Streptomycin and *Escherichia coli* K12 MG1655 Cell Death.

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Abstract

Streptomycin is the prototypical aminoglycoside, a class of broad-spectrum antibiotics which inhibit protein synthesis by targeting the ribosome, and remains the agent of choice for treatment of drug-resistant tuberculosis. Nevertheless, little is known about the molecular consequences of streptomycin treatment at the bacterial level. This thesis explores the molecular consequences of streptomycin challenge in *Escherichia coli* K12 MG1655, a key step towards improving our understanding of how this antimicrobial agent works.

Initial work assessed the physiology of streptomycin-induced cell death utilizing traditional medical microbiology techniques: minimum inhibitory concentration, dose-response, and time-kill assays; to develop standardized, minimum information about a microarray experiment (MIAME) -compliant, culture condition for transcriptional studies. Two distinct phenotypes were observed: MIC and 2xMIC challenges led to bacteriostatic physiology, while 4xMIC challenge resulted in cell death within an hour. These data support previous observations that streptomycin works in both a time- and concentration-dependent manner. To observe the key molecular changes elicited by bactericidal challenge, transcriptional studies focused on 10- and 30-minute treatments with 4xMIC.

To investigate the molecular changes induced by streptomycin, spotted cDNA microarrays representing 99.6% of the *E. coli* K12 MG1655 genome were employed.

Changes to the transcriptome were detected within 10 minutes of drug addition. Following 30 minutes of treatment, 172 transcripts representing 4% of the genome were differentially regulated; although *rpsL*, the molecular target of streptomycin, was not affected. A striking, and almost complete, induction of three key stress responses was observed: heat shock, phage shock, and drug sensitivity. Members of the heat shock response were most enriched; *hslS* was consistently the highest up-regulated transcript. The most significant of the few uncharacterized transcripts observed was *yccV*, a hemi-methylated *oriC* DNA-binding protein. Surprisingly, a lack of coordination between the induction of the anaerobic metabolism transcriptional regulator, *fnr,* and its downstream targets was observed. As expected, transcripts associated with the energy-rich processes of motility, chemotaxis, and anaerobic metabolism were repressed.

To determine whether *hslS* or *yccV*, the highest differentially regulated transcripts, played a critical role in streptomycin-induced cell death, unmarked deletion mutants were engineered and characterized. Neither mutant exhibited a reduction in fitness, change in the minimum inhibitory concentration, or dose-response when compared to the parent strain. These two targets may be important in the overall mechanism of streptomycin lethality, but are not solely responsible for it.

Utilizing traditional microbiology techniques in concert with the high throughput screening afforded by cDNA microarrays, a defined molecular response to streptomycin treatment at both bacteriostatic and bactericidal concentrations was

characterized, although deletion of a key member of this response (*hslS*) did not alter the phenotype of cell death. These data agree with previous proteomic studies which highlight the induction of the heat shock response as a key lesion, but suggest that the misregulation of global regulators and their targets (as observed for *fnr*) may actually play a larger role in cell death. The work presented herein provides a detailed picture of the immediate molecular response of *E. coli* K12 MG1655 to streptomycin and suggests that antibiotic action as a whole is more complex than the interaction of a single compound with a single target.

Declaration

The experiments and composition of this thesis are the sole work of the author unless stated otherwise.

Melinda Terace Hough

Dedication

For my Grandfather, Harry Bryan Hough

Acknowledgements

"If I have seen further, it is by standing on the shoulders of giants."

- Sir Issac Newton

I'd like to thank all of my colleagues, both past and present, especially: Professor Jonathan Bard, who gambled to get this Seattle-ite to Scotland and whose guidance over the years has been invaluable; Professor Arkady Khodursky, for making me feel welcome during the depths of the Minnesota winter while showing me some fantastic, cutting-edge science; Jae Ahn my RNA guru; Dr. Martin Simmen for teaching me a thing or two about statistics; Dr. Betsy Martinez-Vaz, Dr. Kyeong Soo, and Dipen Sangurdekar for continued moral support; and Dr. Scott Hartsel for 'M'tumbo' and the art of troubleshooting, both in and out of the lab.

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I am deeply indebted to my family, especially my mom and dad, for their unwavering support throughout my life as I reach for even further stars.

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Abbreviations

°	degree (angle)
°C	degrees Celsius
A	absorbance
ANOVA	Analysis of Variance
ATP	adenosine triphosphate
bp	base pair
cDNA	complementary deoxyribonucleic acid
CFU	colony forming units
cm	centimetre(s)
Ct	control
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanine triphosphate
DM	Davis Mingioli
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate

FDR	false discovery rate
g	gram(s)
ISA	Iso-sensitest agar
Kb	kilobase(s)
kV	kilovolt(s)
L	litre(s)
LB	Luria broth
M	molar
mg	milligram(s)
mg/L	milligram(s) per litre
mg/mL	milligram(s) per millilitre
MIAME	Minimum Information About a Microarray Experiment
MIC	minimum inhibitory concentration
min	minute(s)
mL	millilitre(s)
mM	millimolar
µg	microgram(s)
µg/mL	microgram(s) per millilitre
µL	microlitre(s)
µm	micrometre(s)

µM	micromolar
mRNA	messenger ribonucleic acid
ng	nanogram(s)
nm	nanometre(s)
OD	optical density
PCR	polymerase chain reaction
pfu	particle forming units
ρmol	picomol
RNA	ribonucleic acid
RPM	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
rxn	reaction
SAM	Statistical Analysis of Microarrays
TCA	tricarboxylic acid cycle
tRNA	transfer ribonucleic acid
U	unit(s)
UV	ultraviolet
v/v	volume to volume

VC	viable count
w/v	weight to volume
WHO	World Health Organization
x	times

Standard gene and protein nomenclature was used. Gene names are in lower case italics. Protein names are capitalized in a normal typeface.

Standard IUPAC nomenclature was used for chemicals.

"Medical science and clinical practice have been revolutionized."

-Selman Waksman, discoverer of streptomycin

Nobel Laureate for Medicine, 1952

1. Introduction

1.1 Overview

Arguably the single most important medical discovery of the 20th century, the introduction of antibiotics decreased death from infectious diseases by over 50% during the first 10 years of use (Wainwright, 1990). Prior to their introduction, death from minor infections, childbearing, and communicable diseases was common due to poor sanitation and growing urban populations. Enhanced by advancements in urban infrastructure, the widespread use of antibiotics has dramatically decreased morbidity and mortality while increasing life expectancies worldwide. The dramatic, life-saving cures that ensued have shaped the public's view of antibiotics as wonder drugs, creating a culture demanding treatment for minor ailments. Rampant overuse has led to the highly publicized emergence of extremely- and/or multiply- resistant strains, including methicillin-resistant *Staphylococcus aureus* (MRSA) and *Mycobacterium tuberculosis*, as well as previously unreported pathogens such as *Acinetobacter baumannii*. These developments represent a major threat to public health and are set to challenge many of the medical advances of the past century.

Given the declining efficacy of available antibiotics, it is now more important than ever to understand their detailed mechanisms of action in order to design new and more potent agents. Despite their ubiquitous nature, the precise mechanism of bacterial cell death has remained illusive. Ominously, no new classes of compounds

have been introduced since the 1970s. The financial burden associated with drug discovery has severely limited basic research. Potential drugs currently take upwards of 12 years at a cost of over $16 billion to reach the clinic (personal correspondence, D. Payne). A detailed understanding of the molecular consequences of antibiotic treatment will identify novel targets and potentially a universal death program providing the much needed catalyst for directed drug development exploiting previously overlooked aspects of bacterial metabolism.

1.2 Antibiotic Classes

Antibiotics, as defined by Webster, are substances produced by, or semi-synthetic substance derived from, a microorganism that, in dilute solution, is able to inhibit or kill another microorganism (Merriam-Webster Collegiate Dictionary, 2002). These are naturally occurring metabolic by-products whose role in nature remains enigmatic; Salyers and Whitt suggest they may protect their producer from invading bacteria (a type of primitive germ warfare) or act as a signalling molecule (Salyers and Whitt, 2002). In recent years, the definition has expanded to encompass both these natural products and their synthetic counterparts.

Antibiotics are broken into two main classes – those that kill bacteria (bactericidal) and those that inhibit bacterial growth and/or replication (bacteriostatic). Current antibiotics target five key bacterial cell processes: cell wall biosynthesis, DNA synthesis, RNA synthesis, protein synthesis, and folic acid biosynthesis (Figure 1.1).

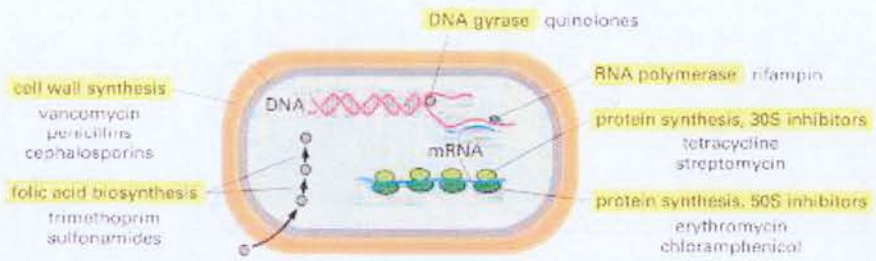


Figure 1.1. Antibiotic Targets.

Antibiotics target five key bacterial processes (yellow): cell wall biosynthesis, DNA synthesis, RNA synthesis, protein synthesis, and folic acid biosynthesis. Adapted from Alberts *et al.*, 2002.

With the exception of rifampin, which blocks RNA synthesis, the majority of antibiotics are bactericidal. The quinolones and 4-quinolones, or fluoroquinolones, are bactericidal antibiotics that target prokaryotic DNA gyrase and topoisomerase IV resulting in the inhibition of DNA synthesis (Levine *et al.*, 1998). Representatives of this class of antibiotic include norfloxacin and ofloxacin. Trimethoprim and the sulfonamides are diaminopyrimidines which block folic acid production through the inhibition of dihydrofolate reductase (Amyes and Smith, 1974). β-lactams, such as penicillin, and vancomycin comprise a group of bactericidal antibiotics which inhibit peptidation steps in cell well biosynthesis. Finally, protein synthesis can be inhibited by the aminoglycosides, such as streptomycin, macrolides, such as erythromycin, or related cyclic compounds including chloramphenicol; the later two groups being bacteriostatic rather than bactericidal.

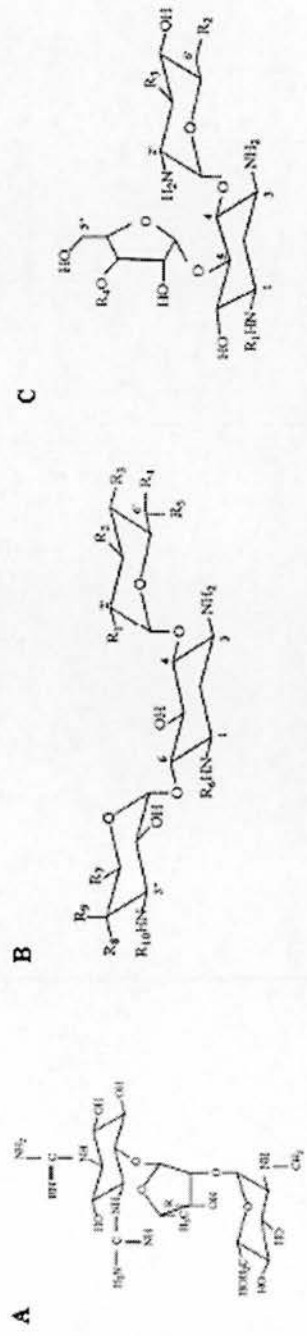
1.3 Aminoglycosides

1.3.1 Discovery

A vast majority of antibiotic discovery can be attributed to serendipity and persistence, rather than hypothesis driven programs. Fleming's 1928 discovery of penicillin occurred as a result of what Pasteur called a 'prepared mind' given that microbiologists had known bacteria and fungi could inhibit bacterial growth since the 1870s (Wainwright, 1990). Waksman and colleagues, on the other hand, discovered the bactericidal metabolite streptomycin, from *Streptomyces griseus*, in 1944 through a systematic screen of previously isolated *Actinomyces* and *Streptomyces* species (Waksman, 1964). The first resistant strain was reported in 1947, driving the discovery of additional aminoglycosides including neomycin by the Waksman group (Waksman, 1964). The first semi-synthetic derivatives appeared in the 1970s.

1.3.2 Structures

Aminoglycosides are multifunctional hydrophilic molecules containing one or more aminated sugars joined by glycosidic bonds to a dibasic cyclitol (Mingeot-Leclercq *et al.*, 1999). In the majority of aminoglycosides, 2-deoxystreptamine is the dibasic cyclitol, whereas streptomycin contains streptidine. Subsequent generations contain substitutions at either positions 4 and 6, as is the case for gentamicin, kanamycin, and tobramycin, or 4 and 5, as is the case for neomycin, of the 2-deoxystreptamine (Figure 1.2).



A. Aminocyclitols

Antibiotic	R
Streptomycin	CHO

B. 4,6- Disubstituted deoxystreptamine

Antibiotic	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	R_{10}
Amikacin	OH	OH	OH	H	NH_2	COR	CH_2OH	OH	H	H
Gentamicin C1	NH_2	H	H	CH_3	$NHCH_3$	H	H	CH_3	OH	CH_3
Gentamicin C1a	NH_2	H	H	H	NH_2	H	H	CH_3	OH	CH_3
Genatmicin C2	NH_2	H	H	CH_3	NH_2	H	H	CH_3	OH	CH_3
Gentamicin C2b	NH_2	H	H	H	$NHCH_3$	H	H	CH_3	OH	CH_3
Gentamicin B	OH	OH	OH	H	NH_2	H	H	CH_3	OH	CH_3
Kanamycin A	OH	OH	OH	H	NH_2	H	CH_2OH	OH	H	H
Kanamycin B	NH_2	OH	OH	H	NH_2	H	CH_2OH	OH	H	H
Kanamycin C	NH_2	OH	OH	H	OH	H	CH_2OH	OH	H	H
Tobramycin	NH_2	H	OH	H	NH_2	H	CH_2OH	OH	H	H

C. 4,5- Disubstituted deoxystreptamine

Antibiotic	R_1	R_2	R_3	R_4
Neomycin	H	NH_2	OH	X
Paramycin	H	OH	OH	X

X= R=CHOH$(CH_2)NH_2$

Figure 1.2. Structure of Related Aminoglycosides.

Structural formulae of some common aminoglycosides. (A) The aminocyclitol streptomycin with the dibasic cyclitol streptidine. (B) Common 4,6-disubstituted deoxystreptamines. (C) Common 4,5-disubstituted deoxystreptamines. Figure adapted from Mingeot-Leclercq *et al.*, 1999.

1.3.3 Clinical Uses

Aminoglycosides are among the most commonly prescribed antibiotics for critically-ill patients with nosocomial or resistant infections due to their high potency, broad-spectrum, and concentration-dependent activity making them both efficacious and cost effective. Alternatively, they can be used in conjunction with β-lactams to treat serious life-threatening infections such as septicaemia caused by *S. aureus* or *Enterococci* endocarditis (Gonzalez *et al.*, 1998). Despite these advantages, oto- and nephro- toxicity often result from prolonged exposure (Kahlmeter and Dahlager, 1984).

Streptomycin, in particular, is used as the first line of defence against brucellosis, tularaemia, plague, and drug-resistant tuberculosis (Vakulenko and Mobashery, 2003). Both tularaemia and plague were recently classified in the top five most likely engineered bioterrorism agents (WHO, http://www.who.int). The World Health Organization also highlighted the emergence of drug- and multi-drug-resistant tuberculosis strains as a growing public health problem due to the high incidence of co-infection with HIV exasperated by expensive, toxic, and prolonged treatment (WHO, http://www.who.int). Patients often fail to complete treatment regimes thus increasing the spread of what was once an almost eradicated disease. Consequently, streptomycin therapy has re-emerged in an attempt to prevent a return to the high mortality rates of the pre-antibiotic era when plague and tuberculosis patients were treated through isolation.

1.4 Streptomycin – The Prototypical Aminoglycoside

The aminoglycosides, streptomycin in particular, have been more extensively studied than any other class of antibiotic since their discovery in 1944 (Davis, 1987). These investigations have lead to the chronicling of an exceptionally pleiotropic set of effects (reviewed by Hancock, 1981 and Davis, 1987). Such a breadth of cellular changes, spanning over six decades of research, has led to several proposed mechanisms of action which account for some but not all of the effects. Herein we focus on the key effects of streptomycin, the prototypical aminoglycoside, as a model for this bactericidal class.

1.4.1 Effects on Bacterial Cells

1.4.1.1 Membrane Damage and the Requirement for Protein Synthesis

Early studies noted the activity of streptomycin was dependent on the nutritional environment, salt concentration, and pH. Sodium, lithium, and potassium ions had no effect on streptomycin's bactericidal activity; magnesium, calcium, and phosphate ions all antagonized the activity; and increasing pH stimulated the activity (Donovick *et al.*, 1948). Cell death was directly proportional to the degree of active metabolism and led to an increase in reducing agents (Paine and Clark, 1953; Henry *et al.*, 1949). Wasserman and colleagues observed that the effects of streptomycin injury were rapid, within 2.5 minutes, and reversible if cells were sub-cultured on antibiotic-free medium within 15 minutes of exposure, but once 30 minutes of exposure had passed cells could no longer be rescued (Wasserman *et al.*, 1954). The molecular changes which commit the bacterial cell to death must occur within the first 30 minutes of exposure.

A breakthrough came in 1960 when Anand and colleagues traced the uptake of radio-labelled [^{14}C]-streptomycin in *Escherichia coli* (Anand *et al.*, 1960). The uptake is biphasic - initial uptake was rapid, and could be reversed by washing the cells suggesting that the highly polar streptomycin can either passively diffuse across the membrane or use a small molecule transporter. This was followed by a lag then secondary influx larger than the initial absorption. Bryan and Van den Elzen described the slow uptake associated with the lag phase as energy-dependent phase I and the subsequent influx as energy-dependent phase II (Bryan and Van den Elzen, 1977). The secondary uptake coincided with a non-specific increase in membrane permeability and the extracellular accumulation of potassium ions and adenine nucleotides (Dubin and Davis, 1961; Bryan and Van den Elzen, 1976). When combined with the previous observation that an increase in pH (effectively a depletion of H^+) enhances the activity of streptomycin, energy, in the form of proton motive force appears to be central to lethality.

Pretreatment of bacterial cells with chloramphenicol, spectinomycin, or bacteriostatic concentrations of streptomycin all prevented subsequent bactericidal activity (Anand *et al.*, 1960; Plotz and Davis, 1962; Wallace *et al.*, 1974, Hancock, 1981). Blocking protein synthesis through the use of bacteriostatic agents protects cells from the harmful effects of streptomycin indicating there is a requirement of protein synthesis in the mechanism of action. Anand and Davis also noted that chloramphenicol pre-treatment prevented the uptake of streptomycin (Anand and Davis, 1961). The increase in extracellular potassium occurs at the same time as protein synthesis

inhibition suggesting a connection between the ribosome and an increase in cell membrane permeability (Dubin *et al.*, 1963). However, the site of action of streptomycin remained unclear.

Spotts and Stanier deduced that streptomycin blocks the ribosome based on the accumulation of RNA, rather than proteins, in sensitive cells (Spotts and Stanier, 1961). However it remained unclear as to whether streptomycin inhibited translation initiation or altered its processivity. Paradoxically, further studies monitoring the translation of polyuridylic acid during streptomycin challenge demonstrated two distinct effects on the ribosome. Several laboratories verified Spotts and Stanier's hypothesis while Gorini and Kataja demonstrated increased mistranslation (Speyer *et al.*, 1962; Flaks *et al.*, 1962; Gorini and Kataja, 1964). Although illuminating the primary ribosomal lesion, the specific translation step inhibited by streptomycin remained unclear.

1.4.1.2 The Ribosome as the Site of Action

A single molecule of streptomycin binds the ribosome (Chang and Flaks, 1972). Mutant screens subsequently identified two unique phenotypes – resistance or dependence, primarily based on changes to *rpsL* which encodes the ribosomal protein S12, a key component of the decoding and accuracy centre of the 30S subunit of the ribosome (Funatsu and Wittmann, 1972; Momose and Gorini, 1971). This genotype reflects the observed increase in error-prone translation and decreased proof-reading capabilities in sensitive strains upon streptomycin challenge (Ruusala

and Kurland, 1984). Protection and cross-linking studies also indicated involvement of the 16S rRNA (Moazed and Noller, 1987; Gravel *et al.*, 1987).

However, it wasn't until the derivation of the high resolution crystal structure of the ribosomal 30S subunit complexed with streptomycin that the precise nature of the biochemical interactions was observed (Figure 1.3). A single molecule of streptomycin forms salt bridges and hydrogen bonds with the phosphate backbone of the 16S rRNA as well as the S12 protein (Figure 1.3B; Carter *et al.*, 2000). These strong chemical bonds explain the irreversible nature of streptomycin – once bound it is difficult to dissociate. This accounts for the blockade of translation initiation as streptomycin binding would destabilize the formation of functional ribosomes.

Based on the crystal structure, it became easier to understand the mistranslation caused by streptomycin. Under normal circumstances, the A-site recognizes cognate or near-cognate tRNA. The weak hydrogen bonds between the codon (mRNA) and anticodon (tRNA) are based on Watson-Crick base pairing rules and induce a conformational change in the ribosome resulting in peptide bond formation and subsequent translocation (as reviewed by Ogle *et al.*, 2003). This conformational change is critical in identifying inappropriate base pairing and reducing error-prone translation. However, streptomycin irreversibly binds to the S12 protein in the A-site. This binding stabilizes the decoding centre, preventing the conformational change required for accurate base pairing and proof-reading thus allowing non-

cognate tRNAs to pair with the mRNA codon and subsequent peptide bond formation (Figure 1.3C). This accounts for the increase in mistranslated proteins.

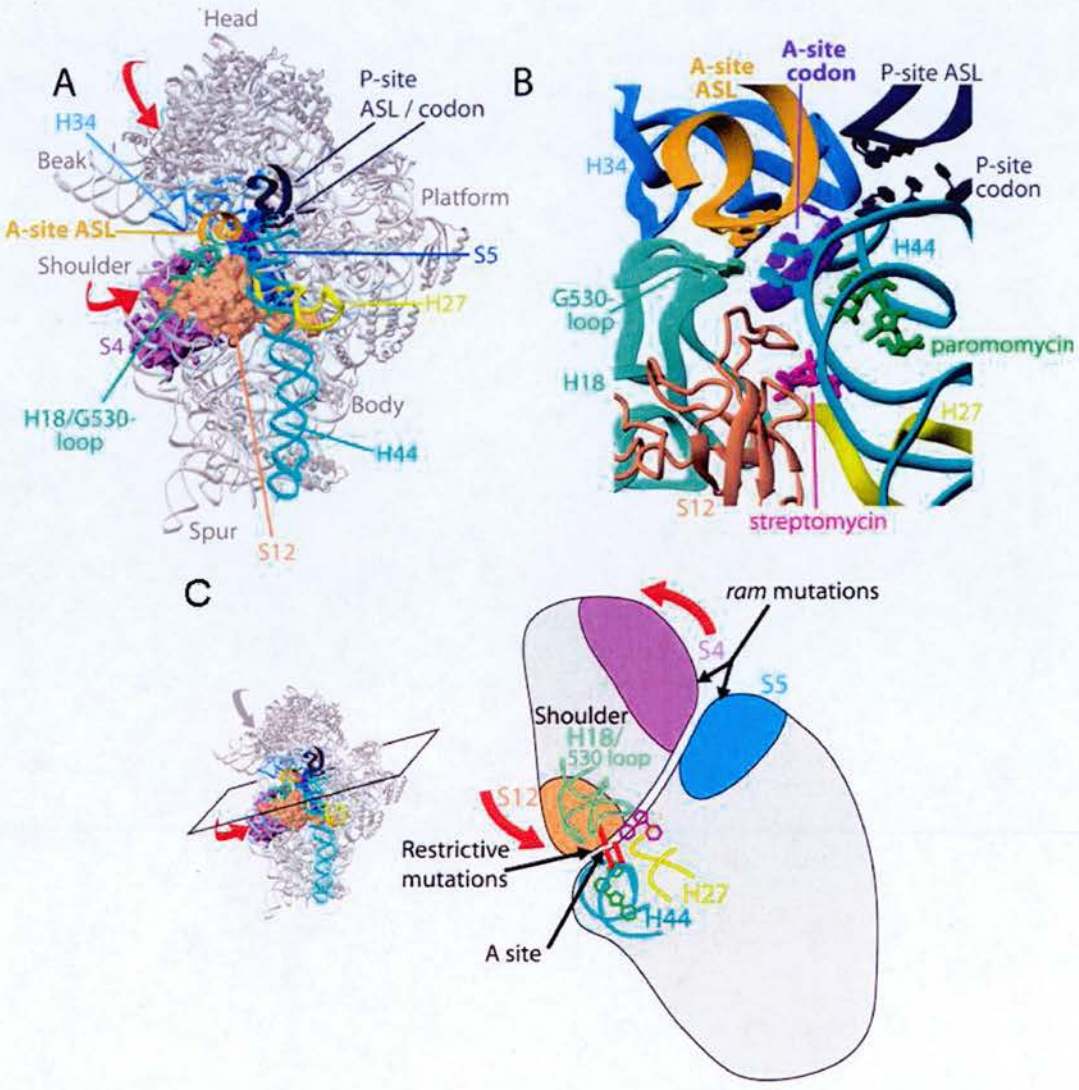


Figure 1.3. Structure of the Ribosome and Streptomycin Binding.

(A) The 30S ribosomal subunit. (B) Magnification of the decoding centre in the 30S subunit showing the binding of streptomycin and paromomycin. (C) Cartoon of the ribosome showing the binding of streptomycin and paromomycin in the decoding centre. Colors represent: the A-site codon (purple); the P-site (black); 16S RNA - H18/530-loop (turquoise), H44 (cyan), H27 (yellow); proteins – S12 (orange), S4 (violet), S5 (dark blue); antibiotics - paromomycin (green), streptomycin (pink). From Olge *et al.*, 2003.

1.4.2 Mode of Action – Davis' Hypothesis

Despite the identification of the ribosome as the site of action for streptomycin, the connection between mistranslation and membrane damage remained unclear until the identification of membrane-bound ribosomes which directly insert proteins into the cytoplasmic membrane (Randall and Hardy, 1977; Smith *et al.*, 1977). Davis and colleagues traced the destination of proteins radio-labelled with [^{35}S]-methionine following streptomycin treatment to the residual fraction (Davis *et al.*, 1986). As membrane components are often found in the residual fraction, Davis inferred the mistranslated proteins where inserted in the membrane and proposed the most widely accepted model of streptomycin action (Davis *et al.*, 1986).

1. Streptomycin enters the cell through small imperfections in the membrane and makes contact with chain-elongating, membrane-bound ribosomes resulting is mistranslation.
2. Aberrant proteins are incorporated into the membrane reducing its tension and increasing its permeability to small molecules, including streptomycin.
3. This cycle of mistranslation and increasing intracellular streptomycin concentration continues until all ribosomes are bound with streptomycin.
4. Cell death occurs due to the irreversible binding of streptomycin to the ribosome and ensuing lack of protein synthesis.

1.4.3 Important Implications

The Davis model (1987) accounts for many of the key pleiotropic effects associated with streptomycin treatment described herein. However, a number of interesting observations remain to be explained.

Hurwitz and colleagues observed that cells were unable to undergo cell division when treated with streptomycin but their mass continued to increase (Hurwitz *et al.*, 1962). Streptomycin treatment of phage-infected *E. coli* resulted in DNA forming nucleoid-like structures in the cytoplasm (Freda *et al.*, 1968). DNA replication can also be inhibited by the aminoglycoside hebakacin (Matsunaga *et al.*, 1986). Given the tight coupling of replication with cell division through the membrane attachment of DNA at the *oriC* by proteins such as SecA, Hancock suggested aminoglycosides may have additional targets (Hancock, 1981). However, the Davis model does not account for the blockade of replication.

Following streptomycin treatment there is an increase in RNA (Dubin *et al.*, 1963). Intuitively, this is expected from a decrease in the translational capacity of bacterial cells in response to streptomycin. Recent transcriptional studies investigating the effect of puromycin on *Streptococcus pneumoniae*, streptomycin on *Yersinia pestis*, gentamicin on *Bacillus subtilis*, and kanamycin on *E. coli* suggest there maybe a hallmark response, specifically the induction of heat shock genes (Ng *et al.*, 2003; Qiu *et al.*, 2005; Lin *et al.*, 2005; Shaw *et al.*, 2003). Such a response cannot be explained by the current model.

VanBogelen and Neidhardt noted that bacterial challenge with the aminoglycosides kanamycin, puromycin, or streptomycin lead to the induction of a protein profile similar to that of the heat shock, however the specific proteins were not identified (VanBogelen and Neidhardt, 1990). Similarly, streptomycin stimulates a marked increase in protease activity specifically DnaK and GroEL (Bakker, 1992). Taken together, these data suggest bacterial cells mount a response to streptomycin treatment which runs counter to the notion of ribosomal blockage induced death. Despite over 60 years of research, the molecular mechanism of streptomycin induced cell death remains enigmatic.

1.5 Aims of This Thesis

The aim of this thesis was to determine the molecular consequences of streptomycin treatment in *E. coli* K12 MG1655 in order to clarify how cell death occurs and identify potentially novel antibacterial targets. This was divided into the following three phases:

1. Determine the physiological effects of streptomycin treatment in different media to establish reproducible culture conditions for transcriptional studies.
2. Assess the global transcriptional response to streptomycin challenge and identify key responses and alternative targets.
3. Investigate the effects of deleting two of the genes differentially regulated in response to streptomycin.

2. Physiology of Streptomycin-Induced Cell Death

2.1 Overview

Since their discovery in 1944, aminoglycosides have been studied more extensively than any other class of antibiotic (Davis, 1987). As a result, their pleiotropic effects have been well characterized. Streptomycin, often considered the class prototype, remains the best characterized of the aminoglycosides. The bactericidal action of this agent is concentration-dependent. Yet, effective killing is reliant on the composition of the media as well as the degree of active metabolism (Berkman *et al.*, 1947; Paine and Clark, 1953). Due to the impact of environmental conditions on metabolism, and ultimately the degree of bactericidal activity, it is imperative to develop standardized and readily reproducible culture conditions prior to further investigations into the mechanism of action.

Microarray transcriptional profiling was chosen to revisit the mechanism of action of streptomycin. However, these studies generate myriad gene expression data, which, if inappropriately annotated, become inaccessible for comparisons across experimental conditions and laboratories. To ensure the interpretability and independent verification of results, the Microarray Gene Expression Data (MGED) group developed the minimum information about a microarray experiment (MIAME) standards (Brazma *et al.*, 2001). These were adopted by the majority of scientific journals as the standard for reporting microarray results in 2002.

MIAME guidelines clearly established the information required for reporting microarray results in order to provide a meaningful context for interpreting raw data and understanding the caveats of their conclusions. This includes the unambiguous reporting of information regarding cell type, culture conditions, and treatments. These requirements, coupled with the variability of the antimicrobial effect of streptomycin, necessitated the physiological characterization of *E. coli* K12 MG1655.

In this chapter, the physiology associated with streptomycin-induced cell death of *E. coli* K12 MG1655 was assessed through traditional medical microbiological methods, primarily minimum inhibitory concentration (MIC) determination, dose-response assessment, and time-kill assays in rich (LB) and minimal (Davis-Mingioli) media. These were used to standardize culture conditions for transcriptional studies and to facilitate comparisons with existing and future data sets.

2.2 *Materials and Methods*

2.2.1 General Microbiological Methods

2.2.1.1 Strains

E. coli K12 MG1655, (courtesy of the Zoonotic and Animal Pathogens group, University of Edinburgh, Edinburgh, Scotland, UK) a non-pathogenic sequenced strain, was used for all physiological experiments (Blattner *et al.*, 1997). This strain was used for the generation of microarray probes; experiments with RNA from this strain will minimize hybridization errors.

E. coli NCTC10418 (courtesy of Molecular Chemotherapy, University of Edinburgh, Edinburgh, Scotland, UK) is an accepted control for minimum inhibitory concentration determination and was used as the positive control (Andrews, 2001).

