolter. 1991. Stationary-phase-inducible "gearbox" promoters: differential effects of *katF* mutations and the role of σ^{70}. *J. Bacteriol.* **173:**4482-4492.

Böhringer, J., Fischer, D., Lucassen, M., Jung, K., and R. Hengge-Aronis. 1995. UDP-glucose is a potential intracellular signal in the control of expression of σ^{s} and σ^{s}-dependent genes in *Escherichia coli. J. Bacteriol.* **177:**413-422.

Bravo, J., Verdaguer, N., Tormo, J., Betzel, C., Switala, J., Loewen, P. C., and I. Fita. 1995. Crystal structure of catalase HPII from *Escherichia coli. Structure.* **3:**491-502.

Breimer, L. H., and T. Lindahl. 1984. DNA glycosylase activities for thymine residues damaged by ring saturation, fragmentation, or ring contraction are functions of endonuclease III in *Escherichia coli. J. Biol. Chem.* **259:**5543-548.

Britton, L., and I. Fridovich. 1977. Intracellular localization of superoxide dismutases of *Escherichia coli*: a reevalution. *J. Bacteriol.* **131:**815 828.

Brunori, M., and G. Rotilio. 1984. Biochemistry of oxygen radical species. *Methods Enz.* **105:**22-35.

Campbell, A. 1965. The steric effect in lysogenization by bacteriophage lambda. I. Lysogenization of a partially diploid strain of *Escherichia coli. Virology.***27**:329-339.

Campbell, J. I. A., Scahill, S., Gibson, T., and R. P. Ambler. 1989. The phototrophic bacterium *Rhodopseudomonas capsulata* sp108 encodes an indigenous class A β-lactamase. *Biochem J.* **260:**803-812.

Carlsson, J., and V. S. Carpenter. 1980. The *recA+* gene product is more important than catalase and superoxide dismutase in protecting *Escherichia coli* against hydrogen peroxide toxicity. *J. Bacteriol.* **142:**319-321.

Chou, J. H., Greenberg, J. T., and B. Demple. 1993. Posttranscriptional repression of *Escherichia coli* OmpF protein in response to redox stress:Positive control of the *micF* antisense RNA by the *soxRS* locus. *J. Bacteriol.* **175:**1026-1031.

Christman, M. F., Morgan, R. W., Jacobson, F. S., and B. N. Ames. 1985. Positive control of a regulon for defenses against oxidative stress and some heat shock proteins in *Salmonella typhimurium*. *Cell* **41**:753-762.

Christman, M. F., Storz, G., and B. N. Ames. 1989. OxyR, a positive regulator of hydrogen peroxide-inducible genes in *Escherichia coli* and *Salmonella typhimurium*, is homologous to a family of bacterial regulatory protein. *Proc. Natl. Acad. Sci. U.S.A.* **86**:3483-3488.

Chiu, J. T., Loewen, P. C., and J. Switala. 1989. Proposed structure for the prosthetic group of the catalase HPII from *Escherichia coli*. *J. Am. Chem.Soc.* **111**:7046-7050.

Chung, C. T., Niemela, S. L., and R. H. Miller. 1989. One-step preparation of competent *Escherichia coli*: transform and storage of bacterial cells in the same solution. *Proc. Natl. Acad. Sci. U.S.A.* **86**:2172-5.

Claiborne, A., and I. Fridovich. 1979. Purification of the ortho-dianisidine peroxidase from *Escherichia coli* B. Physicochemical characterization and analysis of its dual catalytic and peroxidatic activities. *J. Biol. Chem.* **254**:4245-4252.

Claiborne, A., Malinowski, D. P., and I. Fridovich. 1979. Purification and characterization of hydroperoxidase II of *Escherichia coli* B. *J. Biol. Chem.* **254**:11664-11668.

Cunningham, R. P., and B. Weiss. 1985. Endonuclease III (*nth*) mutants of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **82**:474-478.

Cunningham, R. P., Saporito, S.M., Spitzer, S. G., and B. Weiss. 1986. Endonuclease IV (*nfo*) mutant of *Escherichia coli*. *J. Bacteriol.* **168**:1120-1127.

Davies, K. J. A., and M. E. Delsignore. 1987. Protein damage and degradation by oxygen radicals: general aspects. *J. Biol. Chem.* **262**:9908-9913.

Davies, K. J. A., Lin, S. W., and R. E. Pacifici. 1987. Protein damage and degradation by oxygen radicals: degradation of denatured proteins. *J. Biol. Chem.* **262**:9914-9920

Demple, B. 1991. Regulation of bacterial oxidative stress genes. *Ann. Rev. Genet.* **25**:315-337.

Demple, B., and J. Halbrook. 1983. Inducible repair of oxidative DNA damage in *Escherichia coli*. *Nature* **304**:466-468.

Demple, B., Halbrook, J., and S. Linn. 1983. *Escherichia coli xth* mutants are hypersensitive to hydrogen peroxide. *J. Bacteriol.* **153**:1079-1082.