2.2.1.2 Antibiotics

Streptomycin sulphate (Sigma, S6501-5g) was dissolved in distilled water (10mg/mL), filter sterilized (Sartorius Minisart 0.20μm filter, FIL6574), and aliquots were stored at either 4°C (no longer than 7 days) or long-term at –70°C (no longer than 3 months).

2.2.1.3 Media

Liquid cultures were grown in either Davis-Mingioli (DM) minimal media (Davis and Mingioli, 1950), supplemented with 0.4% glucose (Sigma, G7021), or LB Broth (Invitrogen, 12780-052) at 37°C at ~200RPM.

Davis-Mingioli Minimal Media

7.00g	K_2HPO_4
3.00g	KH_2PO_4
0.47g	$Na_3C_6H_5O_7$
0.10g	$MgSO_4 \cdot 7H_20$
1.00g	$(NH_4)_2SO_4$
1L	distilled H_2O

Plate cultures were grown aerobically on MacConkey agar with salt (Oxoid, PO0149A) at 37°C.

2.2.1.4 *Optical Density Measurement*

Optical density (OD) of liquid cultures was determined over a time course by taking the average of three readings at each time point at 600nm using a Cecil CE2041 Spectrophotometer (Cecil Instruments, Cambridge, England, UK). The spectrophotometer was calibrated using fresh media prior to each time point. Experiments were performed in triplicate, the nine data points averaged, the standard error calculated and graphed.

2.2.1.5 *Viable Count Measurement*

Viable counts (VC) of liquid cultures were determined by plating ten-fold serial dilutions, in triplicate, on MacConkey agar with salt. These plates were incubated overnight then only dilutions containing 30-500 colonies were counted. Counts were

automated using a colony counter pen (Sigma, Z367850). Colony forming units (CFU) were determined as:

$$\text{CFU} = \text{number colonies counted} \times \frac{1}{\text{volume plated (mL)}} \times \frac{1}{\text{dilution factor (10^-x)}}$$

Experiments were performed in triplicate, the nine data points averaged, the standard error calculated and graphed.

2.2.1.6 Miscellaneous Formulae

Formulae used to calculate the average, standard deviation, standard error, and colony forming units can be found in Appendix 1.

2.2.2 Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) is the lowest concentration of an antibiotic that inhibits all visible growth of an organism following overnight incubated. The MIC was determined based on BSAC guidelines, with *E. coli* NCTC10418 as a positive control (Andrews, 2001). Antibiotic susceptibility was carried out on freshly prepared plates containing filter sterilized antibiotic solutions.

An antibiotic stock solution was prepared as described in 2.2.1.2. The stock solution was filter sterilized then serially diluted to prepare the following working solutions:

Solution A = 10mg/mL
Solution B = 1mg/mL
Solution C = 0.1mg/mL

Plates were prepared by adding the following volumes of antibiotic working solution to 20mL of molten Iso-Sensitest agar (23.4g per litre Iso-Sensitest Broth, Oxoid CM473; 8g per litre Bacteriological Agar No. 1, Oxoid LP0011) to a final concentration ranging from 0.25mg/L to 128mg/L:

Volume Added	Working Solution	Final Concentration (mg/L)
256µL	A	128
128µL	A	64
64µL	A	32
32µL	A	16
160µL	B	8
80µL	B	4
40µL	B	2
200µL	C	1
100µL	C	0.5
50µL	C	0.25

Plates were cooled at room temperature for 1 to 2 hours then used immediately.

A statically-grown, overnight, LB broth culture was diluted 1:500 in sterile saline and used to inoculate the plates. Plates where inoculated from lowest to highest antibiotic concentration, with a non-antibiotic containing control plate last, using an

automatic multipoint inoculator (Denley; Mast Diagnostics, Bootle, UK), which delivers approximately 1µL of the bacterial suspension to the surface of the agar plate. Alternatively, 1µL of the bacterial suspension was pipetted directly onto the surface of the agar plate without puncturing it. Once the inoculum was absorbed, plates were incubated, aerobically at 37°C for 18 to 24 hours. The MIC was determined based on comparison with a non-antibiotic control plate as the lowest concentration with no visible growth. All experiments were conducted in triplicate.

2.2.3 Dose Response

A shaken, overnight culture was used to inoculate fresh, pre-warmed media at 1:20 for minimal medium (DM), 1:50 for Iso-Sensitest broth, or 1:100 for rich medium (LB) and incubated for 2 hours at 37°C shaking at 200RPM. Ten-mL aliquots of fresh medium containing final streptomycin concentrations from 0.25mg/L to 128mg/L were prepared. Log cultures were used to inoculate each aliquot at the same initial dilution as above (Section 2.2.2). Cultures were grown statically, at 37°C, for three hours, serially diluted into sterile saline, and plated in triplicate. Viable counts were determined as described in 2.2.1.5.

2.2.4 Growth and Kill Curve

A shaken, overnight culture was used to inoculate fresh, pre-warmed medium at 1:20 for minimal medium (DM) or 1:100 for rich medium (LB) and incubated for 2 hours at 37°C shaking at 200RPM. Two hours post-inoculation, appropriate antibiotic concentrations were added to each flask based on the predetermined MIC value: MIC

(2mg/L), 2xMIC (4mg/L), and 4xMIC (8mg/L). Cultures were incubated for a further 4 hours at 37°C at ~200RPM during which time samples were taken hourly for optical density (Section 2.2.1.4) and viable count (Section 2.2.1.5) determination.

2.3 Results

2.3.1 Minimum Inhibitory Concentration

The MIC is the lowest concentration of an antibiotic that inhibits all visible growth of an organism following overnight incubation. The MIC of streptomycin for *E. coli* K12 MG1655 was determined from triplicate experiments following BSAC guidelines (Section 2.2.2).

Concentration (mg/L)	Growth
128	++
64	++
32	++
16	++
8	++
4	+
2	--
1	--
0.50	--
0.25	--

Table 2.1. Minimum Inhibitory Concentration Determination of Streptomycin Against *Escherichia coli* K12 MG1655.

Escherichia coli K12 MG1655 was challenged with increasing concentrations of streptomycin on Iso-Sensitest agar. Growth was accessed after 18 to 24 hours exposure. + = Growth; - = No Growth.

The MIC for *E. coli* K12 MG1655 is 2mg/L. Subsequent experiments were based on multiples of this concentration.

2.3.2 Concentration-Dependent Response

The concentration-dependent activity of streptomycin was determined through dose-response experiments. Mid-log phase *E. coli* were challenged with increasing concentrations of streptomycin for three hours in rich (LB), minimal (DM), and Iso-Sensitest media.

Streptomycin had bactericidal activity in all three media (Figure 2.1). The bactericidal activity in Iso-Sensitest broth was observed at concentrations greater than 0.5xMIC. In rich media, streptomycin was bactericidal at concentrations greater than 2xMIC with a complete loss of viability achieved by concentrations great than 8xMIC. In minimal medium, bacterial cell death was induced by concentrations greater than 2xMIC. However, a complete loss of viability was not observed; viable cells persisted regardless of the concentration tested. *E. coli* was killed in a concentration-dependent manner which is dictated by media composition.

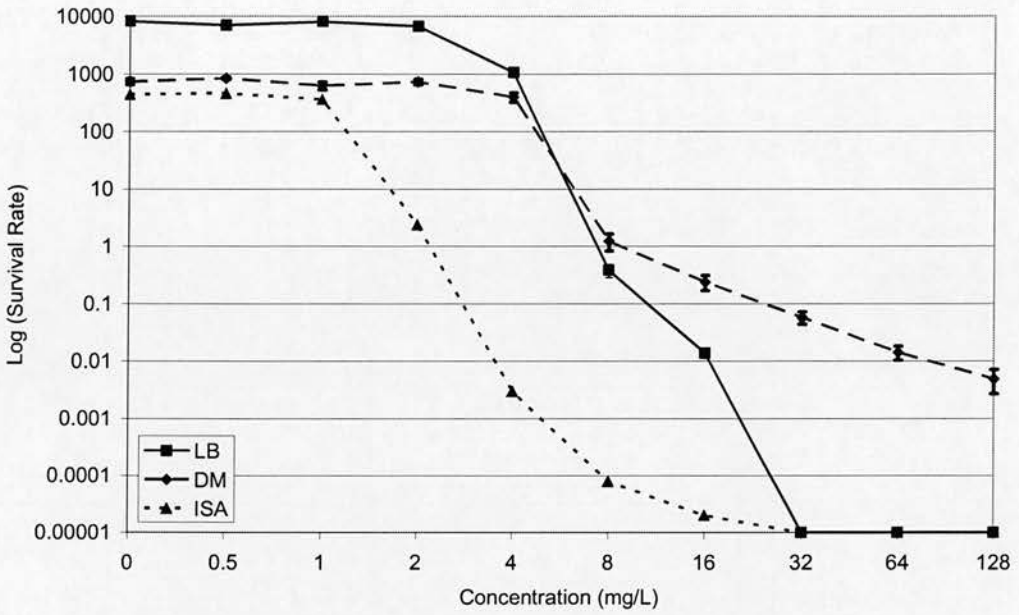


Figure 2.1. Dose-Response of *Escherichia coli* K12 MG1655 to Streptomycin.

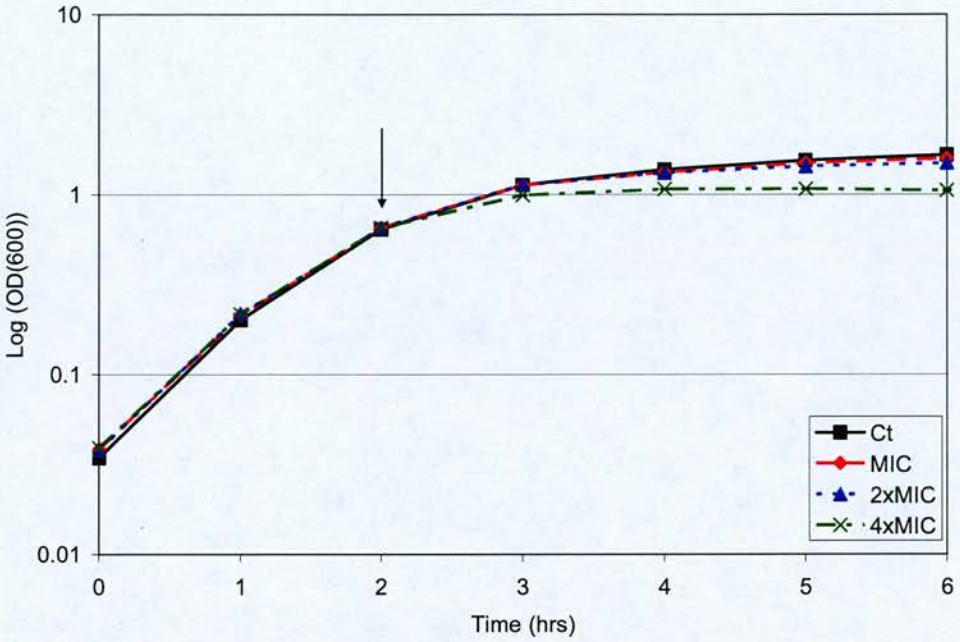
Streptomycin was added to mid-log phase cultures, incubated a further three hours, then serially diluted and plated for viable counts. Results are the average of three trials plated in triplicate. LB – L broth; DM – Davis-Mingioli minimal medium; ISA – Iso-Sensitest broth. 1.00x10-5 is equivalent to zero in these experiments.

2.3.3 Time-Dependent Response

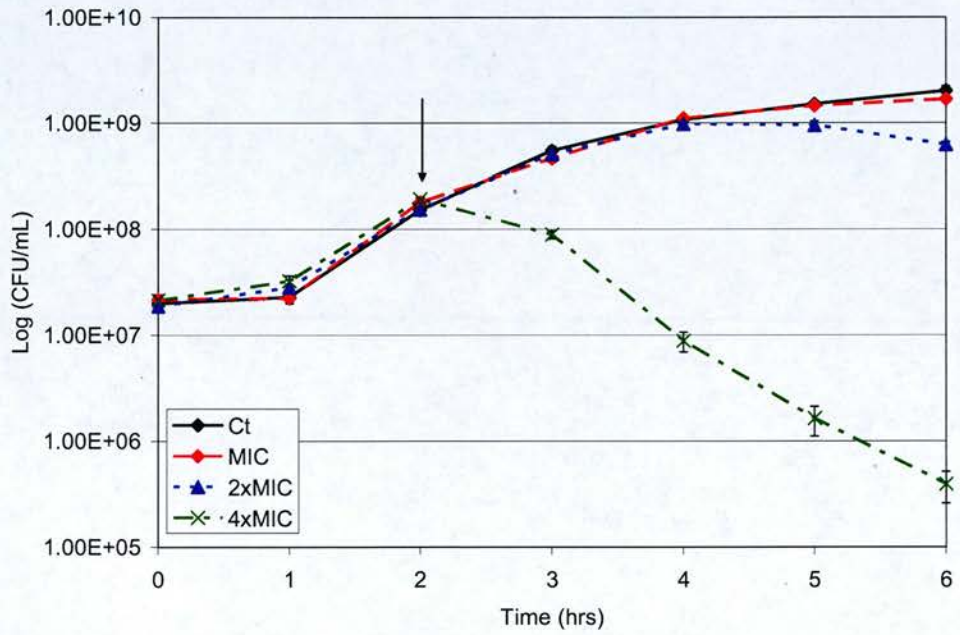
The time-dependent activity of streptomycin was determined through a time-kill assay. Mid-log phase *E. coli* cells were challenged with streptomycin and monitored hourly for a further four hours.

2.3.3.1 Rich Media

Challenge with streptomycin in rich media yields distinct physiological effects depending on the method of observation. Optical density measurements (Figure 2.2A) suggest that streptomycin has little or no inhibitory effect on cell growth. Viable count data (Figure 2.2B), on the other hand, shows a different response. Treatment with MIC and 2xMIC did not decrease viable cell counts. Treatment with 4xMIC is highly bactericidal, resulting in a 64% reduction in cell viability within the first hour of treatment. Consequently, in rich media, *E. coli* was killed in a time-dependent manner at 4xMIC.



(A)



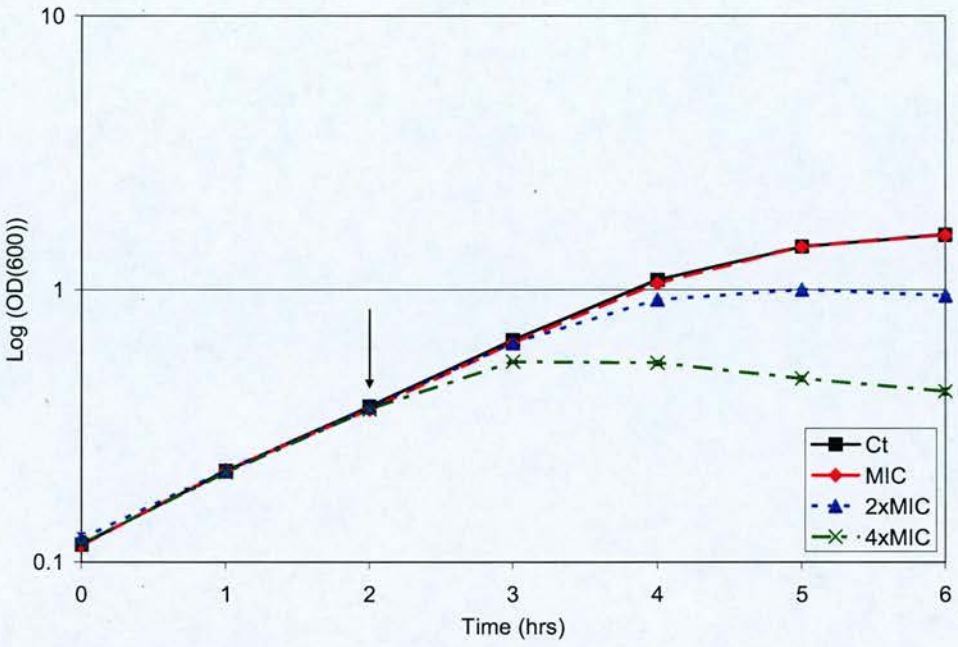
(B)

Figure 2.2. *Escherichia coli* K12 MG1655 Response to Streptomycin in Rich Media.

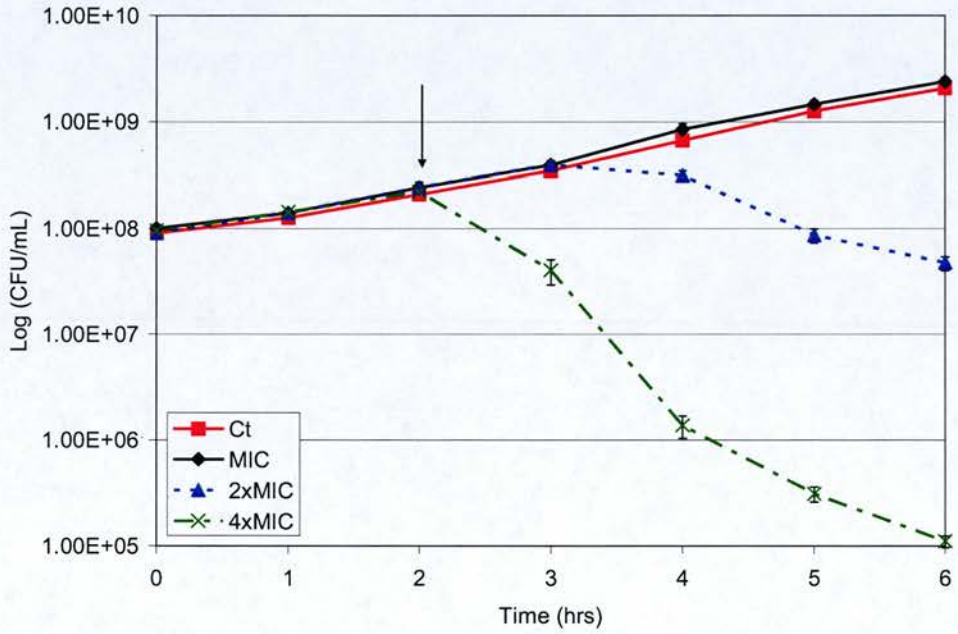
Streptomycin was added to mid-log phase cultures at two hours (arrow) and monitored for 4 hours via (A) optical density and (B) viable counts. Results are the average of three trials plated in triplicate. Concentrations used: Ct – 0mg/L; MIC – 2mg/L; 2xMIC – 4mg/L; 4xMIC – 8mg/L.

2.2.3.2 *Minimal Media*

Challenge with streptomycin in minimal media does not result in a similar disparity between optical density and viable count measurements (Figure 2.3A and B, respectively). Both measures show that treatment with streptomycin at the MIC has no effect on cell growth. Treatment with 2xMIC and 4xMIC both result in a reduction of cell viability. A strong bactericidal effect, similar to that seen in rich media, was observed only following treatment with 4xMIC. The bactericidal activity of streptomycin is reduced at 2xMIC but remains quantifiable. In minimal media, *E. coli* was killed in a time-dependent manner, although the extent of cell death was less robust at 2xMIC than at 4xMIC.



(A)



(B)

Figure 2.3. *Escherichia coli* K12 MG1655 Response to Streptomycin in Minimal Media.

Streptomycin was added to mid-log phase cultures at two hours (arrow) and monitored for 4 hours via (A) optical density and (B) viable counts. Results are the average of three trials plated in triplicate. Concentrations used: Ct – 0mg/L; MIC – 2mg/L; 2xMIC – 4mg/L; 4xMIC – 8mg/L.

2.4 Discussion

The bactericidal activity of streptomycin was well characterized during the first 25 years of research into its mechanism of action. This activity has been shown to be heavily reliant on culture conditions, specifically, the nutritional environment, salt concentration, and pH. This chapter investigated the basic physiology associated with streptomycin-induced cell death in order to establish reproducible culture conditions for subsequent studies.

The MIC is defined as the lowest concentration of an antimicrobial that inhibits visible growth of a microorganism following overnight incubation (Andrews, 2001). The MIC is considered the 'gold standard' for determining the susceptibility and/or resistance patterns of microorganisms and, as such, well defined standards have been established. Concentrations used throughout this study were chosen as multiples of the MIC due to the standardized nature of this method.

The concentration-dependent activity of streptomycin against log-phase *E. coli* was investigated in both rich and minimal media utilizing two different assays – dose response, which varies the dose while maintaining a constant exposure time; and time-kill, which varies the length of exposure while maintaining a constant antibiotic concentration. Both assays indicate that streptomycin exerts a rapid bactericidal effect; however, the magnitude of the kill is directly influenced by the nutritional environment of the challenge.

Streptomycin was shown to exert a bactericidal effect at concentrations greater than 2xMIC in both rich and minimal media, with greater than 99% of cells killed at concentrations greater than or equal to 4xMIC, by dose response experiments (Figure 2.1). Whereas complete loss of viability was observed in rich media, a small number of cells persisted in minimal media. Paine and Clark observed that the antimicrobial activity of streptomycin is directly proportional to the degree of active metabolism of the growing culture (Paine and Clark, 1953). The observed results may be explained due to differences in metabolism dictated by media composition. Under optimal conditions, such as those provided by rich media, bacterial cells are growing at a faster rate than under conditions of nutrient limitation, as presented by minimal media, consequently, less antibiotic is required to kill robustly growing cultures in rich media.

Streptomycin was also observed to exert a rapid (within one hour) killing effect at 4xMIC in both rich and minimal media as determined by viable counts during time-kill assays (Figure 2.2B and Figure 2.3B). Similar results were previously described (Plotz and Davis, 1962). No bactericidal activity was observed at concentrations less than 4xMIC in rich media (Figure 2.2B). This may be explained through the correspondence of antimicrobial activity with metabolism. In cells undergoing rapid cell division, characteristic of logarithmic growth, a higher metabolic rate is frequently observed. As a result, higher concentrations of antibiotic would be required to elicit bacterial cell death due to the generation of new cells – lower concentrations would quickly be diluted as cell numbers increase.

A less robust bactericidal effect was observed when *E. coli* were challenged with 2xMIC in minimal media (Figure 2.3B). This may also be explained by the correlation between antimicrobial activity and metabolism. In nutrient limited conditions, such as those in minimal media, bacterial metabolism is not as robust as in rich media. However, cells remain actively growing and dividing, albeit at a slower rate. As a result, lower concentrations of antibiotic would not be diluted as quickly by new cells, giving it time to exert a bactericidal effect.

The observed bactericidal activity of streptomycin under conditions of nutrient, and subsequently amino acid, limitation contradicts previous studies which suggested active protein synthesis is required for cell death (Plotz and Davis, 1962; Hurwitz and Rosano, 1962). Given this requirement for protein synthesis, one would expect either a reduction or cessation of bactericidal activity during growth in minimal media. This may explain the less robust bactericidal effect at 2xMIC, but cell death proceeds nonetheless. It is reasonable to expect that cell death may proceed despite limiting the availability of amino acids. As the pool of available amino acids, and subsequently charged tRNA, required for protein synthesis, dwindles within the cell, the recycling of aberrant proteins for their components may supply the additional amino acids needed for protein synthesis, cell division, and subsequent bactericidal activity of streptomycin. It remains to be seen if there is a bias in protein degradation or subsequent synthesis as a result of these limiting conditions.

It is interesting to note that treatment with the MIC in either rich or minimal media does not result in bacterial cell death despite being determined as the minimum amount required to elicit such a response. This may be due to the media and culture conditions employed to initially determine the MIC. MIC determinations were carried out as described by BSAC guidelines on the conventional semi-defined media, Iso-Sensitest agar. In addition, the MIC was determined on agarose plates rather than in logarithmically growing cultures. Given these differences, and the relationship between active metabolism and antimicrobial activity, one would expect higher concentrations of antibiotic to be required to kill rapidly dividing liquid cultures, regardless of the media. Interestingly, this was not observed for dose response experiments carried out in liquid Iso-Sensitest broth; a strong bactericidal effect was observed for concentrations greater than 0.5xMIC suggesting that streptomycin's activity either synergizes with one of the components of the medium or that the MIC is a definable hallmark value determined by the specific culture conditions of the experiment.

Traditionally, bacterial cultures are monitored via optical density and viable counts – optical density being immediate and indirect; viable counts being more precise but time consuming. However, these observations are often skewed due to the belief that bacterial cells respond to environmental conditions through filamentation thus increasing optical density without a corresponding increase in cell numbers (Davis, 1987). In this study, two distinct effects were observed depending on the nature of the measurement employed during time-kill assays in rich, and to a lesser extent in

minimal, media. Observations based on optical density (Figure 2.2A and Figure 2.3A) suggest that streptomycin has little or no effect on cell growth which was contradicted by a decrease in viable counts (Figure 2.2B and Figure 2.3B). Henry and colleagues observed a similar disparity and suggested this may be due to an increase in the filamentation index or a heterogeneous population (Henry *et al.*, 1949). Unlike other antibiotics, which have been shown to increase the filamentation index (Davis, 1987), streptomycin does not induce filamentation (Hurwitz *et al.*, 1962). Hurwitz and colleagues demonstrated microscopically that cells were either functionally dead or healthy and able to undergo cell divisions without producing elongated progeny following streptomycin treatment (Hurwitz *et al.*, 1962). These observations lend support to the hypothesis that bacterial cells are heterogeneous populations of susceptible and persister individuals rather than a homogeneous matrix of clones that respond similarly to antibiotic challenge.

Recent work by Hoffman and colleagues may further explain the disparity between optical density and viable count measures. They found that *Pseudomonas aeruginosa* and *E. coli* form biofilms in response to tobramycin, another aminoglycoside (Hoffman *et al.*, 2005). Further studies indicated that *P. aeruginosa* also formed biofilms in response to amikacin, streptomycin, and gentamicin; although none of these antibiotics were tested against *E. coli*. Cells in the process of aggregating to form biofilms would scatter light in a similar manner as filamenting cells, resulting in no change to the optical density of the sample. Taken together these observations suggest that rather than a homogeneous planktonic culture, liquid

cultures are actually composed of a heterogeneous population of planktonic cells and "biofilms" where each individual in the community may have different antibiotic susceptibilities and respond differently to environmental stimuli.

This study utilized two traditional methods to monitor bacterial cell growth. Optical density is a simple and indirect measure of bacterial growth based on the assumption that an increase in turbidity, as a result of increased light scattering, is proportional to an increase in viable cells. This does not take into account an increase in turbidity due to changes in cell morphology, as a result of filamentation or biofilm formation, or cell death. The total increase in turbidity is assumed to be a result of dividing, viable cells and can lead to an inaccurate determination of the growth phase of bacterial cultures. Consequently, viable counts were also performed. Although a direct method of assessing culture viability, viable counting assumes that each colony arises from a single cell and does not account for aggregation as a result of culture conditions or biofilm formation. Due to the bactericidal nature of streptomycin, alternative methods of assessing cell viability based on cellular metabolites may be more useful in future studies.

Normally, ATP is degraded by endogenous ATPases in dying cells. Adenylate kinase luminescence (Berthold Technologies) and ATP bioluminescence (Promega) are two assays that capitalise on the tight control of ATP to monitor the conversion of ADP to ATP in growing cells. Similarly, the electron carrier NADH is naturally fluorescent, but its oxidized form NAD^+ is not, and can be used to monitor active

electron transport through increases in UV absorbance. Finally, a simple live/dead staining protocol (Molecular Probes) which relies on membrane integrity and the nucleic acid stains SYTO 9 and propidium iodine could be used microscopically or in conjunction with FACS sorting to yield a more accurate picture of the cell death. These methods, although also indirect, are rapid, facile, and utilize cellular components as an indictor of cell viability. When coupled with automated viable cell counts, through the use of spiral plating, these assays would yield a more complete picture of growth and death in antibacterial challenged cultures.

Time-kill experiments focused on characterizing the response of *E. coli* to increasing concentrations of streptomycin in rich and minimal media. It was observed that 4xMIC of streptomycin produced a rapid bactericidal effect under both culture conditions (Figure 2.2 and Figure 2.3). However, previous investigations into the effects of various electrolytes on the activity of streptomycin indicate that the majority of the components of DM minimal medium alter streptomycin's activity. Specifically, K_2HPO_4, $MgSO_4$, glucose, and water all antagonized the bactericidal effect (Donovick *et al.*, 1948; Wasserman *et al.*, 1954). Donovick and colleagues also noted that increases in phosphate ions, either PO_4^{-1} or PO_4^{-2}, increased the MIC while increases in the pH decreased the MIC (Donovick *et al.*, 1948). It is likely that the effect of streptomycin in minimal medium is altered by its components. Multiple transcriptional studies, focusing on each media component, would be required to ascertain their effects prior to studying the global response to streptomycin. Due to the volume of data generated and the arduous task of analyzing each profile, such

studies are in themselves prohibitive. As a result, *E. coli* challenged with 4xMIC in rich media was chosen for further transcriptional studies.

Based on time-kill assays, 4xMIC streptomycin killed over half the bacterial population within the first hour of exposure to streptomycin. This rapid loss of viability suggests that the molecular mechanisms underlying lethality should occur within the first hour. Anand and colleagues observed that the uptake of C^{14}-streptomycin was biphasic – a rapid, initial uptake within 1 to 2 minutes, followed by a lag of approximately 10 minutes, then a secondary uptake (Anand *et al.*, 1960). Recovery studies showed that immediate uptake, within the first 15 minutes of treatment, could be reversed through sub-culturing on antibiotic free media yet, once an half hour or more of treatment ensued, cells could no longer be rescued (Wasserman *et al.*, 1954). Based on these observations, two time-points were chosen for subsequent transcriptional studies – 10- and 30- minutes post antibiotic addition. Transcriptional changes induced during the initial ten minutes of challenge may provide additional clues towards understanding the molecular mechanism of action; while changes observed following thirty minutes may provide further clues as to the nature of cell death.

Taken together, the following MIAME compliant framework for further transcriptional studies was adopted – *E. coli* challenged with 4xMIC in rich media for 10- and 30- minutes. In addition, the repetitive nature of time-kill studies lead to

the development of a readily reproducible culture system where the response of the bacteria is consistent and reliable.

2.5 Summary

1. The minimum inhibitory concentration of streptomycin against *E. coli* K12 MG1655 is 2mg/L.
2. *E. coli* K12 MG1655 is killed in a concentration-dependent manner which is dictated by the medium composition.
3. *E. coli* K12 MG1655 is killed in a time-dependent manner in both rich and minimal media at 4xMIC.
4. Optical density is not an accurate measure of culture growth/death in antibiotic studies. Adenylate kinase luminescence, ATP bioluminescence, NADH fluorescence, live/dead staining, or viable counts more accurately describe the culture's fitness.
5. Further studies will characterize the transcriptional impact of streptomycin at 4xMIC in rich media for 10- and 30- minutes to investigate the molecular mechanism of action and cell death, respectively.

3. Transcriptional Changes Associated With Streptomycin-Induced Cell Death

3.1 Overview

Prior to the development of microarrays, molecular biologists studied transcriptional changes one gene at a time (Shaw and Morrow, 2003). With the completion of the *E. coli* genome sequence in 1997 and subsequent development of this high throughput technology, we are now able to monitor global expression patterns in a rapid and facile manner. As such, microarrays have become as important today as PCR was in the 1980s and 1990s.

Microarrays represent a highly-tractable system to survey the dynamic nature of gene function on a global scale. Initial studies investigated the response of *E. coli* to numerous conditions including thermal stress and rich versus minimal media (Richmond *et al.*, 1999; Tao *et al.*, 1999). Richmond and colleagues examined the effects of heat shock on *E. coli* and demonstrated that, in addition to known transcriptional changes, microarrays could be used to identify new members of stress responses (Richmond *et al.*, 1999). Transcriptional profiles of rapidly dividing (logarithmic) versus adapted (stationary) cultures subsequently showed that developmental and physiological changes are traceable to the transcriptional level (Selinger *et al.*, 2000). These advancements have revolutionized the field of microbial physiology. It is now possible to understand physiology on a global scale

and to explore the fundamental transcriptional changes induced by diverse environmental stresses.

Consequently, microarrays are now widely used in drug discovery to gain novel insights into the mechanism of action of antibiotics. Shaw and colleagues observed the transcriptional profile of *E. coli* in response to four different bactericidal agents (Shaw *et al.*, 2003). These gene signatures reflected the accepted mechanism of action for 3 of the antibiotics examined. However, when *E. coli* was challenged with kanamycin, genes associated with the heat shock response, rather than the ribosome, were detected (Shaw *et al.*, 2003). Similar results were obtained when *Y. pestis* was challenged with streptomycin; *S. pneumoniae* was challenged with puromycin, but not other translation inhibitors; and *Bacillus subtilis* was challenged with gentamicin and streptomycin, although no data was presented for the latter. (Qiu *et al.*, 2005; Ng *et al.*, 2003; Lin *et al.*, 2005). Taken together, these studies challenge the traditional wisdom of 'one drug, one target' and suggest bacteria may mount a fundamental stress response to aminoglycosides.

This chapter aims to investigate the transcriptional response of *E. coli* K12 MG1655 to streptomycin utilizing cDNA microarrays and the culture conditions developed in the previous chapter. This study provides a detailed picture of the extent to which the genome is differentially regulated in response to streptomycin. In addition, two new targets were identified for further study.

3.2 *Materials and Methods*

The microarray methods described were developed by Khodursky *et al.* (2003). These techniques are highly specialized and summarized below.

3.2.1 Reagents

All chemicals were purchased from Sigma. Reagents which were not purchased commercially as RNase-free were made with DEPC-treated water.

3.2.2 Microarray Composition

Non-commercial, *E. coli* K12 MG1655 cDNA microarrays were used for this study. The microarray contained approximately 6000 PCR product spots representing over 99.6% of the known open reading frames and stable RNA genes, as well as over 1500 control and replicated elements (Khodursky *et al.*, 2003). In addition to the original Sigma-Genosys primer pairs, approximately 700 additional primer pairs were redesigned to decrease the potential for cross hybridization (Richmond *et al.*, 1999; Khodursky *et al.*, 2003). Once amplified, PCR products were spotted on poly-L-lysine-coated glass microscope slides using the GeneMachines OmniGrid 100 then stored under nitrogen until hybridizations were performed.

3.2.3 Culture Conditions

All experiments in this chapter were carried out utilizing single, purified colonies of *E. coli* K12 MG1655. Colonies were purified through sequential plating of frozen

stocks onto LB agarose (LB broth (Invitrogen, 12780-052 and 15g Bacto Agar (Difco, 0140-01), isolation of a single colony onto a new LB agarose plate, then subsequent selection of a single colony for liquid overnight cultures.

Once a single colony was purified, it was then used to inoculate a 10mL LB broth culture and incubated overnight at 37°C, 250RPM. The overnight bacterial culture was used to inoculate a further 10mL fresh, pre-warmed LB broth at 1:100 and incubated overnight at 37°C, 250RPM.

For each microarray comparison, two glass flat-bottomed Erlenmeyer flasks containing 150mL fresh, pre-warmed LB broth were inoculated 1:100 from the previously prepared overnight culture, covered with aluminium foil, and immediately placed in a rotary shaker at 37°C, 250RPM. Two hours post-inoculation, both flasks were removed from the incubator, streptomycin (Sigma, S6501-5g) added to the experimental flask, and both replaced in the rotary shaker. Streptomycin treatment concentrations reflected multiples of the determined minimum inhibitory concentration, 2mg/L (Section 2.3.1), as follows: 1xMIC = 2mg/L; 2xMIC = 4mg/L; and 4xMIC = 8mg/L. Each experiment was performed four times.

3.2.4 Cell Harvesting

Samples were collected at 10-, 30-, and 60- minutes following streptomycin addition. Cells were harvested by transferring 10mL aliquots from the Erlenmeyer flask to a 50mL Falcon tube containing 1mL of a freshly prepared, ice-cold, 5% water-

saturated phenol (Invitrogen/GibcoBRL, 15509) in ethanol stop solution and inverted 3 to 5 times. Cells were collected by centrifugation using a Beckman JA-20 rotor at 4°C, 8000RPM, for 5 minutes. The supernatant was decanted and cell pellets frozen at -80°C.

3.2.5 Total RNA Extraction

3.2.5.1 Cell Lysis

The cell pellet was resuspended in 600µL of TE, pH 8.0 (100mM Tris-chloride pH 8.0, 10mM EDTA pH 8.0, autoclaved for 20 minutes at 15 pounds per square inch liquid cycle) with 40µL of a 1mg/mL lysozyme solution. The cloudy lysate was transferred to a 1.5mL RNase-free eppendorf tube, 80µL 10% weight/volume (w/v) SDS (Ambion, 9822) was added, and the eppendorf tube vortexed for 30 seconds. The sample was placed in a 64°C water bath until the cloudy lysate cleared, approximately 1 to 2 minutes. Following water bath incubation, 88µL 1M sodium acetate pH 5.2 was added and the eppendorf tube inverted 3 to 5 times.

3.2.5.2 Hot Phenol Extraction

Water-saturated phenol, pH < 7.0, was pre-warmed in the 64°C water bath for a minimum of 30 minutes prior to use in extractions. Once warmed, 1mL water-saturated phenol was added to the cell lysate, the mixture inverted 3 to 5 times and immediately transferred to the 64°C water bath for six minutes during which time the eppendorf tubes were inverted 10 times every 40 seconds. Following water bath

incubation, samples were placed on ice for two minutes then centrifuged at 14,000RPM in a cold (4°C) bench top centrifuge for 10 minutes.

3.2.5.3 *Chloroform Extraction*

Following the hot phenol extraction, two distinct layers were visible – a clear organic lower layer with white fibrous interface and an aqueous top layer. Samples were placed on ice and the aqueous layer pipetted into a new, 1.5mL RNase-free eppendorf tube. An equal volume of chloroform was added, samples inverted 10 times, then centrifuged at 14,000RPM in a cold (4°C) bench top centrifuge for 5 minutes.

3.2.5.4 *Ethanol Precipitation*

Chloroform extraction yielded two distinct layers – a clear organic lower layer with a clear, yet visible emulsion interface, and an aqueous top layer. Samples were placed on ice and the aqueous layer pipetted into a new 1.5mL RNase-free eppendorf tube. Once the entire aqueous layer was collected, it was split between two 1.5mL RNase-free eppendorf tubes. Each sample was then combined with 1/10 the volume of a solution containing 3M sodium acetate, pH 5.2, and 1mM EDTA and inverted 5 times. 2.5 times the volume of 100% ice-cold ethanol (SDA Formula 3A, VWR Scientific, MK701904) was added, and the tubes immediately chilled at -80°C for 20 minutes. Following freezer incubation, samples were centrifuged at 14,000RPM in a cold (4°C) bench top centrifuge for 25 minutes. An opaque pellet was now visible in each eppendorf tube and the ethanol decanted. The pellet was washed with 1mL

70% ice-cold ethanol and centrifuged at 14,000RPM in a cold (4°C) bench top centrifuge for 5 minutes. The ethanol was decanted from the pellet. Any residual liquid was removed via pipetting and the pellet allowed to air dry for 20 minutes in the fume hood. Once dried, each pellet was resuspended in 100µL of RNase-free DEPC-treated water then combined to yield a final volume of 200µL.

3.2.6 DNase I Treatment

The 200µL RNA sample was combined with 0.5µL (20 units) RNase inhibitor (Boehringer Mannheim, 799017), 50µL 5xDNaseI buffer (50mM $MgCl_2$, 50mM Tris-HCl pH 7.5, 5mM EDTA, 5mM DTT), and 1µL (10 units) RNase-free DNaseI (Boehringer Mannheim, 776785) and incubated in a heat block at 37°C for 30 minutes. Following the digest, the reaction was purified via a series of phenol and chloroform extractions – 1 water-saturated phenol pH<7.0 extraction, 1 water-saturated phenol and chloroform (50:50 volume to volume (v/v)) extraction, and 2 chloroform extractions. Briefly, an equal volume of phenol and/or chloroform was added to the digest, the eppendorf tube inverted 10 times then centrifuged in a cold (4°C) centrifuge at 14,000RPM for 3 minutes. The aqueous layer was pipetted to a new eppendorf tube for subsequent extractions.

Following purification of the RNA sample, an ethanol precipitation was performed. To the aqueous layer, 1/10 the volume of 3M sodium acetate pH5.2 was added and the eppendorf tube inverted 3 to 5 times. 2.5 times the volume of ice cold 100% ethanol was added, the eppendorf tube inverted 3 to 5 times, then chilled at -80°C for

20 minutes. Following freezer incubation, samples were centrifuged in a cold (4°C) bench top centrifuge at 14,000RPM for 25 minutes. The ethanol was decanted from the newly formed transparent pellet, a wash of 1mL 70% ethanol was added and centrifuged for 5 minutes at 14,000RPM in a cold (4°C) bench top centrifuge. Again, the ethanol was carefully decanted, any residual liquid removed by pipetting, and the pellet allowed to air dry at room temperature in a fume hood for 25 minutes. Once dried, the RNA was resuspended in 50µL of RNase-free water (Ambion, AM9916).

3.2.7 RNA Quality and Quantity Determination

The amount of RNA obtained from each sample was determined on the basis of UV absorbance at 260nm (Sambrook *et al.*, 1989).

The RNA quality was determined using two methods. First, the ratio of the absorbance at 260nm verses the absorbance 280nm was determined. Samples with ratios less than 2, indicating a low level of residual DNA, were used in subsequent experiments (Sambrook *et al.*, 1989).

Secondly, the state of RNA degradation was assessed through agarose gel electrophoresis (Sambrook *et al.*, 1989). The electrophoresis apparatus (box, gel tray, and combs) was cleaned by soaking in 3% peroxide for 15 minutes. A 1% agarose gel in 1xTBE (90mM Tris-borate, 2mM EDTA) was poured and 1µL RNA electrophoresed at 80 volts for 1 hour. Samples with two distinct bands representing

the 16S and 23S rRNA were deemed of reasonable quality for further experiments. Samples which appeared as a smear of RNA were deemed degraded and discarded.

3.2.8 Direct Labeling of cDNA

3.2.8.1 Annealing of Random Hexamer Primers

RNA samples were directly labelled with Cy-dyes during the generation of cDNA. Briefly, 25 µg total RNA was combined with 10 µg random hexamer primers (Amersham Pharmacia Biotech, 272166) to a volume of 14.8µL. The mixture was incubated in a heat block at 70°C for ten minutes then placed on ice for a further 10 minutes.

3.2.8.2 cDNA Synthesis

To synthesize cDNA, 3µL dNTP mix (Ultrapure dNTP set, Amersham Pharmacia Biotech, 27-2035-01; 10xdNTP stock contained 1mM dATP, 1mM dCTP, 1mM dGTP, 0.4mM dTTP), 6µL 5x1st strand buffer, 3µL 0.1M DTT, 1.2µL 1mM Fluorolink Cy3- or Cy-5 dye (Amersham Pharmacia Biotech, PA53022 and PA55022 respectively) and 2µL Superscript II RNase H^- Reverse Transcriptase (GibcoBRL, 18064-014) were added to the primed random hexamer reaction. The mixture was kept at room temperature for 10 minutes before being transferred to a heat block at 42°C for 1 hour and 50 minutes.

3.2.8.3 Clean Up of cDNA Synthesis Reaction

Unincorporated Cy-dyes were removed through the use of Millipore microcon filters (YM-30, Fisher Scientific, 42410) as described by the manufacturer's instructions. Briefly, microcon filters were assembled using the provided filter and eppendorf tube then 320µL distilled water were added. The labelling reaction was gently pipetted onto the filter, and the sample eppendorf tube washed with 100µL distilled water which was subsequently added to the filter. The filter apparatus was centrifuged at 14,000RPM at room temperature for 8 minutes and the flow through discarded. Each filter was washed twice by adding 450µL distilled water to the filter and centrifuged at 14,000RPM for 5 minutes. Once all washes were completed, the collection eppendorf tube was discarded and filter inverted in a new 1.5mL eppendorf tube. The Cy-dye labelled cDNA was eluted through centrifugation at 14,000RPM for 3 minutes to yield approximately 10µL of probe. The probe was then stored in the dark at 4°C no longer than 24 hours.

3.2.9 Microarray Hybridization

3.2.9.1 Sample Preparation

Labelled cDNA samples were split into roughly equal volumes; one volume used for hybridization, the other covered in aluminium foil and stored at 4°C overnight in case the hybridization needed to be repeated.

Equal volumes of Cy-3 and Cy-5 labelled cDNA samples were combined in a single eppendorf tube and the volume adjusted to 14.6µL with distilled water. Once

combined, 2µL 20xSSC (3M NaCl, 0.3M $Na_3C_6H_5O_7$) and 2µL 10mg/mL salmon sperm DNA were added and mixed by flicking the eppendorf tube. 0.8µL of 5% SDS were added and the sample immediately placed in a boiling water bath for 2 minutes to denature the probes and blocking DNA. Once boiled, samples were collected at the bottom of the eppendorf tube through a short, approximately 5 second, centrifuge pulse.

3.2.9.2 Sample Hybridization

Microarray hybridization was carried out in specially developed hybridization chambers supplied by Monterey Industries (Richmond, California, USA). Printed microarray slides were placed in a clean hybridization chamber and 3µL drops of 3xSSC were placed on each of the four corners of the slide. All of the labelled probe mixture was gently applied as a single spot to the centre of the array making sure to remove any bubbles which may have accumulated. Using forceps, a microscope slide cover slip (No. 1 Corning Brand, Fisher Scientific, UFC30HVNB) was gently lowered over the microarray and sample spot at a 45° angle again taking great care to prevent bubbles from forming. The chamber lid was screwed into place and tightened down to ensure a water-tight seal. Chambers were then placed, taking great pains to keep flat, in a 65°C water bath for 8 to 12 hours.

3.2.10 Post Hybridization Clean Up

Following hybridization, the chambers were removed from the 65°C water bath one at a time, dried, and the lid unscrewed. The glass slide was removed and immersed

in a glass staining dish containing wash 1: 330mL distilled water, 20mL 20xSSC, and 1mL 10% SDS. The slide was gently waved back and forth in the solution until the cover slip dislodged. The slide was then placed in an aluminium slide rack already in the staining dish. Once all slides had had their coverslips dislodged, the slide rack was dunked up and down in wash 1 slowly 30 times.

Slides were removed one at a time and wash 1 wicked off the slide. The slides were not allowed to dry during this process. Immediately the slide was submerged in a second glass-staining dish containing wash 2: 350mL distilled water and 1mL 20xSSC. Once all slides were transferred to wash 2, the slide rack was dunked up and down in wash 2 slowly 30 times.

Again, slides were removed one at a time and wash 2 wicked off the slide. The slides were not allowed to dry during this process. Immediately the slide was submerged in a third-glass staining dish containing wash 3: 350mL distilled water and 0.5mL 20xSSC. Once all sides were transferred to wash 3, the slide rack was dunked up and down in wash 3 slowly 30 times.

Finally, the slides, in their slide rack, were dried via centrifugation at 600RPM for 5 minutes.

3.2.11 Data Acquisition

Microarrays were scanned immediately using a GenePix 4000B scanner (Axon Instruments) with a resolution of 10μm per pixel (Khodursky *et al.*, 2003). Each slide was scanned once at two different wavelengths: 532nm to measure the level of Cy-3 labelling and 635nm to measure the level of Cy-5 labelling. This produced three 16-bit TIFF files: 532_nm.tiff, 635_nm.tiff, and a composit.tiff.

Using the GenePix Pro software (version 3.0) provided with the Axon Scanner, slides were processed. Briefly, a *.gal file corresponding to the layout of the microarray was loaded and used to grid each slide. Once the grid was applied to the slide, the program used a method of adaptive circle segmentation to centre predetermined circles over each microarray spot. The grids were then visually inspected, and any circle which did not match the scanned fluorescence was manually reshaped to reflect the actual area of fluorescence or flag irregularities. The area inside each circle was classified as signal while the area outside was classified as noise. Manually adapting each circle enhanced the feature and ratio extraction process for downstream data analysis.

Once gridded, GenePix Pro analyzed the image to extract feature information and intensities for each spot on the array. This produced two files: *.gps containing the settings of the analysis and *.gpr containing the extracted ratios and features from the analysis. These files were then uploaded into the local, proprietary database, DeaBlu, where they were associated with a master god list which contained the

information required to associate the features with the microarray layout and the experimental conditions annotated.

Finally, the information was uploaded into the local, proprietary database, Gia, where features and intensities could be extracted for data analysis.

3.2.12 Data Analysis

3.2.12.1 Normalization and Significance Testing

All statistical and significance testing were carried out by Professor Arkady Khodursky at the University of Minnesota, St. Paul, USA. Briefly, normalization was performed using the foreground intensities for each wavelength of interest without background subtraction. These were subjected to Lowess smoothing to remove outliers then analysis of variance (ANOVA) to reduce the variance in the dataset at a predetermined p-value. From this a log2 data table was generated and imported into the freely available Statistical Analysis of Microarrays (SAM) program to determine significant changes in gene expression (Tusher *et al.*, 2001). Using a two-class comparison, with a false discovery rate (FDR) at the 90^{th} percentile of 5%, significant genes were determined.

3.2.12.2 Cut-off Values and Database Mining

SAM produced a ranked list of genes based on their computed fold-change. In order to reduce the data set to a manageable volume, these significant genes were

subsequently limited to those with a 2-fold or greater increase or decrease in expression for further mining.

These filtered lists were then used to query a tertiary database, E. coli Entry Point (http://coli.berkeley.edu/ecoli/) to learn about the significant genes. Typically, the name, b number, gene description, and Riley functional group were obtained and used to sort genes based on common functional groups or pathway involvement. For metabolic genes, this was aided by Ecocyc (http://ecocyc.panbio.com) (Keseler *et al.*, 2005). It is important to note that functional groups are not an intuitive labelling method for gene classification as many genes involved in stress responses are either mis- or un-classified. In light of this, analysis of stress responses was generally performed on a gene-by-gene basis guided by the literature rather than through attempts at automated database mining.

3.3 Results

3.3.1 Experimental Design

The global transcriptional changes of logarithmically growing *E. coli* K12 MG1655 cultures in response to streptomycin challenge were determined using spotted cDNA microarrays. In the previous chapter, the MIC of streptomycin against *E. coli* K12 MG1655 was determined as 2mg/L (Section 2.3.1) and the physiology of cell death examined through optical density, viable count, and dose-response measures (Section 2.3.3 and Section 2.3.2). The bactericidal nature of streptomycin was found to be both rapid, within less than one hour at 4xMIC, and concentration-dependent. Consequently, to observe the genes and/or pathways associated with the molecular action of streptomycin, logarithmically growing cultures were initially challenged with 4xMIC streptomycin and sampled at 10-, 30- and 60- minutes post drug addition. Subsequent experiments evaluated the transcriptional changes induced following treatment with increasing concentrations of streptomycin, 1xMIC and 2xMIC, at 30 minutes. Transcriptional changes as a result of sub-MIC concentrations were not evaluated. The lack of bacterial cell death as a result of treatment with 1xMIC was felt to mirror sub-MIC concentrations and utilised instead.

Figure 3.1 provides an overview of the steps involved in the microarray experiments briefly described below.

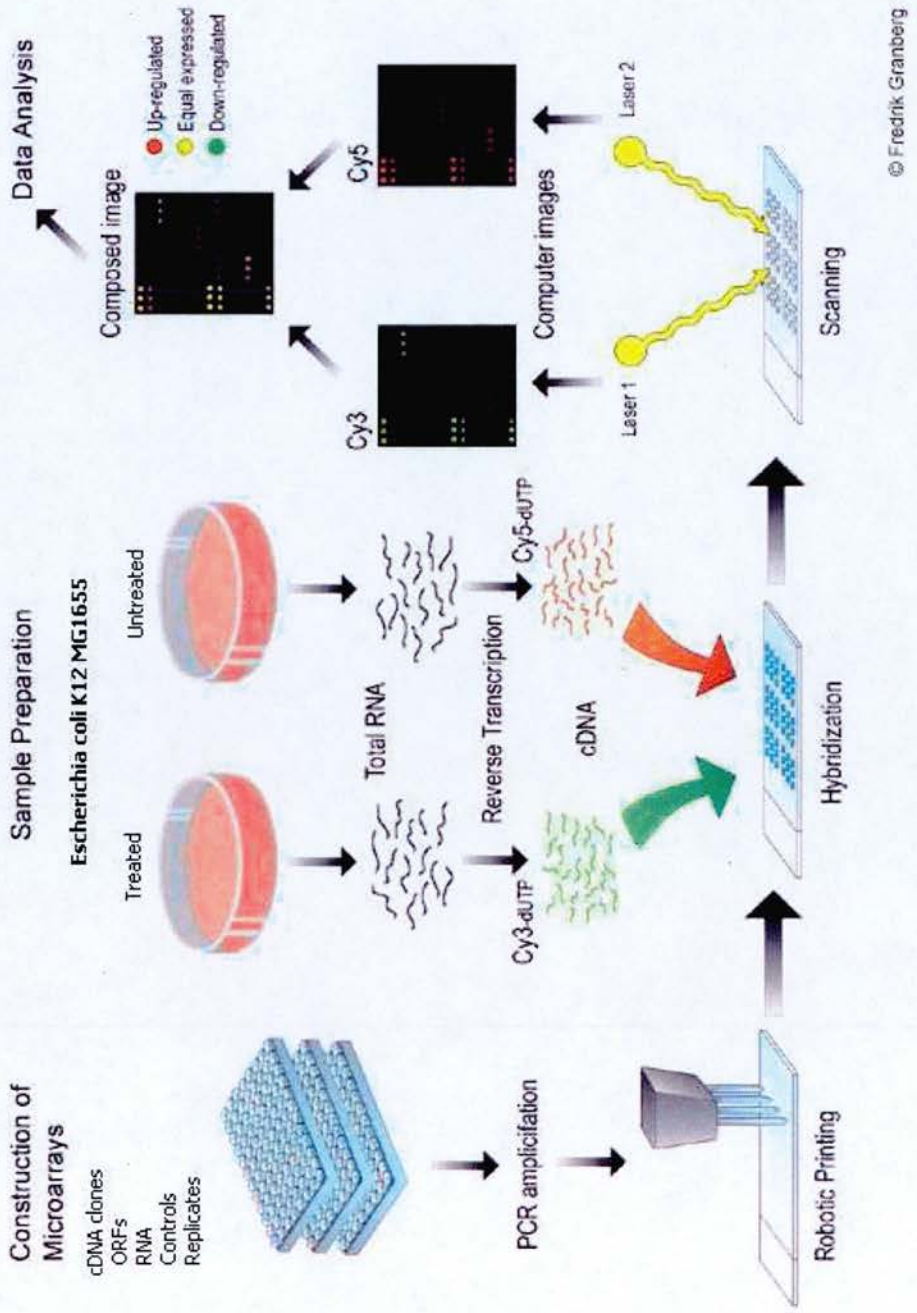


Figure 3.1. Overview of the Microarray Process.

Schematic showing the workflow of a typical microarray experiment. From left to right: cDNA clones are amplified then robotically spotted on poly-lysine coated glass slides; bacterial cultures are challenged, RNA isolated and labelled with fluorophors, then hybridized to microarrays; arrays are washed, fluorophors excited by a laser, and images captured for data analysis. Adapted from http://www.anst.uu.se/frgra677/projekt_eng.html.

An overnight culture was used to inoculate 2 glass-bottomed flasks containing fresh pre-warmed LB media. Once the cultures reached logarithmic growth, two hours after initial inoculation, streptomycin was added to one flask of the pair. Samples were taken at 10-, 30-, and 60-minutes post drug addition and flash-frozen for microarray profiling. Each experiment was performed four times using an individually isolated *E. coli* K12 MG1655 colony to account for biological variation. In addition, the technical variation associated with dye bias during cDNA synthesis was accounted for through a minimum of one dye-swap hybridization for each condition examined. Total RNA was isolated using the hot phenol method (Section 3.2.5.2). RNA concentration was determined by measuring the UV absorbance at 260nm and ranged from 3122.7μg/μL to 11918μg/μL. Exact yields, the ratio A_{260}/A_{280}, and hybridization information can be found in Appendix 2.

RNA quality was assessed on a 1% DEPC-treated, 1xTBE agarose gel. Figure 3.2 presents a representative RNA gel. Upon visualization, 3 discrete bands were observed representing the 23S and 16S rRNA as well as tRNA from top to bottom, respectively. RNA samples with a ratio A_{260}/A_{280} greater than two or a smear via electrophoresis visualization were deemed degraded and discarded (Sambrook *et al.*, 1989). This precaution ensured that only high quality RNA samples were used for hybridization experiments.

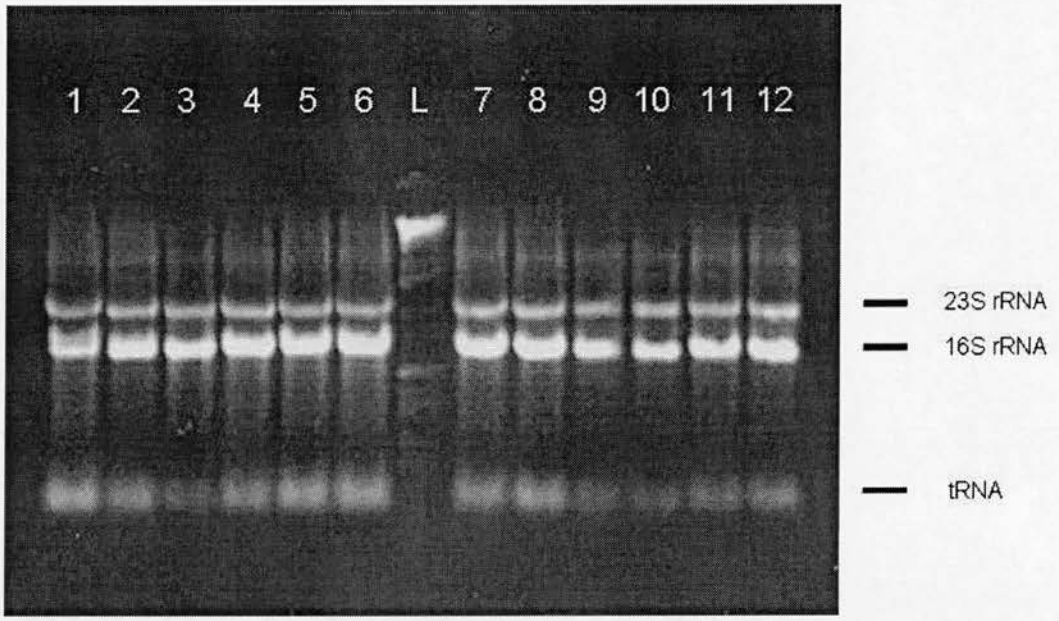


Figure 3.2. Total RNA Extracted from *Escherichia coli* K12 MG1655 Treated and Untreated Cultures.

Representative total RNA gel. RNA was extracted from bacterial cells using the hot phenol method then electrophoresed on a 1% DEPC-treated agarose gel in 1xTBE at 80V for one hour. RNA samples are as follows: 1, 3, 5, 7 – 10 minute control for 4xMIC challenge; 2, 4, 6, 8 – 10 minute sample for 4xMIC challenge; 9, 11 – 10 minute control for 2xMIC challenge; 10, 12 – 10 minute sample for 2xMIC challenge; L – ladder.

Following Cy-fluorophor labelling, two-colour competitive cDNA hybridizations were performed. Figure 3.3 presents a representative microarray hybridization, slide Ec9-25. In this experiment, RNA from 4xMIC streptomycin challenged for 30 minutes was labelled with Cy-3 fluorophor and fluoresced green at 532nm; control RNA from an untreated, 30 minute control was labelled with Cy-5 fluorophor and fluoresced red at 635nm. The image inset provides a closer examination of one sub-grid from the cDNA microarray and allows for cursory image interpretation. Green spots were induced upon exposure to streptomycin (i.e. RNA is more abundant for the target in question when cells are treated with streptomycin). Red spots were repressed upon exposure to streptomycin (i.e. RNA is more abundant for the target in question in the control than in cells treated with streptomycin). Finally, yellow spots represent targets which were detected in equal amounts in both the control and treated samples.

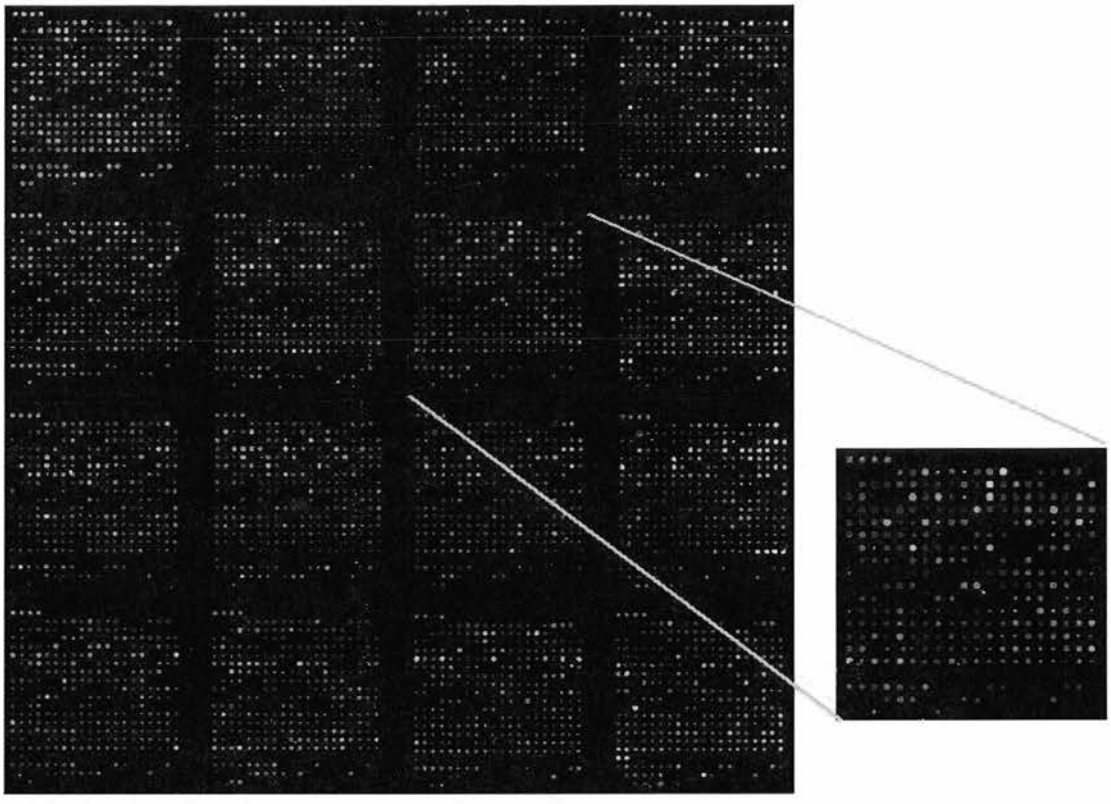


Figure 3.3. *Escherichia coli* K12 MG1655 cDNA Microarray (Ec9-25) of Genes Affected by 4xMIC Streptomycin, 30 Minute Challenge.

Representative cDNA microarray, Ec9-25, showing genes affected by treatment of *E. coli* K12 MG1655 with 4xMIC streptomycin for 30-minutes. cDNA array contains over 6,000 elements corresponding to open reading frames, RNA, and control elements. Inset is a magnification of one of the sub-grids. Each sub-grid contains genomic DNA in the top left corner as a positive control. In this experiment, challenge RNA is labelled with Cy-3 fluorophor (green) and control RNA is labelled with Cy-5 fluorophor (red). Genes which were up-regulated upon challenge appear green; down-regulated appear red; equally produced appear yellow.

Subsequently, the relative fluorescence was measured by a GenePix laser and images processed. Raw data was extracted and statistically analyzed by Professor Arkady Khodursky. Genes identified by SAM as statistically significant (Table 3.1, column one) were then constrained by a $\geq$ 2-fold threshold (Table 3.1, column two). Only those genes found significant by statistical methods and the relative fold-changed cut-off were considered differentially regulated and carried forward to data mining. By filtering data in this way, there was a dramatic reduction from an overwhelming number of features (over 1400 genes in the case of 4xMIC following 30minutes of treatment) to a manageable set (slightly less than 200 for the same case) and numerous false positives were excluded.