Dersch, P., Schmidt, K., and E. Bremer. 1993. Synthesis of the *Escherichia coli* K-12 nucleoid-associated DNA-binding protein H-NS is subjected to growth-phase control and autoregulation. *Mol. Microbiol.* **8**:875-889.

Eichel, J., Chang, Y-Y., Riesenberg, D., and J. E. Cronan, Jr. 1999. Effect of ppGpp on *Escherichia coli* cyclopropane fatty acid synthesis is mediated through the RpoS sigma factor (σ^S). *J. Bacteriol.* **181**:572 576.

Eisenstark, A., Calcutt, M. J., Becker-Hapak, M., and A. Ivanova. 1996. Role of *Escherichia coli rpoS* and associated genes in defense against oxidative stress. *Free Rad. Biol. Med.* **21**:975-993.

Espinosa-Urgel, M., and A. Tormo. 1993. σ^S-dependent promoters in *Escherichia coli* are located in DNA regions with intrinsic curvature. *Nucleic Acids Res.* **21**:3667-3670.

Espinosa-Urgel, M., Chamizo, C., and A. Tormo. 1996. A consensus structure of σ^S-dependent promoters. *Mol. Microbiol.* **21**:657-659.

Fang, R. C., Libby, S. J., Buchmeier, N. A., Loewen, P. C., Switala, J, Harwood, J., and D. G. Guiney. 1992. The alternative σ factor KatF (RpoS) regulates *Salmonella* virulence. *Proc. Natl. Acad. Sci. USA.* **89**:11978-11982.

Fang, R. C., Chen, C-Y., Guiney, D. G., and Y. Xu. 1996. Identification of σ^S regulated genes in *Salmonella typhimurium*: Complementary regulatory interactions between σ^S and cyclic AMP receptor protein. *J. Bacteriol.* **178**:5112-5120.

Farewell, A., Kvint, K., and T. Nystrom. 1998. *uspB*, a new σ^S-regulated gene in *Escherichia coli* which is required for stationary-phases resistance to ethanol. *J. Bacteriol.* **180**:6140-6147.

Farr, S. B., and T. Kogoma. 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. *Microbiol. Rev.* **55**:561-585.

Fridovich, I. 1977. Oxygen is toxic! *Bioscience* **27**:462-466.

Fridovich, I. 1978. The biology of oxygen radicals. *Science* **201**:875-880.

Fridovich, I. 1976. Oxygen radicals, hydrogen peroxide, and oxygen toxicity. In: Pryor, W. A. (ed). Free Radicals in Biology, Vol. I. Academic Press, Inc. New York. 239-277.

Friedman, D. I. 1988. Intergration host factor: a protein for all reasons. *Cell* **55**:545-554.

Gentry, D. R., Hernandez, V. J., Nguyen, L. H., Jensen, D. B., and M. Cashel. 1993. Synthesis of the stationary phase sigma factor σ^s is positively regulated by ppGpp. *J. Bacteriol.* **175**:7982-7989.

Gibson, K. E., and T. J. Silhavy. 1999. The LysR homolog LrhA promotes RpoS degradation by modulating activity of the response regulator SprE. *J. Bacteriol.* **181**:563-571.

Gonzalez-Flecha, B., and B. Demple. 1999. Role for the *oxyS* gene in regulation of intracellular hydrogen peroxide in *Escherichia coli*. *J. Bacteriol.* **181**:3833-3836.

Gort, A. S., Ferber, D. M., and J. A. Imlay. 1999. The regulation and role of the periplasmic copper, zinc superoxide dismutase of *Escherichia coli*. *Mol. Microbiol.* **32**:179-91.

Greenberg, J. T., and B. Demple. 1986. Glutatione in *Escherichia coli* is dispensable for resistance to H_2O_2 and gamma radiation. *J. Bacteriol.* **168**:1026-1029.

Greenberg, J. T., and B. Demple. 1988. Overproduction of peroxide scavenging enzymes in *Escherichia coli* suppresses spontaneous mutagenesis and sensitivity to redox-cycling agents in *oxyR* mutants. *EMBO J.* **7**:2611-2617.

Greenberg, J. T., and B. Demple. 1989. A global response induced in *Escherichia coli* by redox-cycling agents overlaps with that induced by peroxide stress. *J. Bacteriol.* **171**:3933-3939.

Greenberg, J. T., Monach, P., Chou, J.H., Josephy, P. D., and B. Demple. 1990. Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **87**:6181-6185.

Gregory, E. M., Yost,F. J., and I. Fridovich. 1973. Superoxide dismutases of *Escherichia coli*: intracellular localization and functions. *J. Bacteriol.* **115**:987-991.

Groat, R. G., J. E. Schultz, E. Zychlinski, E., Bockman, A. T., and A. Matin. 1986. Starvation proteins in *Escherichia coli*: kinetics of synthesis and role in starvation survival. *J. Bacteriol.* **168**:486-493.