Hybridization Group	Genes with Observed Relative Changes in Transcription				
	After SAM		After Threshold		
	Increase	Decrease	Increase	Decrease	Total
1xMIC, 30-min	174	66	6	0	6
2xMIC, 30-min	125	92	35	10	45
4xMIC, 10-min	33	1	15	0	15
4xMIC, 30-min	723	682	81	91	172
4xMIC, 60-min	665	446	177	182	359

Table 3.1. Summary of Global Transcriptional Changes Induced by Treatment of *Escherichia coli* K12 MG1655 with Streptomycin After Statistical Testing and Threshold Reduction.

The number of genes differentially regulated was determined through statistical analysis of lowess smoothed and variance reduced data by the Statistical Analysis of Microarrays (SAM) program. A false discovery rate (FDR) of 5% was chosen. Only those genes with a ≥2-fold change where subjected to further analysis.

3.3.2 Early Responses to Streptomycin: 30 minutes

3.3.2.1 General Comments

To minimize potentially confounding secondary effects caused by prolonged exposure to any stressor, preliminary analysis focused on changes induced within 30 minutes of streptomycin addition. The relative transcription of 172 genes was altered – 81 genes were up-regulated, 91 were down-regulated; representing 4% of the genome and relatively few functional categories. (The complete list of differentially regulated genes can be found in Appendix 4.)

Figure 3.4 shows the breakdown into functional groups of those genes with altered transcriptional patterns. Genes were grouped by Riley/Labedan categories rather than Blattner groups to limit the number of hypothetical/unclassified products and conforming to the classification schemes currently adopted by emerging *E. coli* databases (Riley, 1998). Of the genes differentially induced, the largest groups were miscellaneous or hypothetical open reading frames (24.7%), amino acid biosynthesis (8.7%), degradation of DNA and adaptation (7.4% each). Of the genes differentially repressed, the largest groups were categorized as cell envelop related (21.9%), aerobic respiration (17.6%), and the degradation of small molecules (14.3%). However, classification via these methods is often misleading due to the involvement of proteins in numerous cellular pathways and stress responses which are not reflected in their functional classification. Consequently, each gene was evaluated in terms of its downstream function rather than its putative functional categorization.

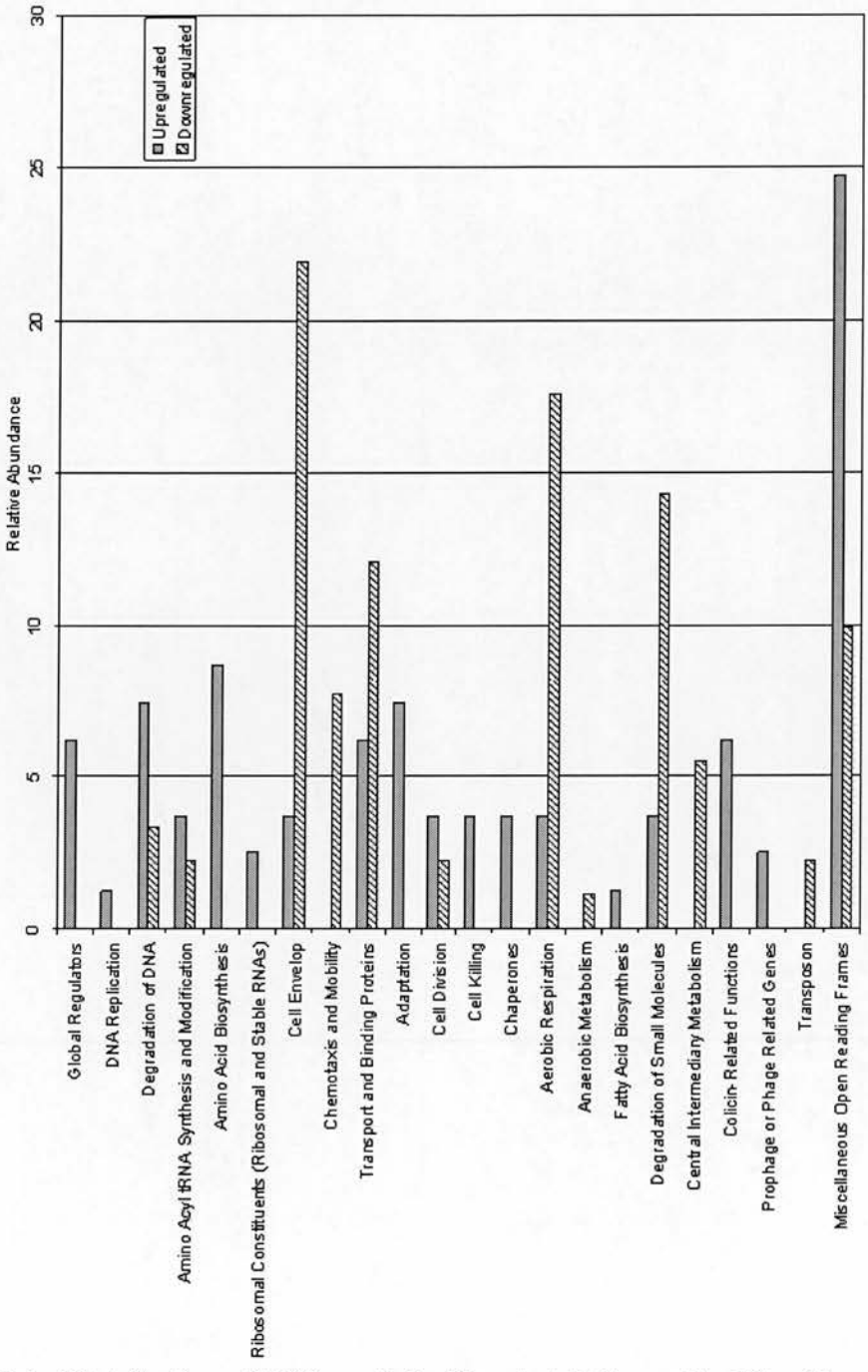


Figure 3.4. Distribution of Differentially Regulated Genes By Riley Functional Categories in Response to 4xMIC Streptomycin Following 30 Minutes of Treatment.

The 172 differentially regulated transcripts in response to 4xMIC streptomycin treatment for 30 minutes were grouped by Riley/Labeden functional categories then expressed as a relative abundance of the total number of differentially regulated transcripts.

3.3.2.2 *Genes Up-Regulated in Response to Streptomycin*

A complete list of differentially up-regulated genes can be found in Appendix 4A sorted by Riley/Labedan functional categories.

3.3.2.2.1 Stress Response Genes

Contrary to the suggestion that hypothetical or unclassified transcripts were the most abundant group of differentially regulated genes (Section 3.3.2.1), an examination of the protein function of each transcript indicated that a number of key stress responses were induced upon treatment with streptomycin. In this study, 17 of the 32 genes associated with the heat shock response were induced including the global regulators *lon* and *rpoH*. When the 2-fold change threshold was removed, a further 5 members of the heat shock regulon were significantly differentially regulated, bringing the total to 23. This accounts for over half of the genes of this regulon which includes both chaperones and proteases responsible for sequestering aberrant proteins for refolding or degradation (Yura *et al.*, 2000). The greatest relative fold-change for a single gene was detected for *hslS* (21.57x relative fold-change), a key member of this response. Similarly, 2 of the 4 genes responsible for drug sensitivity (*marRA*), and 3 of the 6 genes of the phage shock regulon (*pspABC*) were induced to similar levels. The observed expression ratios of these three regulons are detailed in Table 3.2. Taken together, these results suggest that the differential regulation of heat shock, drug sensitivity, and phage shock responses may play a key role in the bacterial response to streptomycin.

Gene Name	B No.	Function	Fold Change				
			1x MIC	2xMIC		4xMIC	
			30min	30min	10min	30min	60min
Heat Shock Response Regulon							
clpA	b0882	ATPase subunit of ClpAP ATP-dependent protease Ti	1.53	2.04	-	1.63	-
clpB	b2592	ATP-dependent ClpB protease	2.96	4.51	3.92	6.55	3.35
clpP	b0437	protease Ti; proteolytic subunit of ClpXP and ClpAP ATP-dependent proteases	1.55	2.25	1.52	2.49	2.25
clpX	b0438	ATPase subunit of ClpXP protease; degrades ssrA-tagged proteins	-	-	-	1.12	-
dnaJ	b0015	heat-inducible DnaK co-chaperone	1.51	2.47	-	4.12	6.74
dnaK	b0014	heat-inducible, ATP-regulated chaperone (HSP-70-like); dnaJ co-chaperone	1.62	3.88	4.84	6.07	4.38
ftsJ	b3179	heat-inducible 23S rRNA U2552 ribose 2'-O-methyltransferase (SAM-dependent)	1.62	2.78	2.43	4.17	3.46
groL	b4143	chaperonin Cpn60; GroESL large subunit	1.72	2.52	2.93	3.11	-
groS	b4142	chaperonin Cpn60; GroESL small subunit	1.95	2.54	3.85	4.58	-
grpE	b2614	nucleotide exchange factor for the dnaKJ chaperone	1.81	3.40	1.90	3.54	4.38
hflB	b3178	essential inner membrane ATP-dependent protease	1.47	1.84	1.52	2.52	2.65
hfq	b4172	HF-I, host factor for RNA phage Q beta replication	1.29	1.99	1.37	2.80	2.08
hslO	b3401	heat induced cytoplasmic chaperone (HSP33)	1.61	2.15	1.78	4.21	3.48
hslR	b3400	abundant heat shock protein that binds RNA & DNA (HSP15)	1.39	2.44	2.09	3.97	6.00
hslS	b3686	heat-inducible chaperone (HSP20)	3.86	16.56	6.38	21.57	21.64
hslU	b3931	heat-inducible ATP-dependent protease HslVU (ATPase subunit, HSP D48.5)	2.23	2.56	2.62	3.32	-
hslV	b3932	heat-inducible ATP-dependent protease HslVU (protease subunit)	1.77	2.45	2.22	3.64	2.98
htpX	b1829	membrane-bound zinc metalloprotease HtpX (Cpr & RpoH regulons)	2.08	5.25	3.11	9.73	7.46
lon	b0439	ATP-dependent protease La; DNA binding	2.03	2.53	2.59	3.95	4.07
miaA	b4171	dimethylallyl diphosphate; tRNA dimethylallyltransferase	1.35	3.21	2.24	4.48	2.79
rpoD	b3067	RNA polymerase σ70 subunit (exponential phase transcription)	1.32	1.74	-	1.45	1.51
rpoE	b2573	RNA polymerase σE subunit (extracytoplasmic/temperature/oxidative stress regulons)	-	-	-	1.11	-
rpoH	b3461	RNA polymerase σ32 subunit (heat shock transcription)	1.35	-	-	2.67	2.54
Drug Sensitivity							
marA	b1531	transcription activator of multiple antibiotic resistance (Mar) locus	-	2.02	-	3.05	6.65
marR	b1530	transcription repressor of multiple antibiotic resistance (Mar) locus	-	-	-	3.39	7.33
Phage Shock Response							
pspA	b1304	negative regulatory gene for phage-shock-protein psp operon	1.63	4.86	1.71	7.55	15.21
pspB	b1305	positive regulatory gene (w/ pspC) for phage-shock-protein operon	1.48	3.05	-	6.33	8.06
pspC	b1306	positive regulatory gene (w/ pspB) for the phage-shock-protein operon	-	-	-	2.84	6.40

Table 3.2. Relative Fold Change of Stress Response Transcripts in *Escherichia coli* K12 MG1655.

* No transcriptional changes were observed for the following transcripts: *fkpA, gapA, htpG, htrA, htrC, lysU, metA, ppiD* (heat shock regulon); *marBC* (drug sensitivity); or *pspDEF* (phage shock).

3.3.2.2.2 Global Regulators

In addition to changes in the relative expression of *lon* and *rpoH*, the induction of three further global regulators was observed – *lexA* (associated with the SOS response to DNA damage), *soxS* (associated with the response to oxidative damage), and *fnr* (associated with anaerobic metabolism). No subsequent alteration to the transcription of the downstream targets of either *lon, lexA,* or *soxS* was observed.

This is not the case for *fnr*. Fnr is a transcriptional dual regulator that induces or represses at least 275 genes primarily associated with anaerobic metabolism and/or the use of nitrogen as an alternative terminal electron acceptor (Keseler *et al.*, 2005). Despite the induction of *fnr*, 22 of its downstream targets are misregulated, 13 specifically associated with anaerobic metabolism. Rather than observing the expected induction of these genes, they are repressed often at the operon level. Only 7 *fnr*-regulated genes were repressed as predicted by functional annotations. Table 3.3 details the observed expression patterns of all 29 genes affected by *fnr*; appropriately regulated transcripts are bold.

Table 3.3. Relative Fold Change of *fnr* Regulated Transcripts in *Escherichia coli* K12 MG1655 After 30 Minute Exposure to Streptomyin.

Gene Name	B No.	Function	Fold Change
aceE	b0114	pyruvate dehydrogenase, decarboxylase component E1	-3.11
aceF	b0115	pyruvate dehydrogenase, dihydrolipoamide acetyltransferase component E2	-3.32
aspA	b4139	L-Aspartate ammonia-lyase; L-aspartase	-2.33
cheB	b1883	methylesterase	-3.03
cheR	b1884	methyltransferase	-2.95
cheY	b1882	response regulator for chemotactic signal transduction	-3.15
cheZ	b1881	CheY-P phosphatase	-4.11
fdnG	b1474	formate dehydrogenase-N (major subunit)	-4.36
focA	b0904	probable bidirectional formate transporter 1	-2.02
frdA	b4154	fumarate reductase flavoprotein subunit	-2.05
G6388	**b0725**	**phantom gene**	**-2.23**
gcvH	b2904	glycine cleavage; aminomethyl carrier	-2.96
gcvP	b2903	glycine dehydrogenase; decarboxylating	-2.62
malP	b3417	maltodextrin phosphorylase	-2.82
napA	b2206	nitrate reductase homolog	-2.14
napB	b2203	cytochrome c homolog	-3.06
narG	b1224	nitrate reductase α-subunit	-2.93
narH	b1225	nitrate reductase β-subunit	-4.26
narK	b1223	nitrate/nitrite antiporter	-3.22
nikA	b3476	nickel-binding periplasmic protein; Tar-dependent Ni-repellant chemosensor	-2.49
nuoC	**b2286**	**NADH-ubiquinone oxidoreductase subunit C, complex I; NADH dehydrogenase I**	**-3.52**
nuoE	**b2285**	**NADH-ubiquinone oxidoreductase subunit E, complex I; NADH dehydrogenase I**	**-4.95**
nuoF	**b2284**	**NADH-ubiquinone oxidoreductase subunit F, complex I; NADH dehydrogenase I**	**-2.98**
nuoG	**b2283**	**NADH-ubiquinone oxidoreductase subunit G, complex I; NADH dehydrogenase I**	**-3.01**
nuoN	**b2276**	**NADH-ubiquinone oxidoreductase subunit N, complex I; NADH dehydrogenase I**	**-2.51**
sucD	**b0729**	**succinyl CoA synthase a-subunit**	**-2.15**
tap	b1885	chemotaxis protein IV (aspartate receptor)	-2.97
tdcA	b3118	transcriptional activator of tdc operon	-2.01
tdcB	b3117	threonine dehydratase	-2.80

* Differentially regulated transcripts are grouped by operon. Transcripts (22) which are misregulated, in this case repressed where they would normally be induced by fnr expression, appear in normal type-set. Transcripts (7) which are appropriately regulated appear in bold type-set.

3.3.2.2.3 Unique Transcripts

Transcription of three unique transporters (*nhaA, zntA,* and *yccA*), two ribosomal components (*yfiA* and *rpoS*), and *prlC* (a gene involved in the degradation of signal peptides) were also observed. Of the few unclassified transcripts, *yccV,* an hypothetical *oriC* DNA binding protein, was the second highest up-regulated gene (5.74x relative fold-change).

Contrary to evidence suggesting an induction of the heat shock response, two genes of the cold shock response were observed – *cspA*, a key cold shock transcription factor; and *deaD*, a RNA metabolism gene. None of the downstream targets of either gene were differentially regulated at 30 minutes streptomycin treatment.

3.3.2.3 Genes Down-Regulated in Response to Streptomycin

A complete list of the differentially down-regulated genes can be found in Appendix 4B sorted by Riley/Labedan functional categories.

3.3.2.3.1 Motility and Chemotaxis

The majority of differentially repressed transcripts (25) observed were associated with cellular motility and chemotaxis – 18 of the 42 genes associated with flagella structure; and 7 of the 10 genes associated with chemotaxis (Macnab, 2003). These are repressed at the operon level in most cases. It is worthwhile to note that there is no observed repression of the master regulators of these operons, *flhDC*. When the 2-fold threshold was removed, 29 of the 42 flagella genes were observed; most

interestingly *flgM, fliZ,* and *motA* were induced 1.36-, 1.09-, and 1.25-fold, respectively. The observed expression ratios are detailed in Table 3.4. These results suggest that the differential regulation of the energy rich processes of motility and chemotaxis are a key response to streptomycin treatment.

Table 3.4. Relative Fold Change of Motility and Chemotaxis Transcripts in *Escherichia coli* K12 MG1655.

Gene Name	B No.	Function	Fold Change	
			30 min	60 min
Chemotaxis				
cheA	b1888	sensor kinase	-2.57	-3.98
cheB	b1883	methylesterase	-3.03	-5.85
cheR	b1884	methyltransferase	-2.95	-4.43
cheW	b1887	signal transduction	-2.94	-6.23
cheY	b1882	response regulator	-3.15	-8.36
cheZ	b1881	CheY-P phosphatase	-4.11	-
tap	b1885	chemotaxis protein IV (aspartate receptor)	-2.97	-5.29
tar	b1886	chemotaxis protein II (aspartate receptor)	-	-2.46
trg	b1421	chemotaxis protein III (ribose receptor)	-1.40	-3.15
tsr	b4355	chemotaxis protein II (serine receptor)	-	-2.05
Flagella synthesis				
flgA	b1072	assembly of P-ring	-1.22	-4.77
flgB	b1073	proximal rod subunit	-1.26	-13.19
flgC	b1074	proximal rod subunit	-2.36	-14.42
flgD	b1075	assembly of hook	-1.84	-11.57
flgE	b1076	hook subunit	-4.62	-19.93
flgF	b1077	proximal rod subunit	-4.61	-15.78
flgG	b1078	distal rod subunit	-5.99	-14.94
flgH	b1079	L-ring protein	-4.67	-8.97
flgI	b1080	P-ring protein	-3.81	-6.37
flgJ	b1081	muramidase	-5.84	-8.38
flgK	b1082	hook-associated protein I	-	-1.50
flgL	b1083	hook-associated protein	-	-3.30
flgM	b1071	Anti-σ28 (FliA) factor; regulator of FlhD	1.36	-
flhB	b1880	hook length control	-	-2.32
fliF	b1938	M-ring protein	-2.30	-5.61
fliG	b1939	rotor component; binds MotA	-4.32	-6.19
fliH	b1940	negative regulator of FliI ATPase	-4.25	-6.61
fliI	b1941	cytoplasmic membrane export ATPase	-3.55	-4.21
fliJ	b1942	rod, hook, & filament chaperone	-4.74	-6.16
fliK	b1943	hook length control	-5.33	-5.34
fliL	b1944	flagellar synthesis	-1.52	-4.13
fliM	b1945	switch component; binds CheY-P	-2.66	-6.76
fliN	b1946	switch component	-2.86	-3.69
fliO	b1947	protein export	-1.29	-1.42
fliP	b1948	protein export	-1.70	-1.63
fliQ	b1949	protein export	-1.77	-1.84
fliR	b1950	protein export	-1.44	-
fliS	b1925	FliC chaperone	-3.92	-7.68
fliT	b1926	FliD chaperone	-3.51	-6.96
fliY	b1920	cystine-binding protein; may regulate *fliA*	-2.05	-3.44
fliZ	b1921	may regulate *fliA*	1.09	-
motA	b1890	force-generator	1.25	-

3.3.2.3.2 Energy Metabolism

Transcripts associated with energy metabolism constitute a diverse set of genes that enable bacteria to respond to a variety of energy-limiting conditions. In this study, 38 genes associated with energy metabolism were differentially repressed, almost exclusively in an operon specific manner. These transcripts are primarily associated with anaerobic metabolism utilizing nitrogen as the terminal electron acceptor; 20 are regulated by *fnr*, a transcriptional dual regulator (Keseler *et al.*, 2005). As discussed in section 3.3.2.2.2, 13 of these 20 transcripts are misregulated – they are repressed despite the presence of *fnr* as a transcriptional activator; the remaining 7 transcripts are appropriately regulated (i.e. repressed). This result suggests that either suppression of the energy rich processes associated with utilizing alternative electron acceptors, such as nitrogen, may be a hallmark of the *E. coli* K12 response to streptomycin. Or, that a fundamental uncoupling of transcription and translation may be induced in response to streptomycin.

3.3.3 Early Response to Streptomycin: 10 minutes

To investigate whether the observed responses of *E. coli* K12 MG1655 to 4xMIC streptomycin challenge for 30 minutes were detectable at any earlier time-points, analysis of the transcriptional profile obtained after 10 minutes of exposure to streptomycin was performed. No transcripts were observed to be repressed at 10 minutes. The relative transcription of 15 genes was induced, all of which were detected at the later 30 minute time-point. Of these 15 genes, 12 belong to the heat shock response and are detailed in Table 3.2. The remaining 3 transcripts included *cpxP*, *yccA*, and *yfiA*. The complete list of differentially regulated genes can be

found in Appendix 3. This result suggests *E. coli* K12 MG1655 may mount an immediate response, which can be detected as early as 10 minutes post challenge, of the heat shock regulon.

3.3.4 Concentration-Dependent Response to Streptomycin at 30 minutes

Having established that there was a defined transcriptional response to streptomycin at the bactericidal concentration, I investigated whether a similar molecular response was elicited at the bacteriostatic concentrations of 1xMIC and 2xMIC streptomycin following 30 minutes treatment.

3.3.4.1 Response to 1xMIC Streptomycin Challenge

The relative transcription of 6 genes (*clpB*, *lon*, *hslS*, *hslU*, *htpX*, and *yfiA*) was induced upon *E. coli* K12 MG1655 challenge with 1xMIC streptomycin for 30 minutes; all of which are found in the 4xMIC response. The first five transcripts are members of the heat shock regulon (see Table 3.2) while the remaining transcript is associated with ribosomal function, the site of the primary lesion of streptomycin. The complete list of differentially regulated genes can be found in Appendix 5. This result suggests that when logarithmically growing cultures are subjected to the statically determined MIC, despite observing no phenotypical response, the bacterium undergo transcriptional changes.

3.3.4.2 *Response to 2xMIC Streptomycin Challenge*

The relative transcription of 45 genes was altered upon *E. coli* K12 MG1655 challenge with 2xMIC streptomycin for 30 minutes – 35 genes were up-regulated; 10 genes were down-regulated; and all but 2 genes (1 each for the up- and down-regulated transcripts) were found in the 4xMIC response. The complete list of differentially regulated genes can be found in Appendix 6.

Similar to the transcriptional alterations observed following bactericidal streptomycin treatment, three key stress responses were induced. At this concentration, 17 of the 32 genes associated with the heat shock response were induced to a similar magnitude as observed following 4xMIC streptomycin treatment for 30 minutes. When the 2-fold change threshold was removed, a further 3 members of the heat shock regulon were observed. The transcript with the greatest induction was again *hslS* (16.56x relative fold-change). The induction of transcripts associated with the heat shock regulon is detailed in Figure 3.5. These data suggest that the 32 induced heat shock transcripts respond proportionally to an increase in streptomycin concentration.

Similarly, *marA,* of the drug sensitivity response, and *pspAB,* of the phage shock regulon, were induced. The observed expression ratios of these three regulons are detailed in Table 3.2.

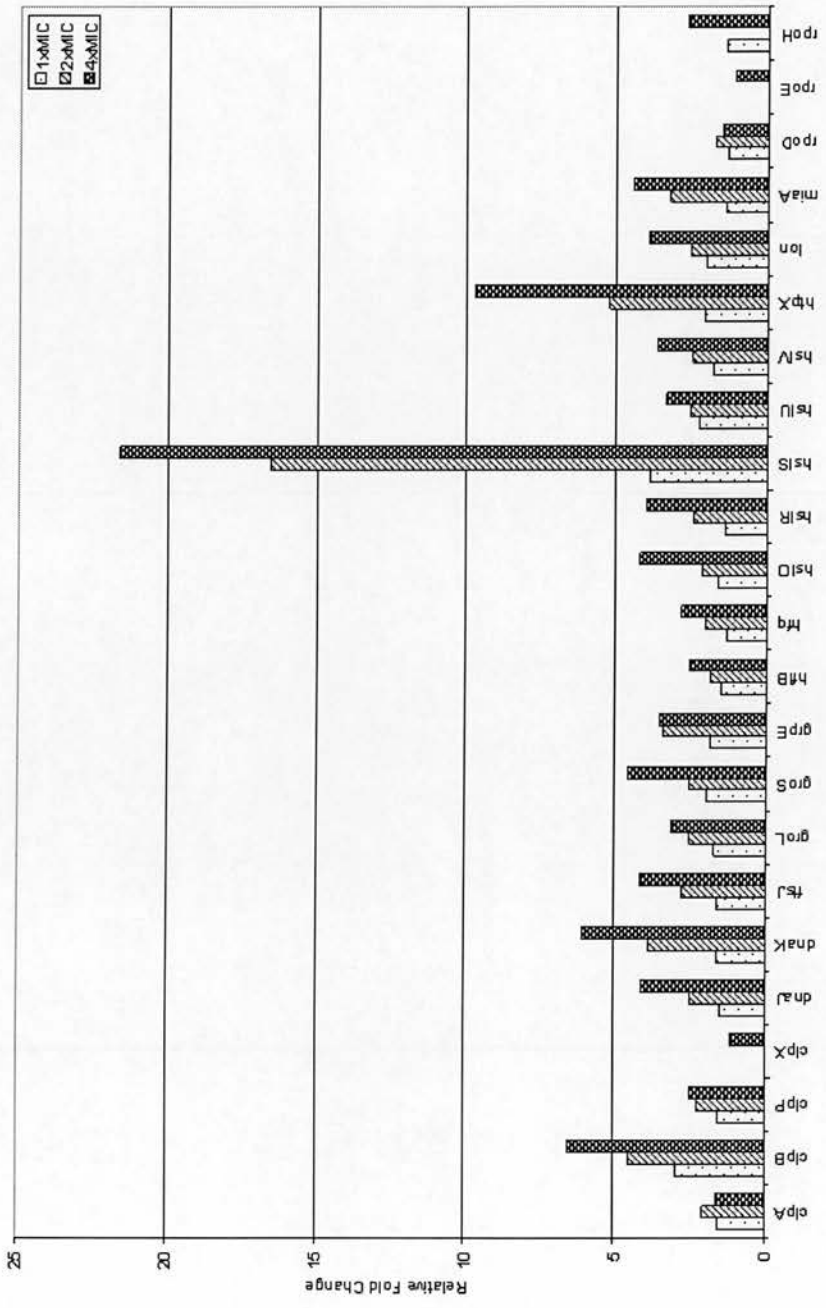


Figure 3.5. Heat Shock Response of *Escherichia coli* K12 MG1655 In Response To Increasing Concentrations of Streptomycin Treatment for 30 Minutes.

The 23 transcripts of the heat shock regulon with observed transcriptional changes following 30 minutes of treatment with increasing concentrations of streptomycin. No transcriptional changes were observed for the following transcripts: *fkpA, gapA, htpG, htrA, htrC, lysU, metA, ppiD* (heat shock regulon).

Ten transcripts were repressed following 30 minutes treatment with 2xMIC streptomycin; all but the *cyo* gene were also observed following 4xMIC streptomycin treatment – 3 transcripts are associated with flagella components (*flgFIJ*); 6 transcripts for maltose transport and degradation (*malKlamBmalE*, *malM*, *malP*). These results suggest that the differential regulation of both flagella synthesis and maltose metabolism (an alternative energy source) may be key responses to both bacteriostatic and bactericidal streptomycin concentrations.

3.3.5 Late Response(s) to Streptomycin: 60 minutes

3.3.5.1 General Comments

To investigate possible secondary effects caused by prolonged exposure to a bactericidal agent, transcriptional changes were observed following 60 minutes of treatment with 4xMIC streptomycin. The relative transcription of 359 genes was altered – 177 genes were up-regulated, 182 genes were down-regulated; representing approximate 8.4% of the genome. When compared to the early responses observed after 30 minutes of streptomycin treatment, 142 genes were previously observed – 70 up-regulated, 72 down-regulated; accounting for 42.3% of the total differentially regulated transcripts. The complete list of differentially regulated genes can be found in Appendix 7.

3.3.5.2 Genes Up-Regulated in Response to Streptomycin

A complete list of the differentially up-regulated genes can be found in Appendix 7A sorted by Riley/Ladedan functional categories.

3.3.5.2.1 Stress Response Genes

Similar to the transcriptional alterations observed after 30 minutes of treatment, three key stress responses were induced. At this time-point, 16 of the 32 genes associated with the heat shock response continued to be induced. When the 2-fold change threshold was removed, one additional member of the heat shock regulon was significantly differentially regulated. The greatest relative fold change for a single gene was detected for *hslS* (21.64x relative fold-change). Surprisingly, this induction level is almost identical to that observed at 30 minutes treatment. The induction of

transcripts associated with the heat shock regulon is detailed in Figure 3.6. These data suggest that despite the dogmatic view that this regulon responds to changes in temperature and/or protein folding in a transient manner, this is not the case in this study (Yura *et al.*, 2000). Transcripts were observed to be induced consistently over the time course.

Similarly, *marRA,* of the drug sensitivity response, and *pspABC,* of the phage shock regulon, were induced. The observed expression ratios of these three regulons are detailed in Table 3.2. Taken together, these three stress responses are induced and sustained for the duration of streptomycin treatment suggesting they represent a fundamental set of responses.

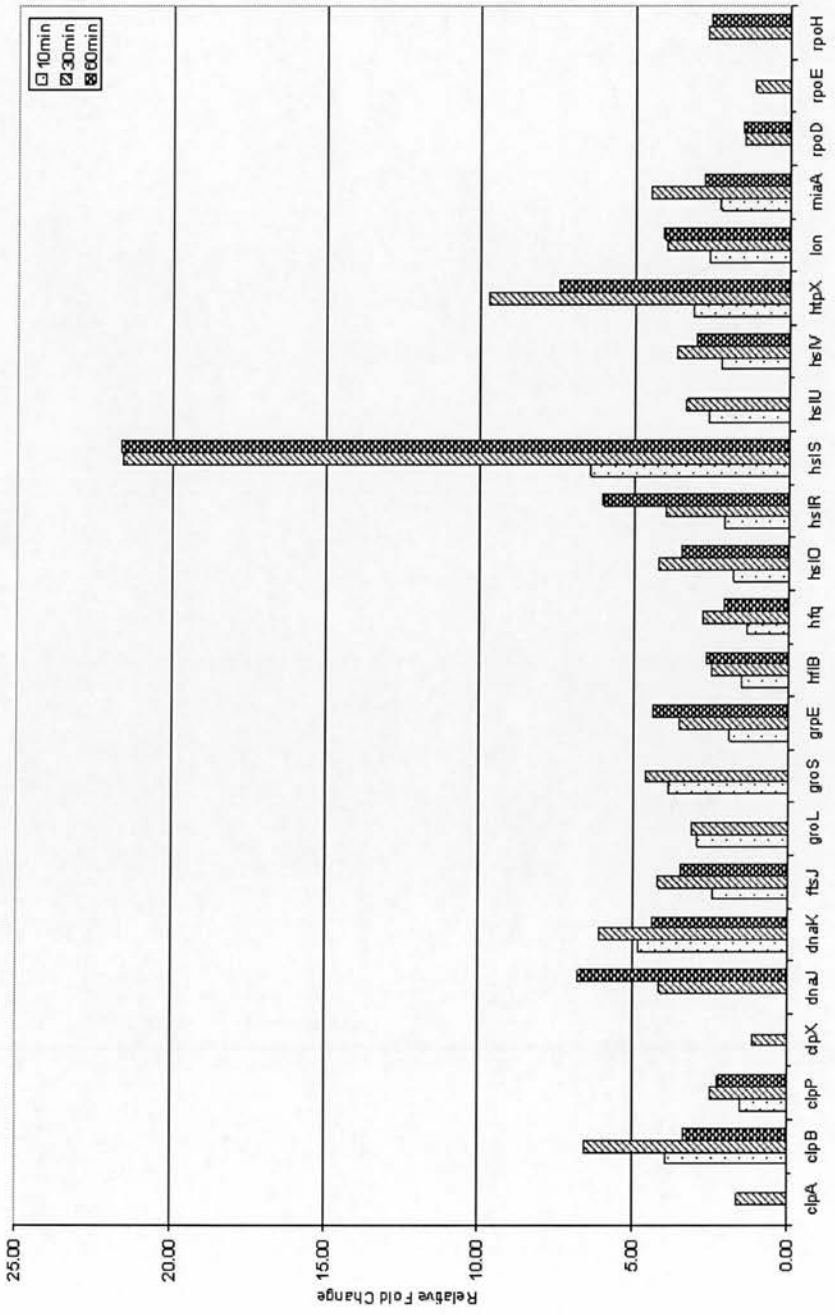


Figure 3.6. Heat Shock Response of *Escherichia coli* K12 MG1655 In Response To Increasing Exposure Time To 4xMIC Streptomycin.