Gruer, M. J., and J. R. Guest. 1994. Two genetically-distinct and differentially- regulated aconitases (AcnA and AcnB) in *Escherichia coli*. *Microbiol.* **140**:2531-2541.

Guyer, M. S., Reed, R. E., Steitz, T., and K. B. Low. 1981. Identification of a sex factor-affinity site in *E. coli* as gamma delta. *Cold Spr. Harb. Symp. Quant. Biol.* **45**:135-140.

Hagensee, M. E., and R. E. Moses. 1986. Repair response of *Escherichia coli* to hydrogen peroxide DNA damage. *J. Bacteriol.* **168**:1059-1065.

Halliwell, B., and J. M. C. Gutteridge. 1986. Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch. Biochem. Biophys.* **246**:501-514.

Halliwell, B., and J. M. C. Gutteridge. 1989. Free Radicals in Biology and Medicine. Oxford University Press, New York.

Hassan, H. M. 1988. Biosynthesis and regulation of superoxide dismutases. *Free Radicals Biol. Med.* **5**:377-385.

Hengge-Aronis, R. 1993. Survival of hunger and stress: the role of *rpoS* in stationary phase gene regulation in *Escherichia coli*. *Cell* **72**:165-168.

Hengge-Aronis, R. 1996. Regulation of gene expression during entry into stationary phase., p.1496-1512. *In* Frederick C. Niedhart (ed.), *Escherichia coli* and Salmonella: Cellular and Molecular Biology. ASM Press, Washington, D.C.

Hengge-Aronis, R, and D. Fischer. 1992. Identification and molecular analysis of *glgS*, a novel growth-phase-regulated and *rpoS*-dependent gene involved in glycogen synthesis in *Escherichia coli*. *Mol. Microbiol.* **6**:1877-1886.

Henikoff, S., Haughn, G. W., Calvo, J., and J. C. Wallace. 1988. A large family of bacterial activator proteins. *Proc. Natl. Acad. Sci. U.S.A.* **85**:6602-6606.

Hillar, A., Van Caeseele, L., and P. Loewen. 1999. Intracellular location of catalase-peroxidase hydroperoxidase I of *Escherichia coli*. *FEMS Microbiol. Lett.* **170**:307-312.

Huisman, G. W., and R. Kolter. 1994. Sensing starvation: a homoserine lactone dependent signaling pathway in *Escherichia coli*. *Science* **265**:537-539.

Ichikawa, J. K., Li, C., Fu, J., and S. Clarke. 1994. A gene at 59 minutes on the *Escherichia coli* chromosome encodes a lipoprotein with unusual amino acid repeat sequences. *J. Bacteriol.* **176**:1630-1638.

Imlay, K. R. C., and J. A. Imlay. 1996. Cloning and analysis of *sodC*, encoding the copper-zinc superoxide dismutase of *Escherichia coli*. *J. Bacteriol.* **178**:2564-2571.

Imlay, J. A., and I. Fridovich, 1991. DNA damage by H_2O_2 through the Fenton reaction *in vivo* and *in vitro*. *Science* **240**:640-642.

Ivanova, A. B., Glinsky, G. V., and A. Eisenstark. 1997. Role of *rpoS* regulon in resistance to oxidative stress and near UV radiation in *ΔoxyR* suppressor mutants of *Escherichia coli*. *Free Radicals Biol. Med.* **23**:627-636.

Ivanova, A., Miller, C., Glinsky, G., and A. Eisenstark. 1994. Role of *rpoS* (*katF*) in *oxyR*-independent regulation of hydroperoxidase I in *Escherichia coli*. *Mol. Microbiol.* **12**:571-578.

Jacobson F. S., Morgan, R. W., Christman, M. F., and B. N. Ames. 1989. An alkyl hydroperoxidase reductase from *Salmonella typhimurium* involved in the defense of DNA against oxidative protein damage: purification and properties. *J. Biol. Chem.* **264**:1488-1496.

Jishage, M., and A. Ishihama. 1995. Regulation of RNA polymerase sigma subunit synthesis in *Escherichia coli*: intracellular levels of σ^{70} and σ^{38}. *J. Bacteriol.* **177**:6832-6835.

Keele Jr., B. B., McCord, J. M., and I. Fridovich. 1970. Superoxide dismutase from *Escherichia coli* B: a new maganese-containing enzyme. *J. Biol. Chem.* **245**:6176-6181.

Klauck, E., Bohringer, J., and R. Hengge-Aronis. 1997. The LysR-like regulator LeuO in *Escherichia coli* is involved in the translational regulation of *rpoS* by affecting the expression of the small regulatory DsrA-RNA. *Mol. Microbiol.* **25**:559-569.

Klebanoff, S. J. 1988. Phagocytic cells products of oxygen metabolism in inflammation: Basic Principles and Clinical Correlations. pp 391-444. Raven, New York.

Kleckner, N., Barker, D. F., Ross, D. B., and D. Botstein. 1978. Properties of the translocatable tetracycline-resistance element – Tn*10* in *Escherichia coli* and bacteriophage lambda. *Genetics.* **90**:427-461.

Kleckner, N., Bender, J., and S. Gottesman. 1991. Uses of transposons with emphasis on Tn*10*. *Methods Enzymol.* **204**:139-180.

Kogoma, T., Farr, S. B., Joyce, K. M., and D. O. Natvig. 1988. Isolation of gene fusions (*soi::lacZ*) inducible by oxidative stress in *Escherichia coli. Proc. Natl. Acad. Sci. U.S.A.* **85**:4799-4803.

Koh, Y-S., and J-H. Roe. 1995. Isolation of a novel paraquat-inducible (*pqi*) gene regulated by the *soxRS* locus in *Escherichia coli. J. Bacteriol.* **177**:2673-2678.

Kohara, Y., Akiyama, K., and K. Isono. 1987. The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. *Cell* **50**:495-508.

Kolter, R., Siegele, D.A., and A. Tormo. 1993. The stationary phase of the bacterial life cycle. *Annu. Rev. Microbiol.* **47**:855-874.

Kowarz, L., Coynault, c., Robbe-Saule, B., and F. Norel. 1994. The *Salmonella typhimurium* katF (rpoS) gene: Cloning, nucleotide sequence, and regulation of *spvR* and *spvABCD* virulence plasmid genes. *J. Bacteriol.* **176**:6852-6860.

Kunkel, T. A., Roberts, J. D., and R. A. Zakour. 1987. Rapid and efficient site specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**:367-382.

Kusano, S., and A. Ishihama. 1997. Stimulatory effect of trehalose on formation and activity of Escherichia coli RNA polymerase $E\sigma^{38}$ holoenzyme. *J. Bacteriol.* **179**:3649-3654.

Landini, P., Hajec, L. I., Nguyen, L. H., Burgess, R. R., and M. R. Volkert. 1996. The leucine-responsive regulatory protein (Lrp) acts as a specific repressor for σ^s-dependent transcription of the *Escherichia coli aidB* gene. *Mol. Microbiol.* **20**:947-955.

Lange, R., and R. Hengge-Aronis. 1991a. Growth phase-regulated expression of *bolA* and morphology of stationary-phase *Escherichia coli* cells are controlled by the novel sigma factor σ^s. *J. Bacteriol.* **173**:4474-4481.

Lange, R., and R. Hengge-Aronis. 1991b. Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. *Mol. Microbiol.* **5**:49-59.

Lange, R., and R. Hengge-Aronis. 1994a. The *nlpD* gene is located in an operon with *rpoS* on the *Escherichia coli* chromosome and encodes a novel lipoprotein with a potential function in cell wall formation. *Mol. Microbiol.* **13**:733-743.

Lange, R., and R. Hengge-Aronis. 1994b. The cellular concentration of the subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation and protein stability. *Genes Dev.* **8**:1600-1612.

Lange, R., Barth, M., and R. Hengge-Aronis. 1993. Complex transcriptional control of the σ^s-dependent stationary-phase-induced and osmotically regulated *osmY* (*csi-5*) gene suggests novel roles for Lrp, cyclic AMP (cAMP) receptor protein-cAMP complex, and integration host factor in the stationary-phase response of *Escherichia coli*. *J. Bacteriol.* **175**:7910-7917.

Lange, R., Fischer, D., and R. Hengge-Aronis. 1995. Identification of transcriptional start sites and the role of ppGpp in the expression of *rpoS*, the structural gene for the σ^s subunit of RNA polymerase in *Escherichia coli*. *J. Bacteriol.* **177**:4676-4680.

Lease, R. A., Cusick, M. E., and M. Belfort. 1998. Riboregulation in *Escherichia coli*: DsrA RNA acts by RNA:RNA interactions at multiple loci. *Proc. Natl. Acad. Sci. U.S.A.* **95**:12456-12461.

Link, A. J., Phillips, D. R., and G. M. Church. 1997. Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: Application to open reading frame characterization. *J. Bacteriol.* **179**:6228-6237.

Liochev, S. I., and I. Fridovich. 1992a. Effects of overproduction of superoxide dismutases in *Escherichia coli* on inhibition of growth and on induction of glucose-6-phospate dehydrogenase by paraquat. *Arch. Biochem. Biophys.* **294**:138-143.

Liochev, S. I., and I. Fridovich. 1992b. Fumarase C, the stable fumarase of *Escherichia coli*, is controlled by the *soxRS* regulon. *Proc. Natl. Acad. Sci. U.S.A.* **89**:5892-5896.

Liochev, S. I., Hausladen, A., Beyer, Jr., W. F., and I. Fridovich. 1994. NADPH:ferredoxin oxidoreductase acts as a paraquat diaphorase and

is a member of the *soxRS* regulon. *Proc. Natl. Acad. Sci. U.S.A.* **91**:1328-1331.