The 23 transcripts of the heat shock regulon with observed transcriptional changes during exposure to 4xMIC streptomycin for 10-, 30-, and 60-minutes. No transcriptional changes were observed for the following transcripts: *fkpA, gapA, htpG, htrA, htrC, lysU, metA, ppiD* (heat shock regulon).

3.3.5.2.2 Global Regulators

The induction of the same five global regulators (*fnr*, *lexA*, *lon*, *soxS*, and *rpoH*) was observed after both 30- and 60-minutes of streptomycin treatment. However, key differences were observed in their downstream targets at the later time-point. A lack of coordination between global regulator and target(s) were observed for both *fnr* and *lexA*. In the case of *fnr*, the number of genes misregulated dramatically increased, often at the operon level. Nine genes were correctly regulated (1 induced, 8 repressed); 47 no longer displayed the expected regulation. Eleven genes were induced rather than repressed. Thirty-six genes were repressed rather than induced. Table 3.5 details the observed expression patters of all 56 differentially regulated *fnr* transcripts; appropriately regulated transcripts are bold.

In the case of the transcriptional repressor *lexA*, associated with the SOS response to DNA damage, four genes were induced rather than repressed (*lexA*, *recN*, *ruvA*, and *sulA*).

In addition, the induction of the global regulator *fur* (associated with the response to oxidative damage and iron depletion) was observed. The induction of this global regulator is induced by *soxS* and may represent a possible transcriptional response cascade.

Table 3.5. Relative Fold Change of *fnr* Regulated Transcripts in *Escherichia coli* K12 MG1655 After 60 Minute Exposure to Streptomycin.

Gene Name	B No.	Function	Fold Change
*aceE**	b0114	pyruvate dehydrogenase, decarboxylase component E1	-4.66
*aceF**	b0115	pyruvate dehydrogenase, dihydrolipoamide acetyltransferase component E2	-4.81
cyoA	b0432	cytochrome o oxidase subunit II; cytochrome bo(3) ubiquinol oxidase subunit II	4.46
cyoB	b0431	cytochrome o oxidase subunit I; cytochrome bo(3) ubiquinol oxidase subunit I	2.54
sdhC	b0721	succinate dehydrogenase (SQR) membrane anchor subunit, cytochrome b556	3.84
sdhD	b0722	succinate dehydrogenase (SQR) hydrophobic subunit	2.66
sdhB	b0724	succinate dehydrogenase (SQR) iron-sulfur protein	2.59
moaB	b0782	chlorate resistance protein B, MPT synthesis	-2.99
moaC	b0783	MPT synthesis; chlorate resistance protein C	-2.23
moaE	b0785	molybdopterin (MPT) synthase (large subunit); chlorate resistance	-2.27
dmsB	b0895	DMSO reductase subunit B; apparent Fe-S binding domain	-2.85
focA	b0904	probable bidirectional formate transporter 1	-4.57
ompW	b1256	outer membrane protein; colicin S4 receprtor	-15.76
tpx	b1324	periplasmic thiol peroxidase, antioxidant, thioredoxin-dependent	2.79
fnr	b1334	transcription factor for anaerobic growth; [4FE-4S] cluster	2.81
fdnG	b1474	formate dehydrogenase-N (major subunit)	-4.86
fdnH	b1475	formate dehydrogenase-N Fe-S subunit	-2.02
tar	b1886	methyl-accepting chemotactic signal transducer protein II (aspartate receptor)	-2.46
tap	b1885	chemotaxis protein IV (aspartate receptor)	-5.29
cheR	b1884	methyltransferase	-4.43
cheB	b1883	methylesterase	-5.85
cheY	b1882	response regulator for chemotactic signal transduction	-8.36
yecR	b1904	predicted protein	-2.44
napA	b2206	nitrate reductase homolog	-3.91
nuoG	**b2283**	**NADH-ubiquinone oxidoreductase subunit G, complex I; NADH dehydrogenase I**	**-3.05**
nuoF	**b2284**	**NADH-ubiquinone oxidoreductase subunit F, complex I; NADH dehydrogenase I**	**-2.99**
nuoE	**b2285**	**NADH-ubiquinone oxidoreductase subunit E, complex I; NADH dehydrogenase I**	**-4.95**
nuoC	**b2286**	**NADH-ubiquinone oxidoreductase subunit C, complex I; NADH dehydrogenase I**	**-3.29**
ackA	b2296	acetate kinase (creBC regulon)	-2.80
yfiD	**b2579**	**glycine radical cofactor that reactivates pyruvate formate lyase after oxidative stress**	**-3.54**
rpsP	b2609	30S ribosomal subunit protein S16; endonuclease	3.71
rimM	b2608	21-kDa ribosome maturation protein, essential for 16S RNA processing	2.84
trmD	b2607	tRNA m(1)G37 methyltransferase	5.33
rplS	b2606	50S ribosomal subunit protein L19	3.89
hypB	b2727	guanine-nucleotide-binding protein, required for metallocenter assembly in hydrogenases 1	-3.37
hypC	b2728	hydrogenase 3 chaperone-type protein; required for Hyd-3 metallocenter; binds HycE subu	-2.23
gcvT	b2905	aminomethyl transferase (tetrahydrofolate dependent)	-4.58
gcvH	b2904	glycine cleavage; aminomethyl carrier	-6.88
gcvP	b2903	glycine dehydrogenase; decarboxilating	-6.23
ansB	b2957	L-Asparaginase II	-11.45
tdcA	b3118	transcriptional activator of tdc operon	-6.78
tdcB	b3117	threonine dehydratase	-15.58
tdcF	b3113	predicted endoribonuclease	-3.80
tdcG	b3112	anaerobic pathway, L-serine deaminase, L-serine dehydratase	-4.27
gltB	**b3212**	**glutamate synthase (large subunit)**	**-2.47**
malP	b3417	maltodextrin phosphorylase	-4.96
pstS	**b3728**	**high-affinity periplasmic phosphate-specific transport protein**	**3.08**
katG	b3942	catalase hydrogen peroxidase I	-4.02
nrfA	b4070	formate-dependent nitrite reduction; tetraheme cytochrome c552	-6.07
nrfB	b4071	formate-dependent nitrite reduction; pentaheme cytochrome c formate-dependent	-2.61
dcuB	b4123	anaerobic, C4-dicarboxylate transporter	-3.35
aspA	b4139	L-Aspartate ammonia-lyase; L-aspartase	-5.73
frdA	b4154	fumarate reductase flavoprotein subunit	-8.43
frdB	b4153	fumarate reductase iron-sulfur protein subunit	-7.08

* Differentially regulated transcripts are grouped by operon. Transcripts (47) which are misregulated appear in normal type-set. Transcripts (9) which are appropriately regulated appear in bold type-set.

3.3.5.2.3 Unique Secondary Effects

With the subtraction of the transcripts induced at 30 minutes, three unique secondary effects were observed.

3.3.5.2.3.1 Transposition

Thirteen transcripts associated with the IS1 protein, a DNA transposase, and 3 prophage-associated genes were induced. These DNA transposases are responsible for the insertion and excision of foreign DNA into the genome and may have an effect on genome stability.

3.3.5.2.3.2 Ribosomal Components

In none of the previous experiments in this study were constituents of the ribosome induced. This includes the molecular target of streptomycin, *rpsL*, which encodes the S12 protein of the 30S subunit. After 60 minutes of treatment, the induction of 22 ribosomal components was observed. Twelve of the 22 transcripts of the 30S subunit and 18 of the 33 transcripts of the 50S subunit were detected. The observed expression ratios of the ribosomal constituents are detailed in Table 3.6.

3.3.5.2.3.3 Toxin-Antitoxin Pairs

Transcription of two antitoxin genes was observed (*dinJ* and *yefM*). Typically these genes are transcribed as a member of a balanced toxin-antitoxin pair that responds to cellular stresses including DNA damage and stalled ribosomes.

Gene Name	B No.	Function	Fold Change
Ribsome-associated proteins			
rimM	b2608	21-kDa ribosome maturation protein, essential for 16S RNA processing	2.84
30S (small) ribosomal subunit			
rpsB	b0169	30S ribosomal subunit protein S2; binds Zn(II)	3.09
rpsF	b4200	30S ribosomal subunit protein S6; suppressor of dnaG-Ts	2.00
rpsG	b3341	30S ribosomal subunit protein S7	1.68
rpsH	b3306	30S ribosomal subunit protein S8	1.73
rpsI	b3230	30S ribosomal subunit protein S9	1.67
rpsJ	b3321	30S ribosomal subunit protein S10	2.10
rpsK	b3297	30S ribosomal subunit protein S11	1.51
rpsM	b3298	30S ribosomal subunit protein S13	1.46
rpsN	b3307	30S ribosomal subunit protein S14	3.05
rpsO	b3165	30S ribosomal subunit protein S15	3.83
rpsP	b2609	30S ribosomal subunit protein S16; endonuclease	3.71
rpsR	b4202	30S ribosomal subunit protein S18	2.99
50S (large) ribosomal subunit			
rplA	b3984	50S ribosomal subunit protein L1	2.85
rplE	b3308	50S ribosomal subunit protein L5	2.07
rplJ	b3985	50S ribosomal subunit protein L10; streptomycin resistance	2.69
rplK	b3983	50S ribosomal subunit protein L11; kasugamycin sensitivity	3.08
rplN	b3310	50S ribosomal subunit protein L14	2.61
rplS	b2606	50S ribosomal subunit protein L19	3.89
rplY	b2185	50S ribosomal subunit protein L25	1.81
rpmE	b3936	50S ribosomal subunit protein L31	2.36
rpmF	b1089	50S ribosomal subunit protein L32	1.67
rpmI	b1717	50S ribosomal subunit protein A (L35)	2.87

Table 3.6. Relative Fold Change of Ribosomal Transcripts in *Escherichia coli* K12 MG1655.

3.3.5.3 Genes Down-Regulated in Response to Streptomycin

A complete list of the differentially down-regulated genes can be found in Appendix 7B sorted by Riley/Labedan functional categories.

3.3.5.3.1 Motility and Chemotaxis

Similar to the transcriptional alterations observed after 30 minutes of treatment, genes associated with cellular motility and chemotaxis continued to be repressed in an operon specific manner – 24 of the 42 genes associated with flagella structure, and 9 of the 10 genes associated with chemotaxis. When the 2-fold threshold was removed, 28 of the 42 flagella genes were repressed. Neither master regulator *flhDC* was repressed. The observed expression ratios are detailed in Table 3.4. These results suggest that the differential regulation of the energy rich processes of motility and chemotaxis may play a role in the response to streptomycin treatment over the observed time-course.

3.3.5.3.2 Energy Metabolism

The majority of differentially repressed transcripts, 81, were associated with complex sugar and/or anaerobic metabolism. As discussed in section 3.3.5.2.2, a lack of coordination was observed for transcripts associated with the global regulator *fnr*. Of the 36 metabolic genes observed as differentially regulated by *fnr*, 30 of these were misregulated – 25 transcripts were repressed rather than activated, 5 transcripts were activated rather than repressed. The remaining 6 transcripts were appropriately

regulated. A further uncoupling of transcriptional regulator and downstream targets may be a secondary consequence of prolonged streptomycin exposure.

3.3.5.3.3 Unique Secondary Effects

With the subtraction of the previously repressed transcripts, transcription of the quorum sensing regulator, *mqsR*, and regulator of biofilm through signal secretion, *bssR*, were repressed.

In addition to the generalized repression of energy metabolism, the specific repression of 7 of the 8 genes required for the production and assembly of ATP synthase, the cellular machine responsible for maintaining proton motive force and energy, were observed. These genes represent one operon which is differentially repressed and are detailed in Table 3.7.

Table 3.7. Relative Fold Change of ATP Synthase Transcripts in *Escherichia coli* K12 MG1655.

Gene Name	B No.	Function	Fold Change
atpE	b3737	ATP synthase subunit c, membrane-bound, F0 sector; DCCD-binding	-1.41
atpF	b3736	ATP synthase subunit b, membrane-bound, F0 sector	-1.40
atpH	b3735	ATP synthase subunit delta, membrane-bound, F1 sector	-2.05
atpA	b3734	ATP synthase subunit alpha, membrane-bound, F1 sector	-1.93
atpG	b3733	ATP synthase subunit gamma, membrane-bound, F1 sector	-2.20
atpD	b3732	ATP synthase subunit beta, membrane-bound, F1 sector	-2.50
atpC	b3731	ATP synthase subunit epsilon, membrane-bound, F1 sector	-1.71

3.4 Discussion

3.4.1 Methodological Considerations

The bactericidal activity of streptomycin against *E. coli* K12 MG1655 is known to be concentration- and time- dependent. Utilizing these observations, reproducible culture conditions conforming to MIAME guidelines were established in the previous chapter. This chapter aimed to investigate the temporal and concentration-dependent transcriptional changes associated with streptomycin-induced cell death in order to develop additional insights into its pleiotropic mechanism of action and identify additional gene candidates for further study.

Microarrays have become an invaluable tool for simultaneously assessing global mRNA changes in an organism under a variety of environmental conditions. Consequently, they are ideally suited to revisit the pleiotropic mechanism of streptomycin-induced cell death. At the time these studies were conceived, two microarray platforms existed, Affymetrix microarrays or glass-slide cDNA microarrays. Although the Affymetrix technology was available in Edinburgh, the facility was in its infancy and unable to provide training or cost-effective reagents. Due to the ambitious nature of the proposed experimentation, alternative sources were investigated.

Glass-slide cDNA microarrays quickly became the preferred platform due to their affordability ($62 per microarray verses £800 per Affymetrix microarray) leading to

a collaboration with Professor Arkady Khodursky at the University of Minnesota. As a leader in the field, his experience with the complicated statistical issues surrounding transcriptomics and a willingness to foster a hands-on learning environment presented the most attractive avenue for utilizing this technique. This decision provided access to training in all aspects of the methodology from experimental design to management of the workflow including microarray printing and data acquisition.

Despite the overwhelming training advantages of using glass-slide cDNA microarrays, a number of difficulties plagued these experiments. Unlike Affymetrix microarrays, which were commercially available and subject to strict quality control verification, cDNA microarrays were printed locally utilizing PCR products spotted on poly-L-lysine coated slides. During production a number of technical difficulties with the pins used to transfer the PCR products to the glass slides occurred. Consequently, the microarrays represent the majority, but not the entirety, of the genome as can be seen by the rows of missing spots on array Ec9-25 (Figure 3.3). These discrepancies went undetected until actual microarray hybridization experiments had been performed. Due to the consistency of the errors, the high quality data generated, and the overwhelming number of hybridization experiments performed it was decided to use the available data to assess relative rather than absolute global trends and not attempt an additional print.

Once an effective microarray platform was chosen, the ability to isolate pure and intact mRNA was critical to the success of these transcriptional studies. Unlike eukaryotic mRNA, prokaryotic mRNA lacks a poly-adenlyated tail (Alberts *et al.*, 2002). Due to this fundamental difference, methods which isolate total RNA then enrich for mRNA were unsuited for these studies. Commercially available reagents from suppliers such as Ambion or Qiagen were not yet widely available. Therefore, the traditional method of RNA isolation, hot phenol extraction, was chosen for the consistent production of high quality RNA samples seen in Figure 3.2. Sharp, discrete bands corresponding to rRNA and tRNA species are indicative of intact RNA whereas a smear represents significant degradation. Subsequent determination of the A_{260}/A_{280} provided an accurate means of assessing DNA contamination (see Appendix 2). Strict quality control was achieved by only using samples with visually discrete gel bands and an A_{260}/A_{280} below 2 (Sambrook *et al.*, 1989).

Well defined protocols for reporting the experimental methods, MIAME guidelines, have been required for publication since 2002 and adopted for these studies (Brazma *et al.*, 2001). To account for potential technical errors introduced during the printing process or the isolation of RNA species, four independent replicates were performed for each hybridization experiment. To control for the known bias associated with reverse transcription and incorporation of Cy-fluorophors, a minimum of one dye-swap experiment was performed (Khodursky *et al.*, 2003). Taken together, these constraints led to the production of highly reproducible results both visually - array images contain very little background noise - and statistically, where a false

discovery rate of <1 gene per genome was achievable for all experiments except the 4xMIC after 60 minutes challenge. A false discovery rate of 5 genes per genome was achieved for this experiment and is highly indicative of the pleiotropic and secondary effects that result from prolonged exposure to an environmental stressor.

Although MIAIME guidelines have established a pipeline for performing and reporting microarray experiments, statistical methodologies and data mining practices remain in their infancy. Anecdotal evidence has shown large discrepancies in statistical results depending not only on the method used, but also the software version and computing environment they were performed in (personal communication, A. Khodursky). Normalization and statistical analysis were kindly performed by Professor Arkady Khodursky, who has been influential in the development of this field both at Stanford and the University of Minnesota.

Finally, an ad hoc data-mining pipeline was designed. Transcripts deemed statistically significant were subjected to a 2-fold change cut-off. This level was chosen to introduce an element of biological significance into the data mining. The transcripts were then grouped by Riley/Labedan functional categories (Figure 3.4). Riley/Labedan categories group genes together based on their known physiological function and were introduced shortly after Blattner *et al.* (1997) published the completed genome sequence of *E. coli* K12 (Riley, 1998; Blattner *et al.*, 1997). Riley noted that gene products were classified in up to four of the 118 proposed physiological categories (Riley, 1998). Despite their adoption as the standard for

functional group classification in the developing *E. coli* genomics resource Ecocyc, an inconsistent nomenclature persists.

Although a valuable method of assessing global trends, functional group categorization provided little more than a cursory survey of differentially regulated transcripts. This may be explained by the multiple groupings of genes; available tertiary databases only return one functional group when queried. The problem was compounded when genes were examined individually. Genes with the highest levels of differential regulation were often randomly assigned to functional categories which did not accurately represent their physiological role. Specifically, the chaperones and proteases of the heat shock response were assigned to numerous functional groups including adaptation, chaperones, colicin-related functions, degradation of DNA, and global regulators. Disparate category designations only complicated the interpretation of these functional genomic studies. Consequently, differentially regulated genes were evaluated individually based on their defined protein function and pathway involvement.

Unlike recently adopted model organisms, including *B. subtilis, Mycobacterium tuberculosis,* and *S. pneumoniae;* or niche studies, *E. coli* has been intensively studied since the 1950s (Richmond *et al.*, 1999). This has led to the productive development of the available biochemical data and resources, yet, adoption of a common nomenclature for both genes and functional annotation remains poorly embraced as reflected in both the disjointed databases and the multitude of keywords

found in the literature. The usefulness of functional genomics will remain hindered until concrete standards are universally adopted.

In addition to biological and experimental variation, microarray experiments can be influenced by myriad technical factors including array production and image analysis. For this reason, differentially regulated genes identified through microarray studies are traditionally validated through independent methods such as real-time RT-PCR. Real-time RT-PCR measures the accumulation of cDNA from the original mRNA signal with greater sensitivity than microarrays thereby eliminating false positives and independently verifying the expression of key genes of interest. In addition, experiments are gene-specific, quantitative, reproducible, and require 1000-fold less mRNA than the original microarray (Rajeevan *et al.*, 2001A; Rajeevan *et al.*, 2001B). The microarray results presented herein were not independently verified through real-time RT-PCR experiments due to a lack of access to facilities or appropriate reagents. Furthermore, all original mRNA samples were degraded due to a delay in shipping samples by the US Customs department. The use of functional studies in the form of deletion mutants was undertaken instead.

3.4.2 Transcriptional Consequences of Streptomycin Treatment

The molecular mechanism of streptomycin lethality was investigated at 4xMIC streptomycin, a bactericidal concentration, following a 30 minute exposure. Preliminary analysis focused on this early time-point to minimize potential secondary effects as a result of prolonged exposure. Streptomycin was shown to

differentially regulate a number of key responses in *E. coli* including induction of the heat shock response, which encompasses members of the phage shock and drug sensitivity operons, as well as a generalized repression of cellular metabolism, specifically motility and chemotaxis. These primary trends were detected in a temporal and dose-dependent manner.

The heat shock response is a fundamental bacterial response to stress used to adapt to environmental changes in temperature, ethanol and/or heavy metals concentration, osmolarity, and starvation (Yura *et al.*, 2000). These conditions often result in unproductive changes to functional proteins and require the induction of chaperones and proteases to sequester abnormal proteins for degradation or facilitated refolding. Although the precise mechanism of streptomycin lethality remains unclear, the primary ribosomal lesion causes mistranslation resulting in an accumulation of aberrant proteins (Davis, 1987). VanBogelen and Neidhardt observed the addition of certain aminoglycosides, including kanamycin, puromycin, and streptomycin (termed the H group), induced changes to the proteome similar to the heat shock response whereas other protein synthesis inhibitors, including chloramphenicol, erythromycin, and tetracycline (termed the C group), induced changes associated with the cold shock response; although they failed to identify the altered proteins (VanBogelen and Neidhardt, 1990).

The induction of heat shock transcripts was detected within 10 minutes of streptomycin challenge with 4xMIC; over half the regulon, 23 of the 32 genes with

assigned functions, were detectable at 30 minutes (Figure 3.6) which validates VanBogelen and Neidhart's (1990) proteomic studies. Subsequent transcriptional studies showed the response to be concentration-dependent; core genes were detected at both 1xMIC and 2xMIC (Figure 3.5) suggesting that despite a non-lethal physiology *E. coli* may mount a molecular response to bacteriostatic challenges. Similar dose-dependent results were obtained by Shaw and colleagues in *E. coli* challenged with increasing concentrations of kanamycin for 30 minutes suggesting a possible common molecular response for H group aminoglycosides (Shaw *et al.*, 2003).

Of the genes of the heat shock regulon, *hslS*, an inclusion body protein was consistently the highest induced of all genes (Table 3.2.) Unfortunately, *hslT*, the second member of this operon, was one of the PCR products that failed to print on the glass-slide cDNA microarrays. Shaw and colleagues observed a similar result in response to kanamycin treatment; *hslS* and *hslT* where consistently induced 303.58- and 233.73-fold, respectively (Shaw *et al.*, 2003). Based on their results, it can be inferred that *hslT* would have also been induced in response to streptomycin treatment.

Bianchi and Baneyx previously generated an *E. coli* strain containing an *hslTS-lacZ* promoter fusion that produced a functional β-galactosidase upon treatment with streptomycin and neomycin (Bianchi and Baneyx, 1999). Streptomycin has been shown to induce the transcription of *hslTS* that are subsequently translated into

functional proteins rather than degraded. *hslS* may play an integral role in the molecular response to streptomycin and is an ideal candidate gene for further investigation via deletion mutation studies.

Interestingly, Ren and colleagues observed the *hslTS* operon most strongly induced upon biofilm formation (Ren *et al.*, 2004). When coupled with the almost complete repression of transcripts associated with chemotaxis and motility (see below), these data suggest *E. coli* cultures may attempt to form protective biofilms to survive streptomycin challenge. Further studies investigating the transcriptional changes of specific subpopulations of *E. coli*, planktonic versus biofilms, in response to streptomycin may yield new insights into bacterial defence mechanisms.

Whereas the induction of genes associated with the heat shock response appears to be both antibiotic class (aminoglycoside) and lesion (empty P-site) specific, there was a generalized, operon-specific repression of genes associated with chemotaxis and motility (Table 3.4). A similar repression was previously observed across diverse antibiotic classes (Shaw *et al.*, 2003). This may be explained as a general reduction in fitness as the cell diverts energy away from unnecessary processes towards combating cellular injuries. Consequently, it is highly unlikely these transcripts play an integral role in the specific mechanism of streptomycin-induced cell death.

Despite the straightforward nature of the majority of differentially regulated genes identified through these studies, three genes appeared to contradict previous trends.

The most interesting of these was the dual regulator *fnr*. Despite a marked induction of *fnr*, associated with anaerobic metabolism and the use of nitrogen as an alternative electron acceptor, there was little consistency between the induction and repression of its downstream targets (Table 3.3 and Table 3.5). Genes traditionally repressed by *fnr* were repressed. However, genes normally induced were detected as repressed suggesting a fundamental uncoupling of global regulation in favour of repressing anaerobic metabolism. A similar trend was observed in response to kanamycin, rifampin, and ampicillin challenge (Shaw *et al.*, 2003). The overall repression of cellular metabolism and electron transport may be a common response to growth arrest, which responds to more than one intracellular signal during times of extreme stress.

In contrast to the observed induction of the heat shock response, both at the transcript and proteomic levels, two cold shock transcripts were differentially induced in response to bactericidal streptomycin challenge; the global regulator *cspA* of the cold shock response and *deaD,* RNA helicase. *cspA* has been previously reported to be induced in rich media (Tao *et al.*, 1999). The differential regulation of this transcript may be explained as an artefact of the culture conditions chosen for these studies. Similarly, *deaD* was induced in response to rifampin challenge (Shaw *et al.*, 2003). The differential regulation of this transcript is almost certainly a false positive. Taken together, these two transcripts highlight the importance of understanding the statistical challenges associated with manipulating large data sets, the limitations of global studies, and the importance of guided human analysis over automated mining.

Once the global trends were identified and subtracted from the significantly differentially regulated genes, few unidentified open reading frames remained. Of these, the highest differentially induced transcript, 5.73-fold, was for *yccV*, a hemi-methylated *oriC* DNA binding protein (d'Alençon *et al.*, 2003). At the time these studies were performed, a single paper reporting a potential link with DNA replication had been published. Therefore, this uncharacterized transcript may be a potential new target for streptomycin and is ideally suited for further study.

In addition to the similarities observed between streptomycin and kanamycin treatment of *E. coli*, two additional studies highlighted the global transcriptional changes induced by translation inhibitors in the important human pathogens *S. pneumoniae* and *Y. pestis* (Shaw *et al.*, 2003; Ng *et al.*, 2003; Qiu *et al.*, 2005). Ng and colleagues demonstrated the heat shock response was induced by a sub-lethal concentration of puromycin, but not by other translation inhibitors, in *S. pneumoniae* (Ng *et al.*, 2003). Northern blot analysis clarified this observation, indicating both puromycin and streptomycin induced *clpL*, a key member of the regulon (Ng *et al.*, 2003). Similarly, bactericidal streptomycin challenge was shown to primarily induce the heat shock response and repress chemotaxis and mobility in *Y. pestis* (Qiu *et al.*, 2005). The induction of the heat shock response and repression of the energy rich processes of chemotaxis and motility may represent a hallmark set of responses induced by streptomycin challenge irrespective of the bacterium and play a fundamental role in cell death.

However, the higher concentrations of streptomycin studied herein may induce heat shock transcripts as a secondary consequence of mistranslation and protein aggregation thus masking a fundamental response. Given the observed induction of heat shock genes across a range of sub-MIC to bactericidal concentrations by puromycin and gentamicin, it seems unlikely that the induction of heat shock transcripts is solely a result of high levels of streptomycin (Ng *et al.*, 2003; Lin *et al.*, 2005). Further transcriptional studies utilizing sub-MIC concentrations may clarify the key players of this response.

After intensive data mining of the differentially regulated transcripts deemed significant following 30 minutes treatment with streptomycin, it was surprising that transcripts associated with the primary lesion – the S12 (*rpsL*), S4 (*rpsD*) or S5 (*rpsE*) proteins of the small ribosomal subunit – were not observed. Visual inspection of the arrays indicated *rpsL* was not appropriately spotted (as previously observed for *hslT*). However, *rpsD* and *rpsE* could be visualized but were not differentially regulated. As a result, a further study focusing on potential secondary effects following 60 minutes of treatment was undertaken.

Previous physiological studies indicated significant cell death following 60 minutes of treatment (Section 2.3.3.1). Differential gene regulation of such an advanced stage of cell death is more likely to reflect the fundamental metabolic catastrophe rather than an organized response and extreme caution was employed in interpreting the results. As previously observed, transcripts associated with the heat shock

response were induced (Figure 3.6). Traditionally viewed as a transient response, these studies show it is induced immediately upon treatment with streptomycin and sustain through the course of cell-death thus challenging the traditional dogma (Yura *et al.*, 2000).

Ribosomal components were significantly induced at the 60 minute time-point. Ng and colleagues had previously observed marginal induction of ribosomal genes in response to other translational inhibitors (Ng *et al.*, 2003). The observed differences could reflect different lesion locations within the ribosome of various translational inhibitors. Streptomycin induces mistranslation without blocking the processivity of the ribosome (Carter *et al.*, 2000). Chloramphenicol, tetracycline, and erythromycin were all previously shown to induce cold shock proteins and actively prohibit translation by blocking sites within the active ribosome (VanBogelen and Neidhardt, 1990). This may be explained based on primary target differences between translational inhibitors; the primary lesion of streptomycin is not an active blockade of translation and consequently does not induce ribosomal components to replace stalled counterparts.

Two unique potential secondary effects were induced – 13 transposition genes and 2 antitoxin genes. The transposition transcripts encode the smallest mobile genetic element in *E. coli*, IS1, which contains three transposases that cause short DNA duplications at GC-rich genomic sequences (Keseler *et al.*, 2005). Transposons randomly move in the genome resulting in mutations by interrupting genes, which

may enhance bacterial survival. It remains to be determined if transposition occurs and whether there are specific chromosomal hot spots favoured as a result of streptomycin-induced stress.

Antitoxin genes are transcribed as part of an operon that encodes a stable toxin and labile antitoxin responsible for stalling cell division through the degradation of the antitoxin during extreme bacterial stress. The observed induction of two antitoxin genes may be explained as an attempt by the bacterial cell to continue cellular growth which may result in protein repair rather than activate a suicide response. It remains to be seen whether either antitoxin transcript is translated into functional proteins.

An expansion of the global repression of energy rich processes including metabolism, chemotaxis, and mobility was observed following 60 minutes of treatment. Interestingly, this study is the first to report a complete repression of the transcripts required to produce ATPase I, the molecular motor that generates ATP energy within the cell (Table 3.7). This observation may account for the rapid cell-death observed – without the generation of ATP cells cannot survive.

The induction of *hslS* has previously been observed during biofilm formation (Ren *et al.*, 2004). When coupled with the almost complete repression of chemotaxis and motility genes, it is plausible that *E. coli* may respond to streptomycin challenge through the aggregation of planktonic cells into a biofilm community. However, two

key global regulators associated with biofilm formation *mqsR*, a quorum sensing regulator, and *bssR*, a regulator of biofilms through signal secretion, were both repressed. It remains to be seen if there is an increase in biofilm formation as a result of streptomycin challenge, although given the repression of these two regulators such an observation appears unlikely.

Taken together, these studies suggest *E. coli* mounts a molecular response to streptomycin-induced cell death. Transcriptional induction and subsequent translation of the heat shock response is specific to streptomycin and was observed to be dose-dependent and temporal but species independent. However, previous studies have failed to identify the specific components of this response. A repression of anaerobic metabolism, motility and chemotaxis were observed and may be a response to impaired cellular metabolism rather than specific antibiotic-induced cell death.

These transcriptional studies investigated the molecular consequences of streptomycin-induced cell death and suggest the potential new leads of *hslS*, of the heat shock response, and uncharacterized *yccV*. Future deletion mutant studies may elucidate their role in cell death.

3.5 Summary

1. Transcriptional studies are well suited for the generation of novel hypothesis for further exploration.
2. Streptomycin challenge induces differential gene regulation at both bacteriostatic and bactericidal concentrations.
3. Induction of the heat shock response is specific to streptomycin and was observed as time- and concentration- dependent. This correlates with the previously observed physiology of cell death.
4. Repression of anaerobic metabolism, motility, and chemotaxis may be a generalized response to growth arrest rather than specific streptomycin lesions.
5. Further studies will characterize the influence of *hslS* and *yccV* on streptomycin-induced cell death through the generation of deletion mutants.