Ljungquist, S. 1977. A new endonuclease from *Escherichia coli* acting at apurinic sites in DNA. *J. Biol. Chem.* **252**:2808-2814.

Loewen, P. C. 1984. Isolation of catalase-deficient *Escherichia coli* mutants and genetic mapping of *katE*, a locus that affects catalase activity. *J. Bacteriol.* **157**:622-626.

Loewen, P. C. 1997. Oxidative stress and the molecular biology of antioxidant defenses. Cold Spring Harbour Lab Press, New York.

Loewen, P. C., and R. Hengge-Aronis. 1994. The role of the sigma factor σ^S (KatF) in bacterial global regulation. *Annu. Rev. Microbiol.* **48**:53-80.

Loewen, P. C., and J. Switala. 1986. Purification and characterization of catalase HPII from *Escherichia coli* K12. *Biochem. Cell Biol.* **64**:638-646.

Loewen, P. C., and B. L. Triggs. 1984. Genetic mapping of *katF*, a locus that with *katE* affects the synthesis of a second catalase species in *Escherichia coli. J. Bacteriol.* **160**:668-675.

Loewen, P. C., Hu, B., Strutinsky, J., R. Sparling. 1998. Regulation in the *rpoS* regulation of *Escherichia coli. Can. J. Microbiol.* **44**:707-717.

Loewen, P. C., Switala, J., and B. L. Triggs-Raine. 1985a. Catalases HPI and HPII in *Escherichia coli* are induced independently. *Arch. Biochem. Biophys.* **243**:144-149.

Loewen, P. C., Triggs, B. L., George, C. S., and B. E. Hrabarchuk. 1985b. Genetic mapping of *katG*, a locus that affects synthesis of the bifunctional catalase-peroxidase hydroperoxidase I in *Escherichia coli. J. Bacteriol.* **162**:661-667.

Loewen, P.C., Switala, J., von Ossowski, I., Hillar, A., Christie, A., Tattrie, B., and P. Nicholls. 1993a. Catalase HPII of *Escherichia coli* catalyzes the conversion of protoheme to cis-heme d. *Biochem.* **32**:10159-10164.

Loewen, P. C., von Ossowski, I., Switala, J., and M. R. Mulvey. 1993b. KatF (σ^S) synthesis in *Escherichia coli* is subject to posttranscriptional regulation. *J. Bacteriol.* **175**:2150-2153.

Majdalani, N., Cunning, C., Sledjeski, D., Elliott, T., and S. Gottesman. 1998. DsrA RNA regulates translation of RpoS message by an

anti-antisense mechanism, independent of its action as an antisilencer of transcription. *Proc. Natl. Acad. Sci. U.S.A.* **95:**12462-12467.

McCann, M. P., Kidwell, K. P., and A. Matin. 1991. The putative σ factor KatF has a central role in development of starvation-mediated general resistance in *Escherichia coli. J. Bacteriol.* 173:4188-4194.

McCann, M. P., Fraley, C. D., and A. Matin. 1993. The putative σ^s factor KatF is regulated posttranscriptionally during carbon starvation. *J. Bacteriol.* **175:**2143-2149.

McFall, E., and M. C. Heincz. 1983. Indentification and control of synthesis of the *dsdC* activator protein. *J. Bacteriol.* **153:**872-877.

Mead, D. A., Skorupa, E. S., and B. Kemper. 1985. Single-stranded DNA SP6 promoter plasmids for engineering mutant RNAs and proteins: synthesis of a "stretched" preparathyroid hormone. *Nuc. Acids Res.* **13:**1103-1118.

Meister, A., and M. E. Anderson. 1983. Glutathione. *Annu. Rev. Biochem.* **52:**711-760.

Miller, J. H. 1972. Experiments in Molecular Genetics, Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

Mito, S., Zhang, Q-M., and S. Yonei. 1993. Isolation and characterization of *Escherichia coli* strains containing new gene fusions (*soi::lacZ*) inducible by superoxide radicals. *J. Bacteriol.* **175:**2645-2651.

Morgan, R. W., Christman, M. F., Jacobson, F. S., Storz, G., and B. N. Ames. 1986. Hydrogen peroxide-inducible proteins in *Salmonella typhimurium* overlap with heat shock and other stress proteins. *Proc. Natl. Acad. Sci. U.S.A.* **83:**8059-8063.

Muffler, A., Fischer, S., Altuvia, S., Storz, G., and R. Hengge-Aronis. 1996a. The response regulator RssB controls stability of the σ^s subunit of RNA polymerase in *Escherichia coli. EMBO J.* **15:**1333-1339.

Muffler, A., Fischer, D., and R. Hengge-Aronis. 1996b.The RNA binding protein HF-1, known as a host factor for phage Qβ RNA replication, is essential for *rpoS* translation in *Escherichia coli. Genes Dev.* **10:**1143 1151.

Muffler, A., Traulsen, D. D., Lange, R., and R. Hengge-Aronis. 1996c. Posttranscriptional osmotic regulation of the σ^s subunit of RNA polymerase in *Escherichia coli*. *J. Bacteriol.* **178:**1607-1613.

Muffler, A., Barth, M., Marschall, C., and R. Hengge-Aronis. 1997. Heat Shock regulation of σ^s turnover: a role for DnaK and relationship between stress responses mediated by σ^s and σ^{32} in *Escherichia coli*. *J. Bacteriol.* **179:**445-452.

Mukhopadhyay, S., and H. E. Schellhorn. 1994. Induction of *Escherichia coli* hydroperoxidase I by acetate and other weak acids. *J. Bacteriol.* **176**:2300-2307.

Mulvey, M. R., and P. C. Loewen. 1989. Nucleotide sequence of *katF* of *Escherichia coli* suggests KatF protein is a novel σ transcription factor. *Nucleic Acids Res.* **17:**9979-9991.

Mulvey, M. R., Sorby, P. A., Triggs-Raine B. L., and P. C. Loewen. 1988. Cloning and physical characterization of *katE* and *katF* required for catalase HPII expression in *Escherichia coli*. *Gene* **73:**337-345.

Mulvey, M. R., Switala, J., Borys, A., and P. C. Loewen. 1990. Regulation of transcription of *katE* and katF in *Escherichia coli*. *J. Bacteriol.* **172:**6713-6720.

Neidhart, F. C., 1987. Multigene systems and regulons, p. 1313-1317. *In* F. C. Neidhart, J. L. Ingraham, K. B. Low, B. Magasanik, M Schaechter, and H.E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D. C.

Newman, E. B., D'Ari, R., and R. T. Lin. 1992. The leucine-Lrp regulon in *E. coli*: a global response in search of a raison d'être. *Cell* **68:**617-619.

Nguyen, L. H., and R. R. Burgess. 1997. Comparative analysis of the interactions of *Escherichia coli* σ^s and σ^{70} RNA polymerase holoenzyme with the stationary-phase specific *bolAp1* promoter. *Biochemistry*. **36:**1748-1754.

Norel, F., Robbe-Saule, V., Popoff, M. Y., and C. Coynault. 1992. The putative sigma factor KatF (RpoS) is required for the transcription of the *Salmonella typhimurium* virulence gene *spvB* in *Escherichia coli*. *FEMS Microbiol. Lett.* **99:**271-276.

Nunoshiba, T., Hidalgo, E., Amabile-Cuevas, C. F., and B. Demple. 1992. Two-stage control of an oxidative stress regulon: the *Escherichia coli* SoxR protein triggers redox-inducible expression of the *soxS* regulatory gene. *J. Bacteriol.* **174**:6054-6060.

Olsén, A., Arnqvist, A., Hammar, M., Sukupolvi, S., and S. Normark. 1993. The RpoS sigma factor relieves H-NS-mediated transcriptional repression of *csgA*, the subunit gene of fibronectin-binding curli in *Escherichia coli*. **7**:523-536.

Pearson, M. L. 1972. The role of adenosine 3'5'-cyclic monophosphate in the growth of bacteriophage lambda. *Virology.* **49**:605-609.

Pratt, L. A., and T. J. Silhavy. 1996. The response regulator SprE controls the stability of RpoS. *Proc. Natl. Acad. Sci. U.S.A.* **93**:2488-2492.

Rao, N. N., and A. Kornberg. 1996. Inorganic polyphosphate supports resistance and survival of stationary-phase *Escherichia coli. J. Bacteriol.* **178**:1395 1400.

Rhaese, H-J., and E. Freese. 1968. Chemical analysis of DNA alterations: I. base liberation and backbone breakage of DNA and oligodeoxyadenylic acid induced by hydrogen peroxide and hydroxlyamine. *Biochim. Biophys.Acta.* **155**:476-490.

Richter, H. E., and P. C. Loewen. 1982. Catalase synthesis in *Escherichia coli* is not controlled by catabolite repression. *Arch. Biochem. Biophys.* **215**:72-77.

Rockabrand, D., Livers, K., Austin, T., Kaiser, R., Jensen, D., Burgess, R., and P. Blum. 1998. Roles of DnaK and RpoS in starvation-induced thermotolerance of *Escherichia coli. J. Bacteriol.* **180**:846-854.

Rosner, J. L., and J. L. Slonczewski. 1994. Dual regulation of *inaA* by the multiple antibiotic resistance (Mar) and superoxide (SoxRS) stress response systems of *Escherichia coli. J. Bacteriol.* **176**:6262-6269.

Sak, B. D., Eisenstark, A., and D. Touati. 1989. Exonuclease III and the catalase hydroperoxidase II in *Escherichia coli* are both regulated by the *katF* gene product. *Proc. Natl. Acad. Sci. U.S.A.* **86**:3271-3275.