4. Mutational Studies of the Potential Streptomycin Targets *hslS* and *yccV*

4.1 Overview

The previous chapter investigated the transcriptional profile of *E. coli* K12 MG1655 in response to streptomycin. Induction of genes associated with the heat shock response was observed. Two of the most strongly induced genes, *hslS* and *yccV*, are associated with this response and were chosen for further study. Deletion mutants for these two genes have not previously been reported. Consequently, these genes represent excellent targets for functional studies to validate the current microarray study and further understand the mechanism of streptomycin-induced cell death.

This chapter aims to investigate the significance of these two genes, *hslS* and *yccV*, through the generation of unmarked deletion mutants. Initial attempts utilizing standard gene replacement protocols were unsuccessful therefore a novel scheme was developed. Once generated and validated through sequence analysis, the fitness and streptomycin-induced phenotypes were investigated through growth curves, MIC determination, and dose-response assays.

4.2 Materials and Methods

4.2.1 General Microbiological Methods

4.2.1.1 Strains

Bacterial strains used in this study are listed in Table 4.1. *E. coli* K12 MG1655 (courtesy of the Zoonotic and Animal Pathogens group, University of Edinburgh, Edinburgh, Scotland, UK), a sequenced wild-type strain, was used for the generation of unmarked mutants in a wild-type genomic background (Blattner *et al.*, 1997).

E. coli NH3198 (courtesy of Dr. B. Martinez-Vaz, University of Minnesota, Saint Paul, Minnesota, USA), containing a temperature-sensitive λ prophage, was used for replacement of the gene of interest with a kanamycin resistance gene (Yu *et al.*, 2000).

E. coli BW25141 (courtesy of Dr. B. Martinez-Vaz, University of Minnesota, Saint Paul, Minnesota, USA), a derivative of BD792, contains the 3267 base-pair plasmid pKD4 with a kanamycin resistance gene which served as the PCR template (Datsenko and Wanner, 2000).

E. coli BT340 (courtesy of Dr. B. Martinez-Vaz, University of Minnesota, Saint Paul, Minnesota, USA), a derivative of DH5α, contains the 9.4 kilobase-pair plasmid pCP20, an ampicillin and chloramphenicol resistant plasmid with temperature-

sensitive induction of the yeast FLP gene for FRT-recombination (Cherepanov and Wackernagel, 1995).

MutH1.1.1 is an *E.* coli K12 MG1655 derivative with a deletion of *yccV*. MutY3.1.1 is an *E.* coli K12 MG1655 derivative with a deletion of *hslS*. Both strains were generated in this study.

Table 4.1. Bacterial Strains Used in this Study.

Strain	Genotype	Reference
MG1655	Wild Type	Science 277(5331)1453-1479.
NH3198	W3110 ΔlacU169 gal490 λcI857 Δ(cro-bioA)	PNAS 97(11):5978-5983.
BW25141	BD792 *lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ Δ*phoBR580 hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$ *galU95 endA*$_{BT333}$ *uidA*(Δ*Mlu*I)::*pir*$^{+}$ *recA1* with pKD4	PNAS 97(2):6640-6645
BT340	DH5α with pCP20	Gene 158(1):9-14.
MutH1.1.1	MG1655 Δ*yccV*	This study.
MutY3.1.1	MG1655 Δ*hslS*	This study.

4.2.1.2 Media

Liquid cultures were grown in LB broth (10g tryptone, 5g yeast extract, 10g NaCl in 1L of distilled water), supplemented with the appropriate selection antibiotic when required, at 37°C at ~200RPM unless stated otherwise. Plate cultures were grown aerobically on LB agar (10g tryptone, 5g yeast extract, 10g NaCl, 15g Bacto Agar (Difco, 0140-01) in 1L distilled water), supplemented with the appropriate selection antibiotic when required, at 37°C unless stated otherwise.

4.2.1.3 Antibiotics

Kanamycin sulphate (Sigma, K4000-5g) was dissolved in deionised water to prepare a 50mg/mL stock solution. Ampicillin sodium salt (Sigma, A9518-25g) was dissolved in deionised water to prepare a 100mg/mL stock solution. Stock solutions were filter sterilized (Sartorius Minisart 0.20μm filter, FIL6574) and aliquots stored at either 4°C or long-term at -70°C.

4.2.2 General Molecular Biology Techniques

4.2.2.1 Reagents

All chemicals were purchased from Sigma and prepared with deionised water unless specified otherwise.

4.2.2.2 PCR Primers

PCR primers used in this study are listed in Table 4.2. All primers, except the kanamycin resistance gene from pKD4 pair, were designed using Primer3 software (http://frodo.wi.mit.edu/; Rozen and Skaletsky, 2000). All primers were resuspended in deionised water to a final concentration of 100μM and stored at -20°C.

Table 4.2. Primers Used in this Study.

Name of Primer	Sequence (5' --> 3')
Primer Pairs for Kanamycin Resistance Gene Ampification frompKD4 With Gene Specific Ends.	
ibpbP1H1	GCT TCT TAG AAG GAG AAA TGA CTA TGC GTA ACT TCG ATT TGT GTA GGC TGG AGC TGC TTC
ibpbP2H2	GGG CAA AGA GAA TAG CTA GTT AGC TAT TTA ACG CGG GAC GCA TAT GAA TAT CCT CCT TAG
yccvP1H1	ATT TCC CGG GAG GTG ACT ATG ATT GCC AGC AAA TTC GGT AGT GTA GGC TGG AGC TGC TTC
yccvP2H2	CCG GGG ATT TGT TCA GGG ATT AGT TAC GCA GAC GCG GGG CCA TAT GAA TAT CCT CCT TAG
Primer Pairs for Kanamycin Resistance Gene Verification.	
kancasF	CGG ATT TCC GGT GTA GGC TGG AGC TGC TTC G
kancasR	CGG AAT TCC GCA TAT GAA TAT CCT CCT CCT TAG
Primer Pairs for Gene Deletion Verification.	
hslS-L01_for	ACC GGC AGT GGA ATA TCA AA
hslS-R02_rev	AAT CTG CTG GTG GTG AAA GG
yccV-L01_for	ATT AAT GAG CAA GCG GCA GT
yccV-R01_rev	GGT CTG ATG ACC AGC GAT TT
Primer Pairs for Gene Sequencing.	
hslS-L_seq	GGA AAG AAC GTG CCG AAA TA
hslS-R_seq	CGC CGT ATC GAA ATC AAC TAA
yccV-L_seq	GCC CGG CTA TTG AGA TCC
yccV-R_seq	TCT GAA AGG GTT TGC CTG TC

*Underlined sequences are the 40 base-pair, gene specific homology regions, H1 or H2 depending on the primer.

4.2.2.3 *DNA Isolation and Purification*

Chromosomal DNA was isolated from a single colony which was gently transferred via toothpick to 100µL of deionised water in an eppendorf tube, gently mixed, then boiled for 5 minutes. Plasmid DNA was purified using a QIAprep Spin Miniprep Kit (Qiagen, 27104) per manufacturer's instructions. PCR and enzyme digest products were gel purified following electrophoresis using a QIAquick Gel Extraction Kit (Qiagen, 28706) per manufacturer's instructions. All purified DNA products were resuspended in distilled water, quantified in mg/mL based on the A_{260} then stored at -20°C.

4.2.2.4 *DNA Visualization by Electrophoresis*

PCR products were mixed with loading buffer (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, 30% w/v sucrose) in a 5:1 ratio then loaded on a 1% agarose gel, prestained with 0.5µg/mL ethidium bromide, in 1xTBE (90mM Tris-borate, 2mM EDTA) with a suitable molecular weight ladder. Gels were electrophoresed at 100 volts for 1 hour then visualized on a UV trans-illuminator. Images were captured via CCD camera.

4.2.3 Generation of Linear DNA

4.2.3.1 *Isolation of pKD4 from BW25141*

Plasmid pKD4 (a 3267 base-pair plasmid containing the kanamycin resistance gene) was isolated (Section 4.2.2.3) from on overnight culture of BW25141 grown in LB broth supplemented with 20µg/mL kanamycin.

4.2.3.2 *PCR of Kanamycin Resistance Cassette from pKD4*

The GeneAmp XL PCR system (Applied Biosystems, N808-0192) was used in conjunction with r*Tth* DNA polymerase SL (2U/μL, Applied Biosystems, N808-0187) and specially designed high homology primers (Table 4.2) to amplify the kanamycin resistance gene from pKD4. New reagent aliquots and filter tip pipettes were used for each experiment. Reactions were assembled as follows:

Component	Volume	Final Concentration
3.3XL Buffer II	30μL	1X
GeneAmp 10mM dNTP Blend	8μL	800μM(200μM per dNTP)
Primer 1 (Forward, 10μM)	3μL	30pmol/rxn
Primer 2 (Reverse, 10μM)	3μL	30pmol/rxn
$Mg(OAc)_2$ 25mM	4.8μL	1.2mM
Water	**	
DNA	*	0.5μg
r*Tth* DNA Polymerase, XL	1.5μL	3 Units/rxn
Total Volume	100μL	
Overlay, Mineral Oil	30μL	

*The appropriate volume of DNA, in deionised water, was added to a final DNA concentration of 0.5μg per reaction. For control reactions, 9μL λ DNA (supplied in the kit) were used per manufacturer's instructions.

**The volume of deionised water was adjusted to achieve a final reaction volume of 100μL.

PCR amplification reactions were performed using a Perkin Elmer DNA Thermal Cycler 480 with the following previously optimized, by Dr. B. Martinez-Vaz, PCR cycling parameters:

Step 1:	94°C – 1 minute; 1 cycle
Step 2:	94°C – 30 seconds, 60°C – 5 minutes; 16 cycles
Step 3:	94°C – 30 seconds, 60°C – 5 minutes; 12 cycles
Time Delay:	72°C – 10 minutes
Hold:	4°C – indefinitely

PCR products were visualized via electrophoresis (Section 4.2.2.4) and products of the appropriate length (~1.6 kilobases), were gel purified (Section 4.2.2.3).

4.2.3.3 Template Elimination - DpnI Digestion

Template DNA was eliminated from the kanamycin resistance cassette by DpnI (10U/μL, Promega, R6231) digestion of the amplicon as follows: 0.5μg DNA, 5μL 10xBuffer B, 0.5μL enzyme, and deionised water to a final volume of 50μL; incubated at 37°C for 90 minutes. The enzyme was heat inactivated at 65°C for 20 minutes prior to visualization (Section 4.2.2.4) and purification (Section 4.2.2.3).

4.2.4 Replacement of the Gene of Interest with Kanamycin Resistance Cassette

Initial gene replace experiments were carried out in NH3198 based on the protocol developed by Yu *et al.* (Yu *et al.*, 2000).

4.2.4.1 Induction of λ Recombination and Generation of Electrocompetent NH3198

A shaken, overnight culture of NH3198 grown at 30°C was used to inoculate two 10mL fresh, pre-warmed LB broth 1:4 then grown statically at 30°C for four hours. One culture was transferred to a 42°C water bath for 15 minutes to induce the chromosomal λ recombinase then transferred to an ice slurry and swirled for 10 minutes. The other culture functioned as a control and was not subjected to 42°C recombinase induction. Cultures were transferred to 15mL Falcon tubes and collected by centrifugation using a Beckman JA-20 rotor at 4°C, 8000RPM, for 8 minutes. Following centrifugation, the media was decanted, the pellet resuspended in 1mL ice-cold distilled water, then centrifuged at 4°C, 10,000RPM, for 1 minute. After washing the pellets as described a further two times, cells were resuspended in 100μL ice-cold deionised water and used immediately for electroporation.

4.2.4.2 Introduction of Kanamycin Resistance Cassette by Electroporation

The purified linear DNA (Section 4.2.3.3) was introduced into freshly derived competent NH3198 cells by electroporation. Purified kanamycin resistance cassette DNA (100ng) was added to 50μL competent cells and chilled on ice for 5 minutes before being pipetted into a pre-chilled, disposable 0.1cm electroporation cuvette.

Electroporation was carried out at 200 ohms in a Bio-Rad Gene Pulser set at 1.8kV and 25μF. Electroporated cells were immediately diluted with 900μL LB broth, recovered aerobically for 2 hours at 30°C, and plated neat on LB plates containing 20μg/mL kanamycin. Plates were incubated at 30°C for 48 hours. Transformants visible following 48 hours incubation were colony purified on LB plates containing 50μg/mL kanamycin to reduce further screening of spontaneous resistance clones.

4.2.4.3 PCR Verification of Recombinants

The following standard PCR mix was used in conjunction with Recombinant *Taq* DNA Polymerase (5U/μL, Invitrogen, 10342-053) and kanamycin resistance gene specific primers (Table 4.2):

Component	Volume	Final Concentration
10x PCR Buffer	10μL	1X
50mM $MgCl_2$	8μL	4mM
10mM dNTP (each)	3μL	0.3mM each dNTP
100μM $Primer_F$	0.5μL	0.5μM
100μM $Primer_R$	0.5μL	0.5μM
DNA*	8μL	
Water	60μL	
Recombinant *Taq*	1μL	5U
Total Volume	100μL	
Overlay, Mineral Oil	30μL	

*1.5μL colony DNA was combined with 6.5μL deionised water then added to the above PCR reaction mix.

PCR amplification reactions were performed using a Perkin Elmer DNA Thermal Cycler 480 with the following previously optimized, by Dr. B. Martinez-Vaz, PCR cycling parameters:

Step 1:	98°C – 5 minutes; 1 cycle
Step 2:	95°C – 1 minute, 55°C – 1 min 15 sec, 72°C – 3 minutes; 35 cycles
Step 3:	72°C – 5 minutes
Step 4:	4°C – indefinitely

PCR products were visualized via electrophoresis (Section 4.2.2.4).

4.2.5 Transduction of Kanamycin Resistance Cassette into MG1655

Gene transduction experiments were carried out using a P1 phage adopted Dr. P. Higgins at the University of Alabama, USA (personal communication, P. Higgins).

4.2.5.1 Preparation of P1 Phage Stock from NH3198

NH3198 was grown aerobically overnight at 30°C in Z broth (LB broth, 5mM $CaCl_2$) with 20μg/mL kanamycin. Ten-μL of a 10^7 pfu P1 phage stock (courtesy P. Higgins, University of Alabama, Birmingham, Alabama, USA) was allowed to adsorb to 300μL of the NH3198 overnight culture at room temperature for 20 minutes. Following adsorption, 10mL of Z broth were added, the cells transferred to a 100mL Erlenmeyer flask and aerobically incubated at 30°C until lysis was visible, approximately 5 hours. Remaining cells were killed through the addition of 100μL chloroform and vortexed for 30 seconds. Cultures were transferred to 15mL Falcon

tubes and cells pelletted through centrifugation using a Beckman JA-20 rotor at room temperature, 5000RPM, for 10 minutes. The phage containing supernatant was decanted into a new 15mL Falcon tube, overlaid with 100µL chloroform, and stored at 4°C. Typical preparations yielded 10^8 to 10^9 pfu.

4.2.5.2 P1 Transduction of Cassette into MG1655

MG1655 was grown aerobically overnight in 10mL Z broth. Cells were pelletted via centrifugation using a Beckman JA-20 rotor at room temperature, 5000RPM, for 10 minute. The supernatant was decanted and the pellet resuspended in 1mL Z broth. 100µL of the P1 phage stock containing the kanamycin resistance gene (Section 4.2.5.1) were allowed to adsorb to 300µL of the resuspended MG1655 cells in a total volume of 2mL Z broth in a 15mL Falcon tube in a 37°C water bath for 20 minutes. Following adsorption, 8mL of 1M citrate buffer (0.1M citric acid, 0.1M $Na_3C_6H_5O_7$) were added and cells collected by centrifugation. After decanting the media, the pellet was washed with 10mL 1M citrate buffer and centrifuged for an additional 10 minutes. Cell pellets were washed once more, then resuspended in 10mL LB broth containing 25mM $Na_3C_6H_5O_7$ and allowed to recover in a 37°C water bath for 30 minutes with gentle aeration. Following recovery, cells were collected by centrifugation and washed twice more as described previously. Cells were resuspended in 300µL 1M citrate buffer, serially diluted into sterile saline, and plated on LB plates containing 2.5mM $Na_3C_6H_5O_7$ and 50µg/mL kanamycin. Transformants visible after 24 hours incubation at 37°C were colony purified on LB

plates containing 50μg/mL kanamycin to reduce further screening of spontaneous resistance clones.

4.2.5.3 PCR Verification of Recombinants

Six transformants for each gene were screened for recombination of the kanamycin resistance gene with the chromosome via PCR as described in Section 4.2.4.3.

4.2.6 Eliminating the Kanamycin Resistance Gene

Elimination of the kanamycin resistance gene to generate unmarked deletion mutants was carried out in MG1655 insertion mutants (Section 4.2.5) based on the protocol developed by Datsenko and Wanner (Datsenko and Wanner, 2000).

4.2.6.1 Isolation of pCP20 from BT340

Plasmid pCP20 (a 9.4 kilobase plasmid containing the FLP-recombinase from yeast) was isolated (Section 4.2.2.3) from an overnight culture of BT340 grown in LB broth supplemented with 100μg/mL ampicillin at 30°C.

4.2.6.2 Generation of Chemically Competent MG1655 Strains

MG1655 insertion mutants were made chemically competent based on the protocol developed by Sambrook *et al.* (Sambrook *et al.*, 1989). Briefly, a shaken, overnight culture was used to inoculate fresh, pre-warmed LB media 1:100 and incubated for 3 hours at 37°C shaking. Cultures were transferred to 50mL Falcon tubes, chilled on ice for 10 minute, then centrifuged in a Beckman JA-20 rotor at 4°C, 5000RPM, for

10 minute. The supernatant was decanted, pellet resuspended in 15mL MgCl-CaCl solution (80mM $MgCl_2$, 20mM $CaCl_2$), and cold centrifuged for 10 minutes. The supernatant was decanted, cells resuspended in 2mL 0.1M $CaCl_2$, divided into 200µL aliquots, and used immediately for electroporation.

4.2.6.3 Introduction of pCP20 (Resolvase) by Thermal Transformation

The purified pCP20 plasmid (Section 4.2.6.1) was introduced into freshly derived chemically competent MG1655 insertion mutants (Section 4.2.6.2) via heat shock. Purified pPC20 (50ng) was added to 200µL competent cells and chilled on ice for 30 minutes. Cells were then heat shocked in a 42°C water bath for 90 seconds, returned to ice for 2 minutes, then diluted with 800µL SOC. Transformed cells were recovered aerobically for 45 minutes in a 37°C water bath and plated neat on LB plates containing 100µg/mL ampicillin. Plates were incubated at 37°C for 48 hours. Transformants visible after 24 hours were purified on LB plates. Once purified, the strains were tested for kanamycin and ampicillin sensitivity on LB plates containing 50µg/mL kanamycin or 100µg/mL ampicillin, respectively. Strains susceptible to both antibiotics had lost both the kanamycin resistance gene and the resolvase plasmid and were verified via PCR (Section 4.2.4.3) and sequencing (Section 4.2.7).

4.2.7 DNA Sequencing

Unmarked deletion mutants were sequenced at the Moredun Sequencing Facility by Karina Reinhardt. Chromosomal DNA was isolated from MutH1.1.1 and MutY3.1.1 by transferring a single colony to 100µL deionised water and boiled for 5 minutes.

DNA was isolated from the supernatant using a Qiagen DNA extraction kit (Qiagen, 28706). Purified DNA products were resuspended in distilled water and quantified in mg/mL based on the A_{260}. Sequencing primers (Table 4.2) with a final concentration of 3.2pmol were combined with 150ng DNA for sequencing reactions. Composite sequence alignments were performed using MultAlin (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html; Corpet, 1988).

4.2.8 Physiological Characterization of Deletion Mutants

The relative fitness of deletion strains was assessed by a growth curve assay previously described (Section 2.2.4). Streptomycin induced physiology changes were examined by determining the MIC (Section 2.2.2) and dose response (Section 2.2.3).

4.3 Results

4.3.1 Experimental Design

Figure 4.1 provides an overview of the steps required to generate unmarked deletion mutations in the wild-type strain, *E. coli* K12 MG1655. It was vital to remove the genes of interest in the same genetic background as previous transcriptional studies to ensure any observed transcriptional, translational, or physiological changes were a result of the introduced deletion rather than genomic variations between parental bacterial strains.

Gene replacement was achieved by utilizing the λ Red recombination system in a novel strategy based on two previously described methods (Datsenko and Wanner, 2000; Yu *et al.*, 2000). The λ Red recombination system exploits three genes of λ (*γ, β, exo*) and linear DNA to introduce gene mutations via double strand break repair (Datsenko and Wanner, 2000). In the Datsenko and Wanner protocol, special high homology primers were designed and used to amplify the FRT-flanked kanamycin resistance gene of pKD4 (Section 4.2.3.2) then transferred into *E. coli* BW25113 which harbours the Red recombinase genes on pKD20, a 6078 base-pair plasmid under the control of an arabinose-inducible promoter. Attempts to revive *E. coli* BW25113 were unsuccessful. Consequently, *E. coli* NH3198 which harbours the Red recombinase genes under the control of a temperature-sensitive chromosomal λ cI-repressor mutant (Yu *et al.*, 2000) was used with the original FRT-flanked PCR products. NH3198 transformants were then used as source genomic material for P1 phage transduction of the homology-FRT-flanked kanamycin resistance gene into *E.*

coli MG1655. After selection for kanamycin resistance, the gene was removed using the FLP-recombinase from yeast, encode on pCP20, and mutants sequenced to verify the unmarked deletion in the wild-type background (Datsenko and Wanner, 2000).

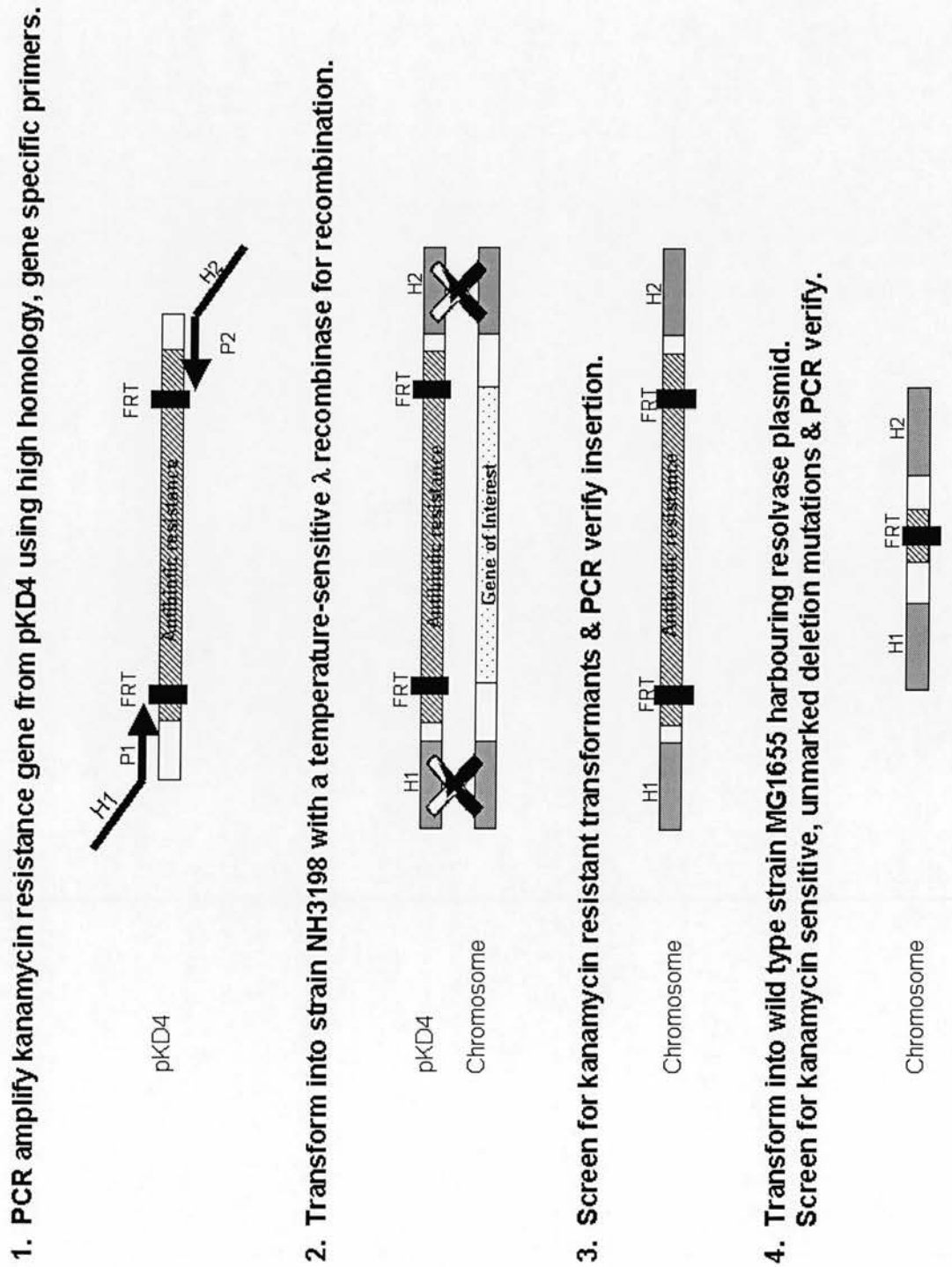


Figure 4.1. Gene Replacement Strategy.

Schematic outlining the gene replacement strategy adapted from Datsenko and Wanner (2000) and Yu *et al.* (2000).

4.3.2 Plasmid pKD4 and Homology Primers

Figure 4.2 shows a linear representation of pKD4, a 3267 base-pair plasmid containing a FRT-flanked kanamycin resistance gene. This template, in conjunction with high homology primers (Table 4.2), was used for the generation of the kanamycin resistance cassette.

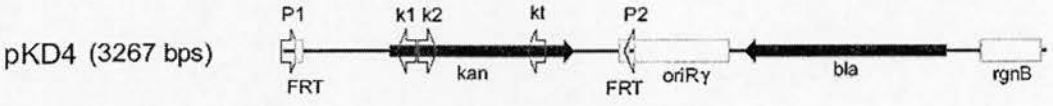


Figure 4.2. Linear Representation of pKD4.
Kanamycin resistance gene template flanked by FRT recombination sites on plasmid pKD4 (from Datsenko and Wanner, 2000).

High homology PCR primers (Table 4.2) were specifically designed for gene replacement experiments based on the work of Datsenko and Wanner (Datsenko and Wanner, 2000). Primers are listed in Table 4.2. Each primer contained a 5' H (homology) region of 40 nucleotides specific to the gene to be replaced, including either the start (H1) or stop (H2) codons, and a 3' P (priming) region of 20 nucleotides identical to the kanamycin P1 and P2 priming sites of pKD4 (Figure 4.2). The transcription of the kanamycin resistance gene was oriented in the same direction as the gene being replaced. PCR using these primers generated a linear DNA fragment containing the FRT-flanked kanamycin resistance gene adjoined by gene specific regions for homologous recombination by the λ recombinase.

4.3.3 Amplification of the Kanamycin Resistance Gene from pKD4

Plasmid pKD4 was isolated from an overnight culture of BW25141. The 1.6 kilobase fragment was successfully amplified (Figure 4.3) using gene specific primers (Table 4.2) and the Gene Amp XL PCR system, specific for extra long PCR amplicons, then gel purified. To eliminate residual template DNA, gel purified amplicons were subjected to DpnI restriction and further gel purification.

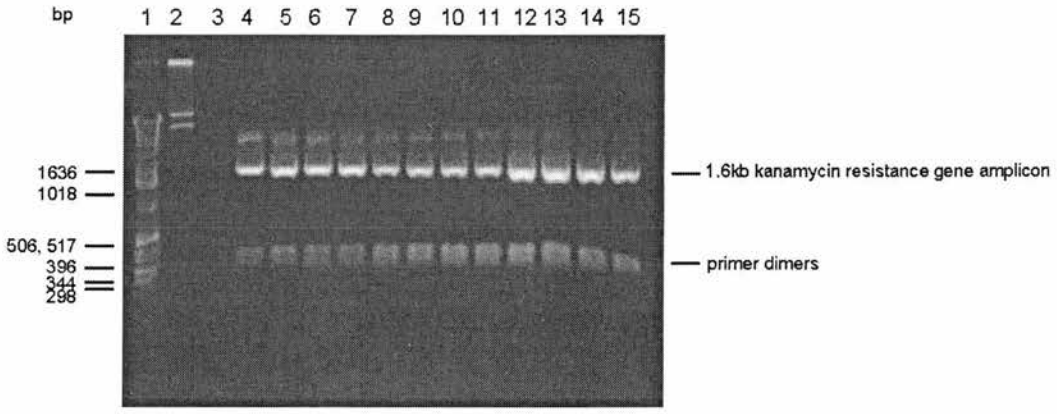


Figure 4.3. Amplification of Kanamycin Resistance Gene from pKD4.

The kanamycin resistance gene was PCR amplified from pKD4 using gene specific primer pairs described in Table 4.2. Lanes: 1) 1Kb DNA Ladder (Invitrogen, 15615-016); 2) + control λ from Gene Amp XL PCR kit; 3) – control λ from Gene Amp XL PCR kit; 4-9) *hslS* specific kanamycin resistance gene; 10-15) *yccV* specific kanamycin resistance gene. Bands visualized below 400 base-pairs are likely primer dimers.

4.3.4 Gene Replacement by Homologous Recombination in NH3198

The kanamycin resistance cassette was electroporated into NH3198 in which the λ recombinase had been thermally induced at 42°C for 15 minutes. Cells which had successfully replaced the gene of interested via homologous recombination were now resistant to kanamycin and grew on selective media. Three transformants for each gene were recovered after 48 hours, colony purified on LB media, then rescreened for resistance to 50μg/mL kanamycin. Survival at the higher concentration reduces the likelihood of further screening spontaneous resistance mutants. One *hslS::kan* strain, MutH1, and three *yccV::kan* strains, MutY1, MutY2, and MutY3 were genotyped via PCR. Homologous recombination was verified with primers specific for the kanamycin resistance gene (Figure 4.4). Only MutH1 (lane 4) and MutY3 (lane 7) strains had successfully replaced the gene of interest with the kanamycin resistance cassette and were used for P1 phage generation.

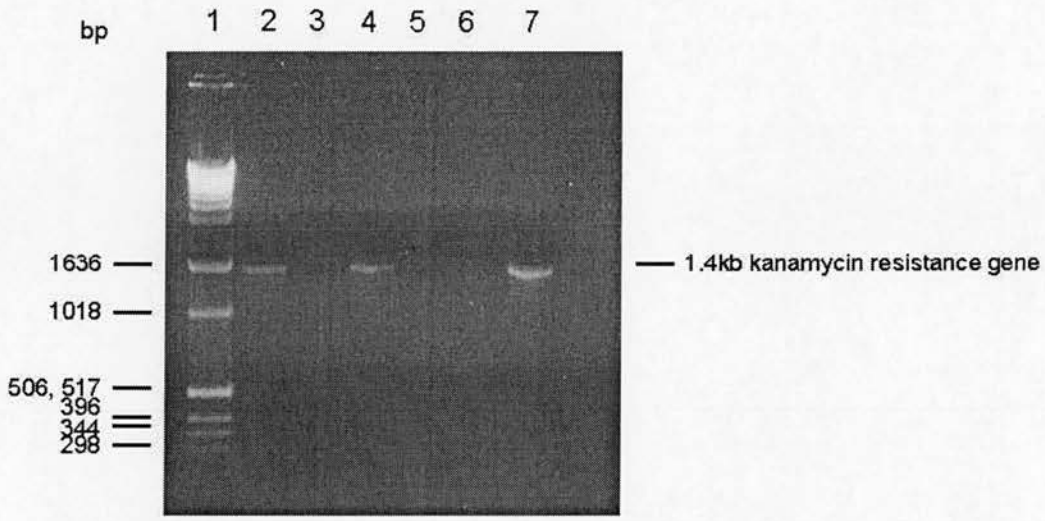


Figure 4.4. Amplification of Kanamycin Resistance Gene from NH3198 Transformants.

The kanamycin resistance gene was PCR amplified from NH3198 transformants using kanamycin resistance gene specific primer pairs. Lanes: 1) 1Kb DNA Ladder (Invitrogen, 15615-016); 2) pKD4 DNA as + control; 3) MG1655 as – control; 4) MutH1; 5) MutY1; 6) MutY2; 7) MutY3.

4.3.5 Gene Replacement by Transfection in MG1655

The kanamycin resistance cassette was introduced to wild-type MG1655 from MutH1 and MutY3 strains via P1 phage transduction. Cells which had successfully received the kanamycin resistance cassette and undergone recombination were now resistant to kanamycin and grew on selective media. Six transformants for each gene transfection were recovered after 24 hours, colony purified on LB media, then rescreened for resistance to 50μg/mL kanamycin to reduce further screens of spontaneous resistance mutants. All 12 transformants were resistant to the higher concentration of kanamycin and genotyped via PCR using primers specific for the kanamycin resistance gene (Figure 4.5). The positive control was faintly visible on the gel. Despite attempts to adjust the exposure, the band was unable to be captured via CCD image. All 12 transformants had successfully integrated the kanamycin resistance gene in place of the gene of interest. Only MutH1.1 (lane 4) and MutY3.1 (lane 10) were used for FLP-resolvase experiments.

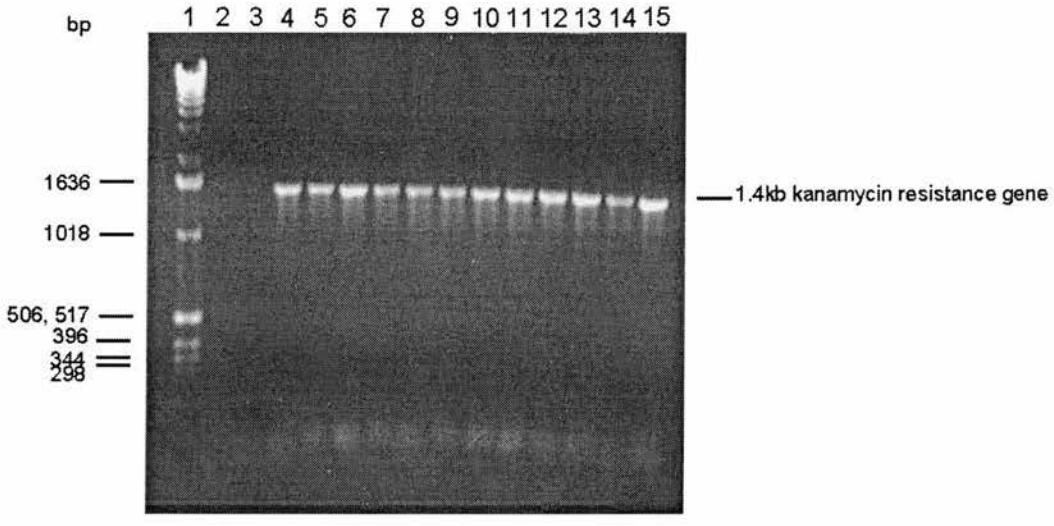


Figure 4.5. Amplification of Kanamycin Resistance Gene from MG1655 Transformants.

The kanamycin resistance gene was PCR amplified from MG1655 transformants using kanamycin resistance gene specific primer pairs. Lanes: 1) 1Kb DNA Ladder (Invitrogen, 15615-016); 2) pKD4 DNA as + control; 3) MG1655 as – control; 4) MutH1.1; 5) MutH1.2; 6) MutH1.3; 7) MutH1.4; 8) MutH1.5; 9) MutH1.6; 10) MutY3.1; 11) MutY3.2; 12) MutY3.3; 13) MutY3.4; 14) MutY3.5; 15) MutY3.6.

4.3.6 Excision of the Kanamycin Resistance Gene by FRT Recombination

The kanamycin resistance cassette was excised from MutH1.1 and MutY3.1 strains by the FLP-recombinase of yeast. The FLP recombinase gene is encoded by pCP20, a 9.4 kilobase ampicillin and chloramphenicol resistant plasmid, under a temperature-sensitive promoter and promotes homologous recombination at specific sequences known as FRT regions (Cherepanov and Wackernagel, 1995). Plasmid pCP20 was isolated from an overnight culture of BT340 grown at the permissive temperature of 30°C. Once isolated, it was thermally transformed into chemically competent MutH1.1 or MutY3.1. Cells were recovered on selective media containing ampicillin to maintain the plasmid and allow for FLP-mediated recombination. Three transformants for each gene were recovered after 48 hours, colony purified on LB media at 42°C (to promote loss of pCP20), then rescreened for kanamycin and ampicillin sensitivity on selective media. Three mutants for each gene were obtained. Initially, one mutant for each gene, MutH1.1.1 and MutY3.1.1, was genotyped using gene specific primers (Figure 4.6). From this gel it was determined that MutH1.1.1 harbours the deletion of the *yccV* gene rather than the *hslS* gene due to the presence of a PCR product from the *hslS*-specific primers (lane 6) and the absence of a PCR product from the *yccV*-specific primers (lane 8). Similarly, MutY3.1.1 harbours the deletion of the *hslS* gene rather than the *yccV* gene due to the absence of a PCR product from the *hslS*-specific primers (lane 7) and the presence of a PCR product from the *yccV*-specific primers (lane 9). As a result of this confusion, greater care must be taken with labelling in future experiments.

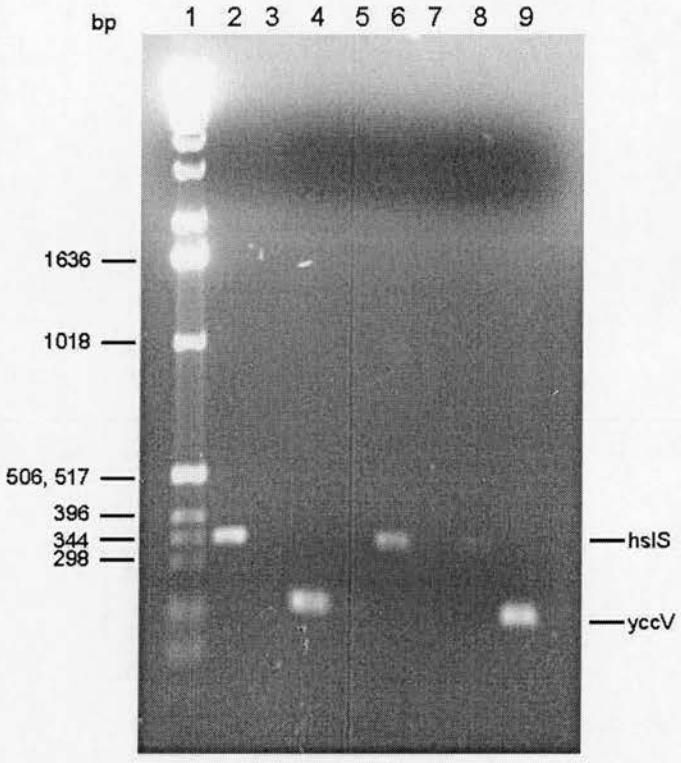


Figure 4.6. PCR Verification of Unmarked Deletion Mutants.

The kanamycin resistance gene was excised from MutH1.1 and MutY3.1 by FLP recombination. Excision of the kanamycin resistance gene was verified using gene specific primer pairs. Lane 1 – 1Kb DNA Ladder (Invitrogen, 15615-016). The other lanes can be interpreted as follows:

Lane	Primer Pair	DNA	
2	hslS	MG1655 (wild-type)	+ control
3	hslS	Deionised Water	- control
4	yccV	MG1655 (wild-type)	+ control
5	yccV	Deionised Water	- control
6	hslS	MutH1.1.1	
7	hslS	MutY3.1.1	
8	yccV	MutH1.1.1	
9	yccV	MutY3.1.1	

4.3.7 Verification of Unmarked Deletion Mutants

To further verify the identity of the deletion strains, gene specific sequencing primers were designed (Table 4.2). While the gene specific primers amplified the region surrounding the gene of interest, the sequencing primers amplified the gene of interest and approximately 100 base-pairs on either side. MutH1.1.1 and MutY3.1.1 were reverified via PCR prior to sequencing (Figure 4.7).

From this gel it was determined that MutY3.1.1 contains a deletion of *hslS*. This is apparent when lane 3, the wild-type control, is compared to lane 5, the mutant amplification, noting the difference between these two lanes was equivalent to the length of the predicted *hslS* deletion, 494 base-pairs. A similar result was obtained by comparing lanes 4 and 6. Finally, lane 6 validated the use of the *hslS_seq* primer pair for sequencing the region of the gene deletion.

Similarly, MutH1.1.1 contains a deletion of *yccV*. This is apparent when lane 9, the wild-type control, is compared to lane 11, the mutant amplification which appears as a very faint band, noting the difference between these two lanes was equivalent to the length of the predicted *yccV* deletion, 354 base-pairs. A similar result was obtained by comparing lanes 10 and 12. Finally, lane 12 validated the use of the *yccV_seq* primer pair for sequencing the region of the gene deletion.

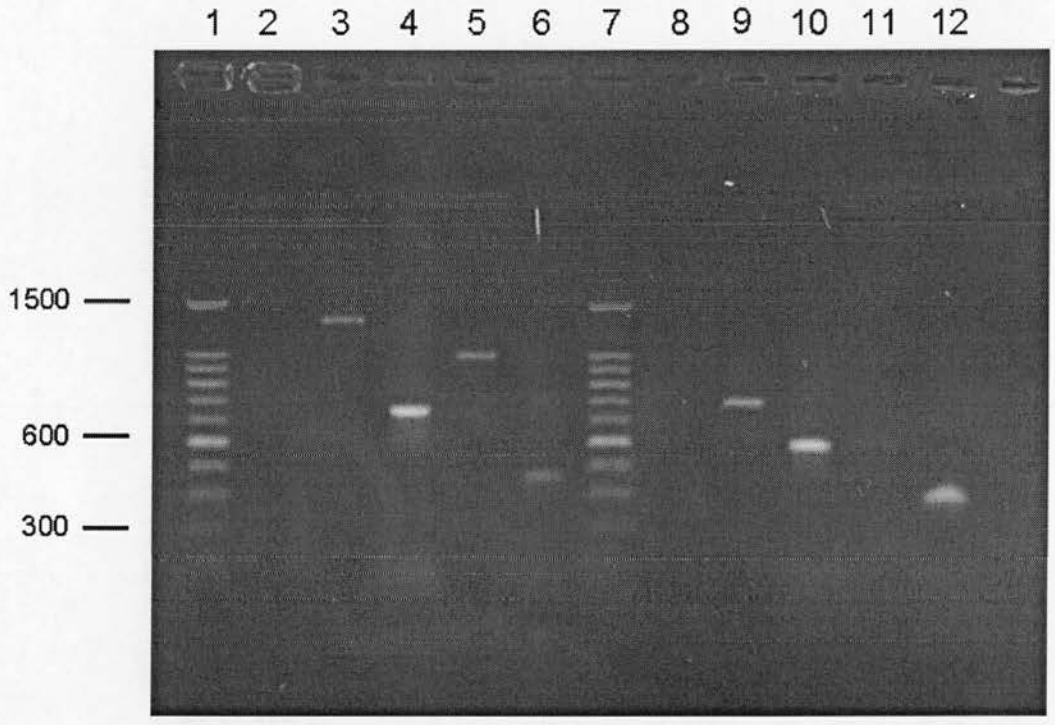


Figure 4.7. PCR Verification of Unmarked Deletion Mutants Using Sequencing Primers.

The genotype of the unmarked deletion mutants was further verified through the use of gene specific and gene sequencing primer pairs. Lane 1 & 7 – 1Kb DNA Ladder (Invitrogen, 15615-016). The other lanes can be interpreted as follows:

Lane	Primer Pair	DNA	Product Length
2	--	MG1655	- control
3	hslS	MG1655	1309bp + control
4	hslS_seq	MG1655	664bp + control
5	hslS	MutY3.1.1	815bp
6	hslS_seq	MutY3.1.1	170bp
8	--	MG1655	- control
9	yccV	MG1655	689bp + control
10	yccV_seq	MG1655	490bp + control
11	yccV	MutH1.1.1	335bp
12	yccV_seq	MutH1.1.1	136bp

Based on these results, MutY3.1.1 was sent for scar sequencing verification using the *hslS_seq* primer pair. MutH1.1.1 was sent for scar sequence verification using the *yccV_seq* primer pair.

4.3.8 Sequence of Unmarked Deletion Mutant Scar Region

MutH1.1.1 and MutY3.1.1 were sequenced by Karina Reinhardt at the Moredun Sequencing Facility (Edinburgh, Scotland, UK) to verify deletion of *yccV* or *hslS*, respectively. Sequences were aligned (Figure 4.8) with both the high homology primer pairs and the predicted Datsenko and Wanner "scar" sequence using MultAlin (Datsenko and Wanner, 2000; Corpet, 1988).

From the alignment with the deletion primers, it was deduced that the kanamycin resistance cassette was successfully introduced into the chromosome of both MutH1.1.1 and MutY3.1.1 in *yccV* or *hslS*, respectively. Similarly, based on the alignment with the scar sequence, it was deduced the kanamycin resistance cassette was cleanly excised by the FLP-recombinase at the engineered FRT-recombination site. Therefore, MutH1.1.1 and MutY3.1.1 contain the proper scar sequence. Sequencing results confirmed *yccV* was knocked-out in MutH1.1.1 and *hslS* was knocked-out in MutY3.1.1.

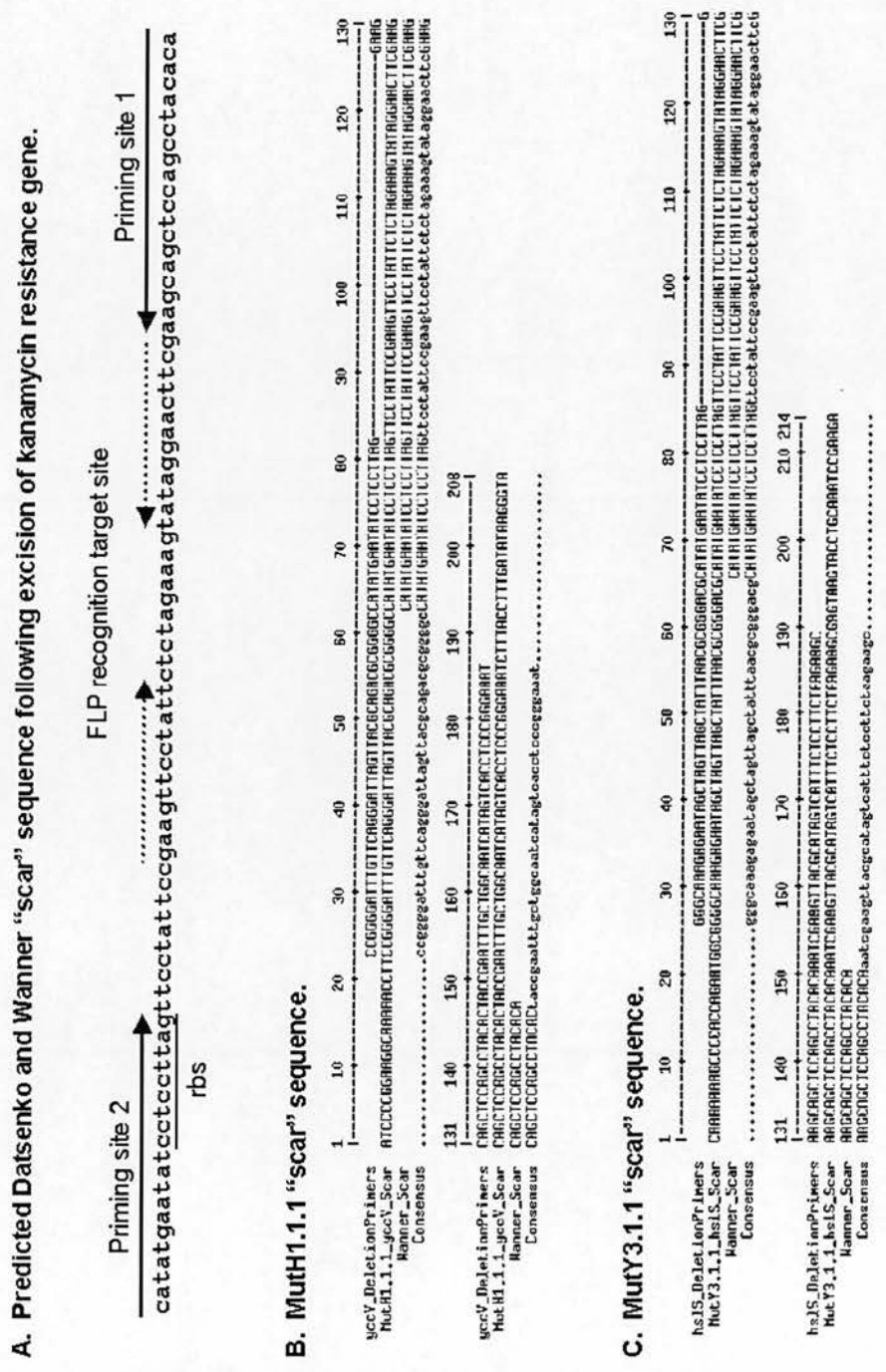


Figure 4.8. Unmarked Deletion Mutant Scar Sequences.
MutH1.1.1 and MutY3.1.1 were sequenced to verify deletion of *yccV* or *hslS*, respectively. (A) Predicted Datsenko and Wanner "scar" sequence after FLP-mediated recombination. The sequences includes priming sites 1 and 2, the FRT-recognition site, and the ribosome binding site (rbs) and is shown as the minus strand for direct comparison with the mutant sequences. (B) Alignment of MutH1.1.1 with the *yccV* high homology deletion primers and the Wanner scar from part A. (C) Alignment of MutY3.1.1 with the *hslS* high homology deletion primers and the Wanner scar from part A.

4.3.9 Physiological Characterization of MutH1.1.1 and MutY3.1.1

Following sequence verification, the physiological consequences of the engineered deletions in the knock-out strains compared to the parental *E. coli* MG1655 strain were determined by growth curve, MIC determination, and dose-response assay.

4.3.9.1 Fitness Determination by Growth Curve

The fitness of MutH1.1.1 and MutY3.1.1 was determined through a growth curve assay in LB broth (Section 2.2.4) versus the wild-type strain MG1655. Both strains exhibited robust growth similar to that measured for the wild type strain. Consequently, neither the deletion of *yccV* in MutH1.1.1 or *hslS* in MutY3.1.1 results in reduced fitness.

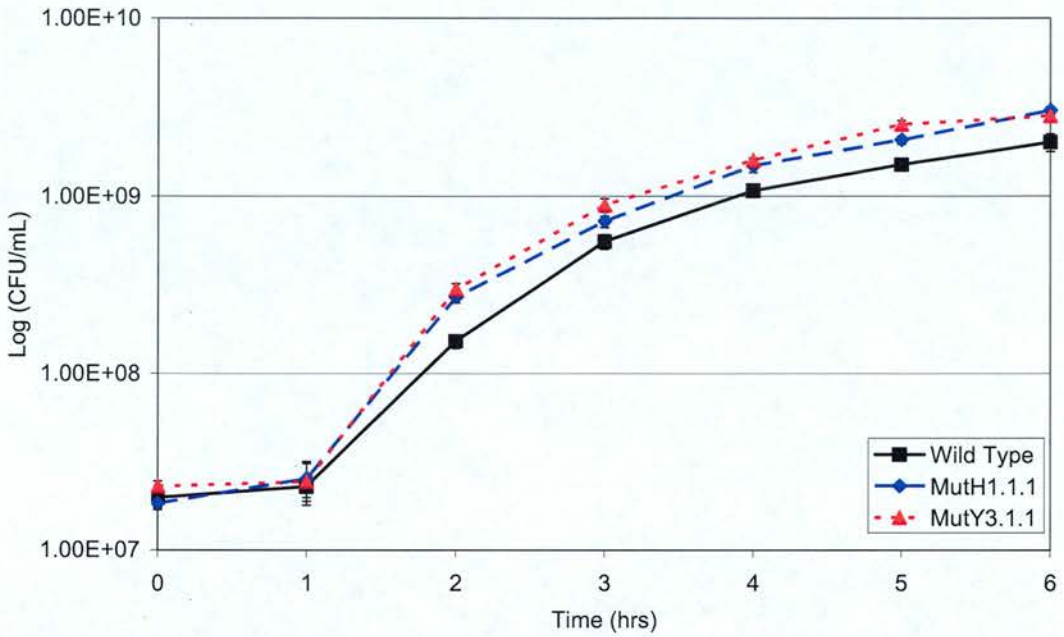


Figure 4.9. Growth Curve of *Escherichia coli* K12 MG1655, MutH1.1.1, and MutY3.1.1 in LB Broth.

Cultures were grown at 37°C with aeration. Samples were taken hourly, serially diluted, and plated for viable counts. Results are the average of three trials plated in triplicate.

4.3.9.2 *Minimum Inhibitory Concentration*

The MIC of streptomycin for *E. coli* K12 strains was determined in triplicate (Section 2.2.2). The MIC for *E. coli* K12 MG1655 is 2mg/L. The MIC for both *E. coli* K12 MutH1.1.1 and *E. coli* MutY3.1.1 is 2 mg/L. Consequently, neither the deletion of *yccV* in MutH1.1.1 or *hslS* in MutY3.1.1 resulted in a change to the minimum amount of streptomycin required to kill these strains.

4.3.9.3 *Concentration-Dependent Response*

The concentration-dependent activity of streptomycin was determined through a dose-response assay in LB media (Section 2.2.3). Mid-log phase cultures were challenged with increasing concentrations of streptomycin. This result indicated there was no change in the concentration-dependent bactericidal activity of either MutH1.1.1 or MutY3.1.1 compared to wild-type (Figure 4.10). Consequently, neither the deletion of *yccV* in MutH1.1.1 or *hslS* in MutY3.1.1 resulted in an increase or decrease to the concentration-dependent activity of streptomycin.

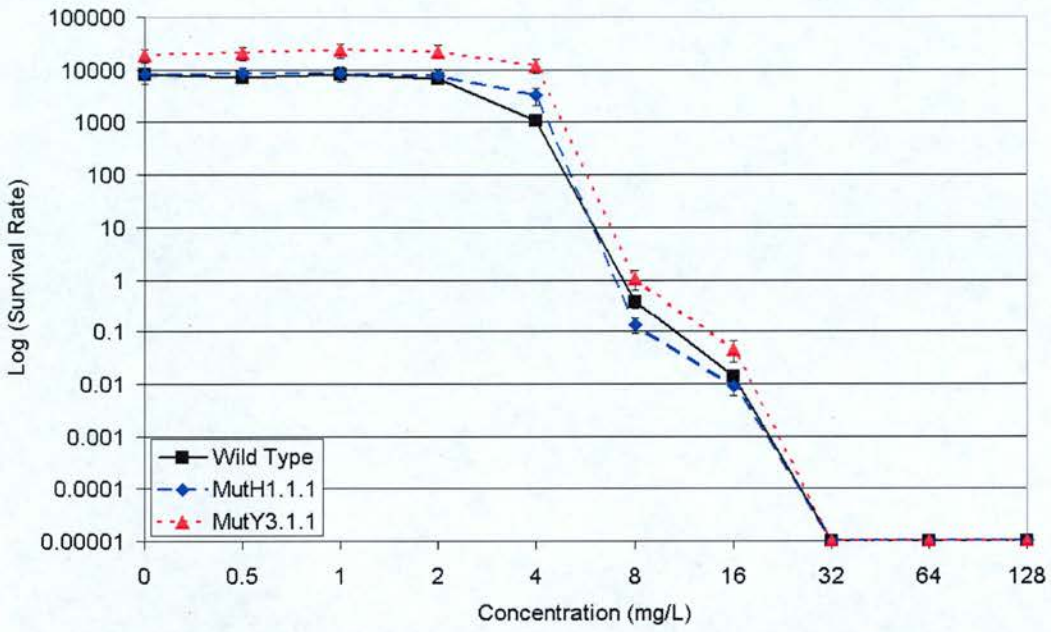


Figure 4.10. Dose-Response of *Escherichia coli* K12 MG1655, MutH1.1.1, and MutY3.1.1 to Streptomycin.

Streptomycin was added to mid-log phase cultures, statically incubated a further three hours, then serially diluted and plated for viable counts. Results are the average of three trials plated in triplicate.

4.4 Discussion

In the previous chapter, the transcriptional profile of *E. coli* K12 MG1655 in response to streptomycin challenge was examined. The observed differential gene regulation in response to bactericidal concentrations induced genes associated with the heat shock response as well as numerous uncharacterized open reading frames. Two genes, *hslS* and *yccV*, consistently exhibited the largest induction for their respective groups. Neither single nor unmarked deletion mutants have been described for either gene. Consequently, the generation and characterization of unmarked deletion mutants to functionally validate these transcriptional studies and assess their contribution to the molecular consequence of streptomycin-induced cell death were undertaken.

It was vital to assess the effects of each gene's deletion in the same genetic background as the previous transcriptional studies to ensure that any changes were a direct result of the gene deletion rather than genomic variation among parental strains. For this, the one-step inactivation strategy described by Datsenko and Wanner was initially chosen (Datsenko and Wanner, 2000). This protocol utilizes a plasmid-borne λ Red recombinase under the control of an arabinose-inducible promoter to replace the chromosomal gene of interest with an antibiotic resistance cassette flanked by FRT-recombination regions. The cassette is then removed utilizing the FLP-recombinase of yeast. Initial attempts to revive BW25113, containing the plasmid-borne λ Red recombinase, were unsuccessful thus resulting in the creation of a novel gene replacement scheme combining the ideas of Datsenko

and Wanner with strains from Yu and colleagues (Yu *et al.*, 2000) as outlined in Figure 4.1. The resultant scheme lead to the successful generation of two deletion mutants – MutH1.1.1., an unmarked deletion mutant of *yccV*, and MutY3.1.1, an unmarked deletion mutant of *hslS*; verified by sequence analysis.

The relative fitness of each mutant was assessed in relation to the wild-type strain by a standard growth curve. Robust growth was observed for each mutant similar to that of the wild-type strain at 37°C suggesting there is no fitness cost associated with the deletion of either gene. Consequently, neither gene is essential for survival and thus a tractable system for further investigation into the consequences of streptomycin challenge. The non-essential nature of each gene suggests it may be induced in response to streptomycin treatment thus allowing us to study the response cascade through its deletion.

The physiological consequences of streptomycin challenge were assessed through the determination of the MIC and dose-response phenotypes associated with each gene deletion. Neither deletion of *hslS* nor *yccV* resulted in changes to the phenotype of streptomycin challenge – the observed MIC for all strains remained 2mg/L and the concentration-dependent kill was identical to the wild-type strain.

Previous studies investigated the effects of an *hslTS* kanamycin resistance insertion mutant (Thomas & Baneyx, 1998; Kuczynska-Wisnik *et al.*, 2002, Mogk *et al.*, 2003). Deletion of either gene had no effect on survival at either 37°C or 50°C

despite a marked increase in the aggregation of unfolded proteins in inclusion bodies which were slow to disaggregate (Mogk *et al.*, 2003). Mogk and colleagues determined this was due to a cooperation between HslTS and the 'major' chaperones ClpB and DnaJKGrpE forming a network to protect and facilitate protein folding in times of severe stress (Mogk *et al.*, 2003). Protein sedimentation studies suggest that HslT and HslS can form dimers which protect unfolded proteins with HslT dominating protein binding (Kuczynska-Wisnik *et al.*, 2002). The effect of performing these experiments in kanamycin containing media, known to induce the heat shock response (VanBogelen & Neidhardt, 1990), remains to be determined. The lack of an observable phenotype as a result of *hslS* deletion in MutY3.1.1 may be the result of increased production of HslT as a means of compensation to bind unfolded proteins or the inherent redundancy of the DnaJKGrpE-ClpB-HslTS protein quality control system neither of which may have a direct effect on the mechanism of streptomycin-induced cell death.

Little is known about *yccV*. d'Alençon and colleagues observed that YccV has an affinity for hemimethylated *oriC* DNA and negatively controls replication initiation through repression of *dnaA* (d'Alençon *et al.*, 2003). Work published after the generation of the *yccV* deletion mutant demonstrated that *yccV* is a heat shock protein under the control of the heat shock promoter, σ^{32}, which stimulates the degradation of particular *dnaA* mutants and may help to regulate the cell cycle (Shimuta *et al.*, 2004). Based on studies of a kanamycin resistance insertion mutant, Shimuta and colleagues suggest *yccV* may function as a minor, homodimeric protease similar to

other heat shock proteases such as ClpXP, ClpAP, or HslUV, however the effect of kanamycin on these studies was not addressed (Shimuta *et al.*, 2004). Consequently, the lack of an observable phenotype as a result of *yccV* deletion in MutH1.1.1 may be attributed to a similar redundancy in the protein quality control systems as seen for *hslS*.

The non-essential nature of *hslS* and *yccV* coupled with their involvement in the heat shock response suggest that the simple growth curve at 37°C may not be an appropriate measure of the fitness costs associated with these deletions. Further physiological studies investigating the effects of a range of temperatures from 30°C to 50°C and other antibiotics may prove useful in understanding the function of these two genes but were out width the scope of this study. Despite high-level transcriptional induction, deletion of neither gene altered the phenotype of streptomycin-induced cell death and further transcriptional studies were abandoned.

4.5 Summary

1. Unmarked deletion mutants were engineered for *hslS* and *yccV*, two strongly induced genes in response to streptomycin challenge, and are reported here for the first time.

2. There is no fitness cost associated with deleting either gene at 37°C. Therefore, neither gene is essential for cell survival.

3. There is no change in the bactericidal activity of streptomycin on these deletion mutants. Therefore, neither gene is essential in the mechanism of streptomycin-induced cell death.

5. Discussion and Future Work

Despite over 60 years of investigation and clinical use, the molecular consequences of streptomycin treatment on the bacterium remain poorly understood. This thesis aimed to investigate the molecular response of *E. coli* K12 MG1655 to streptomycin challenge through a combination of traditional microbiological techniques and emerging transcriptomics to develop a further picture of the mode of action and cell death.

Initial studies assessed the physiology associated with streptomycin-induced cell death using the traditional medical microbiology techniques of MIC, dose-response, and time-kill assays to develop standardized culture conditions for subsequent transcriptional studies. Rapid bactericidal activity was observed in a media-, concentration-, and time- dependent manner. The magnitude of the kill was directly affected by the composition of the media – over 99% of cells were killed in rich media versus a small persister population in minimal media (Section 2.3.2, Figure 2.1). This is most likely due to the direct correlation between bactericidal activity and the degree of active metabolism and protein synthesis of a growing population (Paine and Clark, 1953; Plotz and Davis, 1962; Hurwitz and Rosano, 1962). The molecular consequences of streptomycin treatment may be difficult to separate from the general stress responses and altered metabolism required for cells to adapt to poor nutritional environments or slowed growth in minimal media.

The time required for bactericidal activity was directly effected by the concentration of streptomycin – over half of the bacterial population was killed within the first hour of exposure to 4xMIC in rich media versus continued growth for 1xMIC and 2xMIC (Section 2.3.3.1, Figure 2.2). Wasserman and colleagues noted the effects of streptomycin could be reversed if cells were sub-cultured on antibiotic-free media within 15 minutes of uptake but were unable to be rescued after 30 minutes of exposure (Wasserman *et al.*, 1954). This rapid loss of viability suggested that the molecular changes responsible for streptomycin-induced cell death occur within the first 15 to 30 minutes of treatment leading to visible cell death within an hour. Subsequent transcriptional studies focused on the immediate (10- and 30- minute) changes induced by bactericidal (4xMIC) streptomycin challenge in rich media.