Sanger, F. S., Nicklen, S., and A. R. Coulson. 1977. DNA Sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* **74**:5463 5467.

Sanner, T., and A. Pihl. 1963. Studies on the active -SH group of papain and on the mechanism of papain activation by thiols. *J. Biol. Chem.* **238:**165-171.

Sambrook, J., Fritsch, E. F., and T. Maniatis. 1989. Molecular Cloning, a Laboratory Manual, Second Edition. Cold Spring Harbour Laboratory Press. Cold Harbour, New York.

Schell, M. A. 1993. Molecular Biology of the LysR family of transcriptional regulators. *Annu. Rev. Microbiol.* **47:**597-626.

Schellhorn, H. E. 1994. Regulation of hydroperoxidase (catalase) expression in *Escherichia coli. FEMS Microbiol. Lett.* **131:**113-119.

Schellhorn, H. E., and H. M. Hassan. 1988. Transcriptional regulation of *katE* in *Escherichia coli* K-12. *J. Bacteriol.* **170:**4286-4292.

Schellhorn, H. E., and V. L. Stones. 1992. Regulation of *katF* and *katE* in *Escherichia coli* K-12 by weak acids. *J. Bacteriol.* **174:**4796-4776.

Schweder, T., Lee, K-H., Lomovskaya, O., and A. Matin. 1996. Regulation of *Escherichia coli* starvation sigma factor (σ^s) by ClpPX protease. *J. Bacteriol.* **178:**470-476.

Shiba, T., Tsutsumi, K, Yano, H., Ihara, Y., Kameda, A., Tanaka, K., Takahashi, H., Munekata, M., Rao, N. N., and A. Kornberg. 1997. Inorganic polyphosphate and the induction of *rpoS* expression. *Proc. Natl. Acad. Sci. U.S.A.* **94:**11210-11215.

Sledjeski, D., and S. Gottesman. 1995. A small RNA acts as an antisilencer of the H-NS-silenced *rcsA* gene of *Escherichia coli. Proc. Natl. Acad. Sci. U.S.A.* **92:**2003-2007.

Sledjeski, D. D., Gupta, A., and S. Gottesman. 1996. The small RNA, DsrA, is essential for the low temperature expression of RpoS during exponential growth in *Escherichia coli. EMBO J.* **15:**3993-4000.

Stauffer, C. E., and D. Etson. 1969. The effect of subtilisin activity of oxidizing a methionine residue. *J. Biol. Chem.* **244:**5333-5338.

Storz, G., Jacobson, F. S., Tartaglia, L. A., Morgan, R. W., Silveria, L. A. and B. N. Ames. 1989. An alkyl hydroperoxide reductase induced by oxidative stress in *Salmonella typhimurium* and *Escherichia coli*: genetic characterization and cloning of *ahp*. *J. Bacteriol.* **171:**2049 2055.

Storz, G., Tartaglia, L. A., and B. N. Ames. 1990. Transcriptional regulator of oxidative stress-inducible genes: Direct activation by oxidation. *Science* **248:**189-194.

Takayanagi, Y., Tanaka, K., and H. Takahashi. 1994. Structure of the 5' upstream region and the regulation on the *rpoS* gene of *Escherichia coli*. *Mol. Gen. Genet.* **243:**525-531.

Tanaka, K., Takayanagi, Y., Fujita, N., Ishihama, A., and H. Takahashi. 1993. Heterogeneity of the principal factor in *Escherichia coli*: the *rpoS* gene product, σ^{38}, is a second principal σ factor of RNA polymerase in stationary-phase *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **90:**3511-3515.

Tao, K., Makino, K, Yonei, S., Nakata, A., and H. Shinagawa. 1989.Molecular cloning and nucleotide sequencing of *oxyR*, the positive regulator gene of a regulon for an adaptive response to oxidative stress in *Escherichia coli*: homologies between OxyR protein and a family of bacterial activator proteins. *Mol. Gen. Genet.* **218:**371-376.

Tao, K., Makino, K, Yonei, S., Nakata, A., and H. Shinagawa. 1991. Purification and characterization of the *Escherichia coli* OxyR protein, the positive regulator for a hydrogen peroxide-inducible regulon. *J. Biochem.* **109:**262 266.

Tartaglia, L. A., Storz, G., and B. N. Ames. 1989. Identification and molecular analysis of *oxyR*-regulated promoters important for the bacterial adaptation to oxidative stress. *J. Mol. Biol.* **210:**709-719.

Touati, D. 1988. Molecular genetics of superoxide dismutases. *Free Radicals Biol. Med.* **5:**393-402.

Trépanier, S., Prince, A., and A. Huletsky. 1997. Characterization of the *penA* and *perR* genes of *Burkholderia cepacia* 249 which encode the chromosomal class A penicillinase and its Lys-R type transcriptional regulator. *Antimicrob. Agents. Chemother.* **41:**2399-2405.

Triggs-Raine. 1987. Physical characterization of *katG* encoding HPI of *Escherichia coli*. University of Manitoba, Winnipeg, Manitoba.

Triggs-Raine, B. L., Doble, B. W., Mulvey, M. R., Sorby, P. A., and P. C. Loewen. 1988. Nucleotide sequence of *katG*, encoding catalase HPI of *Escherichia coli*. *J. Bacteriol.* **170:**4415-4419.

Tsaneva, I. R., and B. Weiss. 1990. *soxR*, a locus governing a superoxide response regulon in *Escherichia coli* K-12. *J. Bacteriol.* **172:**4197-4205.

Tuveson, R. W. 1981. The interaction of a gene (*nur*) controlling near-UV sensitivity the polA1 gene in strains of *E. coli* K12. *Photochem. Photobiol.* **33:**919-923.

Vieira, J., and J. Messing. 1987. Production of single-stranded plasmid DNA. *Methods Enzymol.* **153:**3-11.

Volkert, M. R., Loewen, P. C., Switala, J., Crowley, D., and M. Conley. 1994. The Δ(*argF-lacZ*)*205*(U169) deletion greatly enhances resistance to hydrogen peroxide in stationary-phase *Escherichia coli. J. Bacteriol.* **176:**1297-1302.

Volkert, M. R., and D. C. Nguyen. 1984. Induction of specific *Escherichia coli* genes by sublethal treatments with alkylating agents. *Proc. Natl. Acad. Sci. USA.* **81:**4110-4114.

von Ossowski, I., Mulvey, M. R., Leco, P. A., Borys, A., and P. C. Loewen. 1991. Nucleotide sequence of *Escherichia coli katE*, which encodes catalase HPII. *J. Bacteriol.* **173:**514-520.

Walkup, L. K. B., and T. Kogoma. 1989. *Escherichia coli* proteins inducible by oxidative stress mediated by the superoxide radical. *J. Bacteriol.* **171:**1476-1484.

Way, J. C., Davis, M. A., Morisato, D., Roberts, D. E., and N. Kleckner. 1984. New Tn10 derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. *Gene.* **32:**369-379.

Weichart, D., Lange, R., Henneberg, N., and R. Hengge-Aronis. 1993. Identification and characterization of stationary phase-inducible genes in *Escherichia coli.* **10:**407-420.

Weiss, B. 1976. Endonuclease II of *Escherichia coli* is exonuclease III. *J. Biol. Chem.* **251:**1896-1901.

Wilson, R. L., and Steiert, P. S., and G. V. Stauffer. 1993. Positive regulation of the *E. coli* glycine cleavage system. *J. Bacteriol.* **175:**902-904.

Wise, A., Brems, R. L., and M. Villarejo. 1996. Sequences in the –35 region of *Escherichia coli rpoS*-dependent genes promote transcription by $E\sigma^S$. *J. Bacteriol.* **178:**2785-2793.

Wu, J., and B. Weiss. 1991. Two divergently transcribed genes, *soxR* and *soxS*, control a superoxide response regulon of *Escherichia coli*. *J. Bacteriol.* **173**:2864-2871.

Wu, J., and B. Weiss. 1992. Two-stage induction of the *soxRS* (superoxide response) regulon of *Escherichia coli*. *J. Bacteriol.* **174**:3915-3920.

Xu, J., and R. C. Johnson 1995. Identification of genes negatively regulated by Fis: Fis and RpoS co-modulate growth-phase-dependent gene expression in *Escherichia coli*. *J. Bacteriol.* **177**:938-947.

Yamashino, T., Ueguchi, C., and T. Mizuno. 1995. Quantitative control of the stationary phase-specific sigma factor, σ^S, in *Escherichia coli*: involvement of the nucleoid protein H-NS. *EMBO J.* **14**:594-602.

Yanisch-Perron, C., Vieira, J., and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene.* **33**:103-119.

Yonei, S., Yokota, R., and Y. Sato. 1987. The distinct role of catalase and DNA repair systems in protection against hydrogen peroxide in *Escherichia coli*. *Biochem. Biophys. Res. Comm.* **143**:638-644.

Yost F. J, and I. Fridovich. 1973. An iron-containing superoxide dismutase from *Escherichia coli*. *J. Biol. Chem.* **248**:4905-4908.

Zgurskaya, H. I., Keyhan, M., and A. Matin. 1997. The σ^S level in starving *Escherichia coli* cells increases solely as a result of its increased stability, despite decreased synthesis. *Mol. Microbiol.* **24**:643-651.

Zhang, A., Altuvia, S., Tiwari, A., Hengge-Aronis, R., and G. Storz. 1998. The *oxyS* regulatory RNA represses *rpoS* translation by binding Hfq (HF-I). *EMBO J.* **17**:6061-6068.

Zhou, Y., and S. Gottesman. 1998. Regulation of proteolysis of the stationary phase sigma factor RpoS. *J. Bacteriol.* **180**:1154-1158.