Based on this MIAME compliant framework, transcriptional studies using glass-slide *E. coli* K12 MG1655 cDNA microarrays were undertaken. Only a small percentage (4%) of the transcriptome was observed to be differentially regulated; however, a number of primary responses were detected. The induction of heat shock transcripts was observed within 10 minutes of streptomycin addition; with most heat shock transcripts detectable at 30 minutes (Section 3.3.2.2.1, Table 3.2). Surprisingly, core heat shock transcripts were detected for bacteriostatic challenge with 1xMIC and 2xMIC (Section 3.3.2.2.1, Table 3.2) suggesting a molecular response may be mounted despite the non-lethal insult. This observation was novel at the time; the study reported herein was the first to observe both a time- and concentration-dependent response. A similar time- and concentration- dependent response was

later observed in response to the aminoglycosides kanamycin and gentamicin (Shaw *et al.*, 2003; Lin *et al.*, 2005). The induction of heat shock transcripts was not observed following exposure to other classes of bactericidal antibiotics (Shaw *et al.*, 2003; Kaldalu *et al.*, 2004). Although initially surprising, the recent emergence of similar results for other aminoglycosides suggest the heat shock response may be a fundament response to aminoglycoside challenge. Taken together, these transcriptional studies validated previous proteomic studies by VanBogelen and Neidhardt who observed that bactericidal H group antibiotics, including kanamycin, puromycin, and streptomycin induced changes similar to those elicited by a heat shock, but failed to identify the effected proteins (VanBogelen and Neidhardt, 1990).

Whereas the induction of heat shock transcripts appears to be specific to aminoglycosides, the specific repression of transcripts associated with metabolism and movement has been observed across diverse classes of antibiotics. Despite the induction of *fnr*, a transcriptional regulator associated with anaerobic metabolism, an uncoupling of the regulation of its downstream targets was observed (Section 3.3.2.2.2, Table 3.3). Genes normally repressed, including *nuoCEFGN* for NADH dehydrogenase I of the electron transport chain, were correctly regulated; however, genes normally induced were repressed suggesting a generalized repression of metabolism. Similar results have been observed for ampicillin, kanamycin, and rifampin (Shaw *et al.*, 2003). This generalized repression of cellular metabolism and electron transport may be a universal response to antibiotic challenge.

Shaw and colleagues noted the repression of ten transcripts – *frdB, gatB, gcvH, glpB, malK, narGHJ, ompF,* and *yhaF*, by four different bactericidal antibiotics – ampicillin, kanamycin, norfloxacin, and rifampin (Shaw *et al.*, 2003). Interestingly, a more robust repression of metabolism- (including 4 of Shaw's proposed genes) and motility- associated transcripts were observed in a study limited to two bactericidal agents – ampicillin and ofloxacin (Kaldalu *et al.*, 2004). In the present study, 7 of Shaw's proposed commonly repressed transcripts were observed - *frdB, gatB, gcvH, malK, narGH,* and *ompF*; whereas, 20 similarities with the Kaldalu data were observed (*flgC, flgE, flgF, flgG, flgI, fliF, fliH, fliI, fliJ, fliK, fliM, fliN, fliS, gatA, gatB, gatD, malK, nuoG, nuoN,* and *treC*). These similarities may reflect a generalized reduction in cellular metabolism as the cell diverts energy away from extraneous pathways towards survival responses. However, the inability to generate similar lists of differentially regulated genes in these studies reflects the dynamic nature of the transcriptome and the statistical challenges inherent in large data sets. Therefore, considerable caution should be used when comparing microarray results.

While this thesis was in preparation, a study examining the transcriptional changes of *E. coli* in response to three bactericidal antibiotics (ampicillin, kanamycin, and norfloxacin, all of which have been the subject of previous studies) was published. Kohanski and colleagues suggest there may be a fundamental response to bactericidal antibiotic challenge that includes the induction of 30 genes and the repression of 142 (Kohanski *et al.*, 2007). A comparison of their results with those of the present study revealed 6 similarities – *asnA, cpxP, dnaK, groLS,* and *hslU*; the

last 4 of which are members of the heat shock response. Kohanski *et al.* also noted the induction of 4 genes, *murF* and *nuoCEF*, which have previously been reported as repressed (Shaw *et al.*, 2003; Kaldalu *et al.*, 2004; present study). It remains to be seen if the results of Kohanski *et al.* are reproducible as no replicates or real time RT-PCR experiments were performed to validate their results. These discrepancies further highlight the need for stringent standards in statistical reporting and experimental protocols in order to allow cross-study comparisons.

In addition to the transcriptional changes, Kohanski and colleagues observed the liberation of hydroxyl radicals and an immediate increase in NAD^+/NADH in response to all three antibiotics (Kohanski *et al.*, 2007). This was subsequently shown to be the result of intracellular ferrous iron reduction potentially related to an increase in NADH dehydrogenase I, which transfers electrons from NADH via an iron-sulfur cluster (Kohanski *et al.*, 2007). Deletion mutants for key enzymes of the tricarboxylic acid (TCA) cycle linked the increase in NAD^+ to an increase in central metabolism which magnified the bactericidal effects of antibiotic challenge. Despite the transcriptional contradictions, the induction of heat shock transcripts and the production of reactive oxygen species are significant discoveries and help to further clarify the mechanism of streptomycin-induced cell death.

The most widely accepted hypothesis of streptomycin's mechanism of action was proposed by Davis in 1987 (Davis, 1987). Upon streptomycin challenge, a small amount of the antibiotic enters the cell and irreversibly binds to membrane-bound

ribosomes inducing misreading. Due to their proximity to the membrane, these mistranslated proteins are directly incorporated into the membrane increasing permeability and promoting further antibiotic influx. As the intracellular concentration of streptomycin increases, translation initiation is prohibited causing a decrease in protein synthesis resulting in death. Davis rightly points out that the lethal event is not the induction of leaky membranes but the effect of increasing concentrations of intracellular streptomycin. However, the sequence of events connecting the observed increased permeability, requirement for protein synthesis, and other pleiotropic effects has remained a mystery for almost 20 years. Based on the present study, the following sequences of events may present a plausible link between mistranslation and cell death.

Following an increase in permeability, streptomycin diffusion increases across the membrane. Due to this influx, cytoplasmic as well as membrane-bound ribosomes are now irreversibly bound with the antibiotic inducing the misreading of nascent mRNAs. The resultant aberrant proteins are quickly sequestered for recycling by the cellular chaperone/protease triad DnaJKGrpE-ClpB-HslTS, responsible for protein quality control (Mogk *et al.*, 2003). Under normal conditions, not only is DnaJKGrpE responsible for protein quality control but also efficient degradation of σ^{32}, the alternative σ factor responsible for transcription of heat shock genes (Arsene *et al.*, 2000). As the levels of aberrant proteins increase, the level of free DnaJKGrpE will decrease and the level of active σ^{32} will increase resulting in an increased transcription of heat shock genes. All three of these processes –

transcription of heat shock genes, mistranslation of existing mRNAs, and proteolysis are energy-rich processes (Alberts *et al.*, 2002). Consequently an active metabolism, or at least the electron transport chain and oxidative phosphorylation, would be required to generate the vast quantities of energy required to combat streptomycin injury. This would generate high levels of ATP as well as oxidized electron acceptors, such as NAD^+, and intracellular oxygen.

As the cycle of transcription, mistranslation, degradation, and energy consumption continues, nascent proteins are unable to be processed appropriate in order to replace proteins which have naturally undergone conformational deformation causing an increase in both free cofactors and poorly functioning proteins. The rise in intracellular oxygen from increased electron transport naturally could react with the free intracellular iron (possibly from unformed cofactors) generating reactive oxygen species thus speeding up the natural process of oxidative damage to DNA, proteins and lipid membranes. Ultimately, this synergism would increase membrane permeability destroying the proton gradient responsible for the generation of energy. Consequently, cell death may be via a lack of energy and leaky membranes rather than a well orchestrated death cascade.

While the Davis model eloquently accounts for many of the pleiotropic effects of streptomycin treatment, it fails to account for the effects of the drug on cytoplasmic ribosomes prior to cell death. The updated model presented here suggests a link between mistranslating cytoplasmic ribosomes and previous observations that both

active metabolism and protein synthesis are required for lethality. In particular, this study has identified the induction of heat shock genes in response to streptomycin treatment. This validates the suggestion that the protein quality control chaperone/protease triad DnaJKGrpE-ClpB-HslTS is mobilized to combat aberrant proteins leading to an increase in σ^{32} and target transcription. Moreover, although previous proteomic studies suggest the response for kanamycin and streptomycin is similar to the heat shock response, no protein species were identified. Can we trace the transcriptional changes to an increase or decrease in protein levels? Proteome studies determining the identity of translated proteins as well as their level of mistranslation may provide clues about the role of heat shock proteins as well as further insights into the extent of protein damage – mistranslation, oxidative, or a combination thereof, to further develop our understanding of cell death as a result of bactericidal antibiotics.

This updated model may also explain the recent observation of Kohanski and colleagues that there is an immediate rise in NAD^+ following kanamycin treatment that results in an increase in reactive oxygen species (Kohanski *et al.*, 2007). Presumably, these increases are the result of increased electron transport in an attempt to cope with the increasing energy demands of transcription, mistranslation, and proteoloysis where NAD^+ and oxygen radicals are the by-products. A similar increase in level of FAD^+ relative to FADH should also be observable and would help to clarify whether a generalized or specific oxidation of electron carriers is required for lethality.

In addition to shedding light on some of the pleiotropic effects of streptomycin, this model raises further questions. In the present study there was a marked repression of metabolic genes, specifically *nuoCEF*, part of the operon required for the production of NADH dehydrogenase I, the first enzyme of the electron transport. However, Kohanski and colleagues observed their induction (Kohanski *et al.*, 2007). This discrepancy is further complicated by the increase in NAD^+ relative to NADH following bactericidal antibiotic treatment as NADH dehydrogenase I is responsible for the conversion of NADH to NAD+ and establishing the proton motive force required for ATP generation. Further studies to determine whether the *nuo* transcripts result in functional proteins are needed to clarify these conflicting observations.

The observed increases in transcription and mistranslation are energy-rich processes. Yet the source of this energy remains ambiguous. Under normal conditions, NADH is generated as a by-product of the TCA cycle then used by the electron transport chain to generate a proton gradient and drive ATP synthesis. Deletion mutants for components of the TCA cycle had a lower susceptibility to bactericidal antibiotics (Kohanski *et al.*, 2007). This supports previous observations that active metabolism is a prerequisite for cell death (Paine and Clark, 1953). However, the current study indicates metabolic genes are repressed in response to streptomycin. It remains to be seen if there is a turn over of TCA cycle and ATP synthesis enzymes during streptomycin treatment or if the noted repression is a response to the cessation of cell division.

On a related note, it would be interesting to determine whether there is an increase in ATP to fuel the proposed molecular responses and accompany the observed increase in NAD^+. Such experiments would help to clarify if the proposed response is active as well as if the energy requirements of cell death are required throughout the process or solely for its induction.

Neither the Davis model (1987) nor the updated model suggested here account for the almost complete repression of both chemotaxis and motility genes in the current study. This immediately raises the questions – What are the physiological consequences of streptomycin treatment on mutants for these pathways? Is there a change in MIC? Could the energy required to combat the cellular injuries caused by streptomycin treatment be diverted from these pathways in addition to that generated through active metabolism? Is the transcriptome altered in response to streptomycin? Interestingly, these are the most commonly repressed genes across independent transcriptional studies. Is this a product of monitoring changing environmental conditions? Or, is this an artefact of *in vitro* model systems? Clearly the role of chemotaxis and motility remains un-quantified as a possible stress response.

Finally, most transcriptional studies, the present study included, investigate the global changes associated with planktonic cells. However, a growing body of evidence suggests bacteria live in complex communities of planktonic cells and biofilms. Very little is known about the molecular responses of bactericidal antibiotics on these communities. Are higher concentrations of antibiotics required

to kill biofilms? Do planktonic cells form biofilms in response to antibiotic treatment? Are the transcriptional changes induced by streptomycin in biofilms the same as those induced in planktonic cells? Such information would aid in our understanding of how cell death may occur in complex communities which often define infections.

In conclusion, this thesis supports an emerging body of evidence suggesting that the induction of the heat shock response may be a key component of streptomycin-induced cell death. Aberrant proteins synthesized as a result of the mistranslation observed by Davies may now be linked with the appearance of leaky membranes observed by Anand and Davis through the induction of chaperones and proteases (Davies *et al.*, 1964; Anand and Davis, 1960; present study). This includes the DnaJKGrpE-ClpB-HslTS triad of the heat shock response which sequesters and degrades aberrant proteins rather than allowing them to replace their naturally degraded membrane and cytoplasmic counterparts. These ATP-dependent proteases require active metabolism, as observed by Paine and Clark, and a functional electron transport chain for the generation of ATP in order to degrade nascent, abnormal proteins sequestered by chaperones (Paine and Clark, 1953). While ATP fuels proteolysis, its generation leads to additional membrane damage through the oxidation of members of the electron transport change, such as NADH I dehydrogenase, and the accumulation of hydroxyl radicals as observed by Kohanski and colleague (Kohanski *et al.*, 2007). Consequently, it is the induction of heat shock proteases and their energy requirements that may be the trigger of cell death.

When coupled with the self-promoted uptake observed by Hancock, whereby streptomycin competitively displaces divalent cations in the lipopolysaccharide of the outer membrane resulting in increased permeability, the bacterial cell's fate is sealed (Hancock, 1997). Consequently, cell death as a result of streptomycin treatment is an active, energy requiring process. Further investigations into the correlation between the transcriptional induction of heat shock proteases and functional proteins, the potential oxidative damage to proteins and nucleic acids, and their energy requirements may further our understanding of the nature of cell death and provide potential new targets for drug discovery.

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Appendix 1. Formulae and Statistics

1. Colony Forming Units

Colony forming units (CFUs) were determined as follows:

$$\text{CFU} = \text{number colonies counted} \times \frac{1}{\text{amount plated}} \times \frac{1}{\text{diltuion factor}}$$

2. Statistics

2.1 Average

$$\text{Average} = \frac{\sum x_1 + x_2 + \ldots x_i}{n-1}$$

Where x_1, x_2, $\ldots x_i$ are the values of random variables, and n is the number of variables.

2.2 Standard Deviation

$$\text{SD} = \sqrt{\frac{(AVERAGE)^2}{(n-1)}}$$

Where n is the number of variables.

2.3 Standard Error

$$\text{SE} = \frac{\text{SD}}{\text{n}}$$

Where SD is the standard deviation and n is the number of variables.

Appendix 2. RNA Sample Details for Microarray Experiments

Sample No.	Control/Sample	xMIC	Time (min)	RNA (ug/mL)	A260/A280	Cy-3/Cy-5	Array No.
1	C	4	10	6343.6	1.89	3	EC9-10
2	S	4	10	4687.3	1.93	5	EC9-10
3	C	4	10	4020.2	1.91	5	EC9-17
4	S	4	10	5025.4	1.92	3	EC9-17
5	C	4	10	5396.6	1.94	5	EC9-03
6	S	4	10	4591.5	1.94	3	EC9-03
7	C	4	10	4505.1	1.76	5	EC9-44
8	S	4	10	4604.6	1.94	3	EC9-44
25	C	4	30	5123.3	1.83	3	EC9-24
26	S	4	30	5098.0	1.86	5	EC9-24
27	C	4	30	4991.3	1.83	5	EC9-25
28	S	4	30	3712.8	1.75	3	EC9-25
29	C	4	30	8871.8	1.99	5	EC9-29
30	S	4	30	5779.8	2.00	3	EC9-29
31	C	4	30	6078.7	1.73	3	EC9-53
32	S	4	30	8138.0	1.80	5	EC9-53
33	C	2	30	9165.6	1.94	3	EC9-51
34	S	2	30	8514.0	1.92	5	EC9-51
35	C	2	30	10192.0	1.90	5	EC9-52
36	S	2	30	9414.7	1.90	3	EC9-52
37	C	2	30	9863.7	1.71	3	EC9-54
38	S	2	30	7232.9	1.77	5	EC9-54
39	C	2	30	6773.9	1.74	5	EC9-55
40	S	2	30	6998.2	1.77	3	EC9-55
41	C	1	30	7669.4	1.90	3	EC9-26
42	S	1	30	5643.2	1.85	5	EC9-26
43	C	1	30	5906.6	1.86	5	EC9-27
44	S	1	30	3122.7	1.73	3	EC9-27
45	C	1	30	6386.0	2.01	5	EC9-30
46	S	1	30	4886.3	1.98	3	EC9-30
47	C	1	30	8184.6	1.78	3	EC9-57
48	S	1	30	7732.0	1.79	5	EC9-57
49	C	4	60	11574.0	1.83	3	EC9-58
50	S	4	60	9791.5	1.87	5	EC9-58
51	C	4	60	11918.0	1.92	5	EC9-60
52	S	4	60	4704.0	1.83	3	EC9-60
53	C	4	60	11457.0	1.86	3	EC9-84
54	S	4	60	6968.3	1.86	5	EC9-84
55	C	4	60	9596.8	1.86	5	EC9-64
56	S	4	60	6322.5	1.86	3	EC9-64

APPENDIX 3. Expression Ratios of Genes with a ≥2-Fold Up Regulation 10-minutes After Addition of 4xMIC Streptomycin.

Gene Name	B Number	Function	Fold Change
Cell processes			
Adaptation			
hslS	b3686	heat-inducible chaperone (HSP20)	6.38
htpX	b1829	membrane-bound zinc metalloprotease HtpX (Cpx & RpoH regulons)	3.11
cpxP	b3914	CpxAR-regulated periplasmic protein (feedback inhibition of Cpx signal transduction?)	2.70
hslU	b3931	heat-inducible ATP-dependent protease HslVU (ATPase subunit, HSP D48.5)	2.62
hslR	b3400	abundant heat shock protein that binds RNA & DNA (HSP15)	2.09
Cell division			
groS	b4142	chaperonin Cpn10; GroESL small subunit	3.85
groL	b4143	chaperonin Cpn60; GroESL large subunit	2.93
ftsJ	b3179	23S rRNA U2552 ribose 2'-O-methyltransferase (SAM-dependent)	2.43
Chaperones			
dnaK	b0014	heat-inducible chaperone ATP-regulated (HSP70); DnaJ co-chaperone	4.84
Global regulatory functions			
lon	b0439	protease LA; ATP-dependent; DNA binding	2.59
Macromolecule metabolism			
Amino acyl tRNA synthesis; tRNA modification			
miaA	b4171	dimethylallyl diphosphate; tRNA hypermodification	2.24
Degradation of DNA			
clpB	b2592	ATP-dependent ClpB protease	3.92
hslV	b3932	heat-inducible ATP-dependent protease HslVU (protease subunit)	2.22
Structural elements			
Inner membrane			
yccA	b0970	membrane-associated protein that binds FtsH(HflB) & HflKC proteins against dissociation at low magnesium concentrations/stationary phase	2.05
Ribosomal and stable RNAs			
yfiA	b2597	Protein Y, associated with 30S ribosomal subunit; stabilizes ribosomal 70S complex	2.75

APPENDIX 4A. Expression Ratios of Genes With a ≥2-Fold Up Regulation 30-minutes After Addition of 4xMIC Streptomycin.

Gene Name	B Number	Function	Fold Change
Cell processes			
Adaptation			
hslS	b3686	heat-inducible chaperone (HSP20)	21.57
htpX	b1829	membrane-bound zinc metalloprotease HtpX (Cpx & RpoH regulons)	9.73
cpxP	b3914	CpxAR-regulated periplasmic protein (feedback inhibition of Cpx signal transduction?)	6.08
hslR	b3400	abundant heat shock protein that binds RNA & DNA (HSP15)	3.97
hslU	b3931	heat-inducible ATP-dependent protease HslVU (ATPase subunit, HSP D48.5)	3.32
cspA	b3556	cold shock protein CS7.4 (transcription factor)	2.39
Cell division			
groS	b4142	chaperonin Cpn10; GroESL small subunit	4.58
ftsJ	b3179	23S rRNA U2552 ribose 2'-O-methyltransferase (SAM-dependent)	4.17
groL	b4143	chaperonin Cpn60; GroESL large subunit	3.11
Cell killing			
marR	b1530	transcription repressor of multiple antibiotic resistance (Mar) locus	3.39
marA	b1531	transcription activator of multiple antibiotic resistance (Mar) locus	3.05
cutC	b1874	copper sensitivity	2.43
Chaperones			
dnaK	b0014	heat-inducible chaperone ATP-regulated (HSP70); DnaJ co-chaperone	6.07
hslO	b3401	heat-induced cytoplasmic chaperone (HSP33)	4.21
dnaJ	b0015	heat-inducible DnaK co-chaperone	4.12
Transport/binding proteins			
nhaA	b0019	pH-dependent Na+/H+ antiporter 1	4.36
alx	b3088	putative membrane transport or efflux protein	3.10
zntA	b3469	Zn(II), Cd(II), and Pb(II) translocating P-type ATPase	2.55
ybeZ	b0660	PhoH paralog	2.33
gntP	b4321	membrane protein homologous to *B. subtilis* gluconate permease	2.21
Extrachromosomal			
Colicin-related functions			
pspA	b1304	negative regulatory gene for phage-shock-protein psp operon	7.55

APPENDIX 4A. Continued.

Gene Name	B Number	Function	Fold Change
pspB	b1305	positive regulatory gene (w/ PspC) for phage-shock-protein operon	6.33
grpE	b2614	nucleotide exchange factor for the DnaKJ chaperone	3.54
pspC	b1306	positive regulatory gene (w/ PspB) for phage-shock-protein operon	2.84
hfq	b4172	HF-I, host factor for RNA phage Q beta replication	2.80
Prophage gene or phage related function			
intF	b0281	CP4-6 prophage	2.46
yffS	b2450	CPZ-55 prophage predicted protein	2.37
Global regulatory functions			
soxS	b4062	soxRS regulon; induces nine-protein sox regulon when superoxide levels increase	7.36
lon	b0439	protease LA; ATP-dependent; DNA binding	3.95
lexA	b4043	repressor of SOS regulon	3.13
rpoH	b3461	RNA polymerase, s32 subunit (heat shock transcription)	2.67
fnr	b1334	transcription factor for anaerobic growth; [4FE-4S] clusters	2.23
Macromolecule metabolism			
Amino acyl tRNA syntesis; tRNA modification			
miaA	b4171	dimethylallyl diphosphate; tRNA hypermodification	4.48
deaD	b3162	RNA helicase translation factor W2; facilitaties translation of mRNAs w/ 5' secondary structures	3.77
ruvA	b1861	Holliday junction recognition; lexA regulon	2.11
Degradation of DNA			
clpB	b2592	ATP-dependent ClpB protease	6.55
hslV	b3932	heat-inducible ATP-dependent protease HslVU (protease subunit)	3.64
ftsH	b3178	essential inner membrane ATP-dependent protease	2.52
clpP	b0437	protease Ti; proteolytic subunit of ClpXP and ClpAP ATP-dependent proteases	2.49
prlC	b3498	zinc metalloprotease oligopeptidase A; degradation of cleaved signal peptides	2.23
hflX	b4173	putative GTPase in hflA operon	2.17
DNA replication			
yccV	b0966	hemimethylated oriC DNA-binding protein (heat shock protein hspQ)	5.73

APPENDIX 4A. Continued.

Gene Name	B Number	Function	Fold Change
Metabolism of small molecules			
Aerobic respiration			
sdhC	b0721	cytochrome b556; succinate dehydrogenase membrane anchor subunit	2.41
maa	b0459	maltose O-acetyltransferase	2.08
aldH	b1300	putative aldehyde dehydrogenase, by homology	2.02
Amino acid biosynthesis			
ilvG	b3767	acetohydroxy acid synthase II (AHAS-II); acetolactate synthase II (ALS-II) large subunit	3.90
asnA	b3744	asparagine synthase A	3.43
ilvB	b3671	acetohydroxy acid synthase I (AHAS-I); acetolactate synthase I (ALS-I) large subunit	2.81
hisG	b2019	ATP phosphoribosyltransferase	2.72
iscS	b2530	cysteine desulfurase used in synthesis of Fe-S cluster; tRNA 4-thiouridine sulfurtransferase	2.49
ilvG_2		acetohydroxy acid synthase II (AHAS-II); acetolactate synthase II (ALS-II) large subunit	2.46
iscR	b2531	transcriptional repressor for isc operon, contains Fe-S cluster; binds RNA in vitro	2.37
Degradation of small molecules			
uxuA	b4322	D-mannonate dehydratase (hydrolase)	3.46
glcC	b2980	regulatory gene for glc operon	2.33
uxuR	b4324	regulatory gene for uxuBA operon	2.09
Fatty acid biosynthesis			
tesA	b0494	acyl-CoA thioesterase I	2.56
Structural elements			
Inner membrane			
yccA	b0970	membrane-associated protein that binds FtsH(HflB) & HflKC proteins against dissociation at low magnesium concentrations/stationary phase	3.77
crl	b0240	regulatory protein for curli (cryptic csgA)	3.30
fxsA	b4140	suppresses F exclusion of phage T7	2.28
Ribosomal and stable RNAs			
yfiA	b2597	Protein Y, associated with 30S ribosomal subunit; stabilizes ribosomal 70S complex	3.40
rpsO	b3165	30S ribosomal subunit protein S15	2.21

APPENDIX 4A. Continued.

Gene Name	B Number	Function	Fold Change
Miscellaneous/unclassified			
yhdN	b3293	concerved hypothetical protein	4.43
ytfK	b4217	conserved protein	3.28
ybaJ	b0461	predicted protein	3.07
yejG	b2181	predicted protein	2.79
yfeK	b2419	predicted protein	2.73
yqjA	b3095	concerved inner membrane protein	2.63
ydeT	b1505	putative outer membrane protein	2.62
tusB	b3343	sulfur transfer protein complex subunit	2.58
yfgG	b2504	predicted protein	2.58
yigI	b3820	concerved hypothetical protein	2.49
ydbA	b1405	function unknown; interrupted by IS2D and IS30C	2.31
slyB	b1641	putative lipoprotein	2.30
ygaU	b2665	predicted protein	2.30
yibQ	b3614	putative nucleoside (IDP) diphosphate	2.12
ydeH	b1535	function unknown	2.12
mqsR	b3022	motility quorum sensing regulator	2.09
yrfG	b3399	purine nucleotidase open reading frame	2.06
qmcA	b0489	putative protease	2.04
ydgK	b1626	conserved inner membrane protein	2.03
yhbE	b3184	conserved inner membrane protein	2.02

APPENDIX 4B. Expression Ratios of Genes With a ≥2-Fold Down Regulation 30-minutes After Addition 4xMIC Streptomycin.

Gene Name	B Number	Function	Fold Change
Cell processes			
Transport/binding proteins			
malE	b4034	periplasmic maltose-binding protein;substrate recognition for transport & chemotaxis	-8.07
lamB	b4036	maltoporin (maltose high-affinity uptake system); phage lambda receptor	-5.39
gatA	b2094	galactitol-specific enzyme IIA of phosphotransferase system (PTS)	-4.10
narK	b1223	nitrate/nitrite antiporter	-3.22
rbsB	b3751	D-ribose periplasmic binding protein	-2.82
malK	b4035	maltose transport complex (ATP-binding subunit)	-2.63
nikA	b3476	nickel-binding periplasmic protein; Tar-dependent Ni-repellant chemosensor	-2.49
malF	b4033	maltose transport complex	-2.36
gatB	b2093	galactitol-specific enzyme IIB of phosphotransferase system (PTS)	-2.32
fliY	b1920	cystine-binding protein (may regulate FliA (σ28))	-2.05
focA	b0904	probable bidirectional formate transporter 1	-2.02
Cell division			
murF	b0086	D-alanyl:D-alanine adding enzyme; UDP-N-acetylmuramoyl-tripeptide:D-alanyl-D-alanine ligase	-2.07
mukB	b0924	required for chromosome partitioning; DNA binding; kinesin-like motor protein?	-2.01
Chemotaxis and mobility			
cheZ	b1881	CheY-P phosphatase	-4.11
cheY	b1882	response regulator for chemotactic signal transduction	-3.15
cheB	b1883	methylesterase	-3.03
tap	b1885	chemotaxis protein IV (aspartate receptor)	-2.97
cheR	b1884	methyltransferase	-2.95
cheW	b1887	signal transduction	-2.94
cheA	b1888	autophosphorylating histidine sensor kinase of chemotactic response	-2.57
Extrachromosomal			
Transposon related			
insH-10	b3218	IS5 transposase & trans-activator	-2.24
insH-6	b1994	IS5 transposase & trans-activator	-2.24

APPENDIX 4B. Continued.

Gene Name	B Number	Function	Fold Change
Macromolecule metabolism			
Amino acyl tRNA synthesis; tRNA modification			
rfaL	b3622	LPS core biosynthesis; O-antigen ligase	-3.00
lysS	b2890	constitutive lysine--tRNA ligase	-2.03
Degradation of DNA			
hsdR	b4350	host DNA restriction endonuclease R	-2.37
rnb	b1286	RNase II; mRNA degradation	-2.26
amyA	b1927	α-amylase	-2.08
Metabolism of small molecules			
2'-Deoxyribonucleotide metabolism			
gcvH	b2904	glycine cleavage; aminomethyl carrier	-2.96
gcvP	b2903	glycine dehydrogenase; decarboxylating	-2.62
aspA	b4139	L-Aspartate ammonia-lyase; L-aspartase	-2.33
deoD	b4384	purine-nucleoside phosphorylase	-2.09
talB	b0008	transaldolase B	-2.07
Aerobic respiration			
nuoE	b2285	NADH-ubiquinone oxidoreductase subunit E, complex I; NADH dehydrogenase I	-4.95
fdnG	b1474	formate dehydrogenase-N (major subunit)	-4.36
narH	b1225	nitrate reductase β-subunit	-4.26
nuoC	b2286	NADH-ubiquinone oxidoreductase subunit C, complex I; NADH dehydrogenase I	-3.52
aceF	b0115	pyruvate dehydrogenase, dihydrolipoamide acetyltransferase component E2	-3.32
aceE	b0114	pyruvate dehydrogenase, decarboxylase component E1	-3.11
napB	b2203	cytochrome c homolog	-3.06
nuoG	b2283	NADH-ubiquinone oxidoreductase subunit G, complex I; NADH dehydrogenase I	-3.01
nuoF	b2284	NADH-ubiquinone oxidoreductase subunit F, complex I; NADH dehydrogenase I	-2.98
narG	b1224	nitrate reductase α-subunit	-2.93
nuoN	b2276	NADH-ubiquinone oxidoreductase subunit N, complex I; NADH dehydrogenase I	-2.51
eno	b2779	enolase; phosphoprotein; component of RNA degradosome	-2.30
sucD	b0729	succinyl CoA synthase α-subunit	-2.15

APPENDIX 4B. Continued.

Gene Name	B Number	Function	Fold Change
napA	b2206	nitrate reductase homolog	-2.14
zwf	b1852	glucose 6-phosphate dehydrogenase	-2.10
frdA	b4154	fumarate reductase flavoprotein subunit	-2.05
Anaerobic metabolism			
yhaS	b3114	2-ketobutyrate formate-lyase/pyruvate formate-lyase 4	-2.77
Degradation of small molecules (amines)			
malM	b4037	periplasmic protein	-6.64
treC	b4239	trehalose-6-phosphate hydrolase, osmoprotectant	-5.10
gatD	b2091	galactitol-1-phosphate dehydrogenase	-3.47
tnaA	b3708	tryptophanase	-3.34
maoC	b1387	putative dehydrogenase	-3.10
lacZ	b0344	β-D-Galactosidase	-2.85
malP	b3417	maltodextrin phosphorylase	-2.82
tdcB	b3117	threonine dehydratase	-2.80
rbsK	b3752	ribokinase	-2.33
tnaC	b3707	regulatory leader peptide for tna operon	-2.30
tdcA	b3118	transcriptional activator of tdc operon	-2.01
gudD	b2787	D-glucarate dehydratase; L-idarate dehydratase; D-glucarate, L-idarate epimerase	-2.00
melA	b4119	α-galactosidase	-2.00
Structural elements			
Inner membrane			
flgG	b1078	flagella distal rod subunit	-5.99
flgJ	b1081	flagella muramidase	-5.84
fliK	b1943	flagella hook length control	-5.33
fliJ	b1942	flagella rod, hook, & filament chaperone	-4.74
flgH	b1079	flagella L-ring protein	-4.67
flgE	b1076	flagella hook subunit	-4.62
flgF	b1077	flagella proximal rod subunit	-4.61
fliG	b1939	flagella rotor component; binds MotA	-4.32

APPENDIX 4B. Continued.

Gene Name	B Number	Function	Fold Change
fliH	b1940	negative regulator of FliI ATPase	-4.25
fliS	b1925	FliC chaperone	-3.92
flgI	b1080	flagella P-ring protein	-3.81
fliI	b1941	flagella cytoplasmic membrane export ATPase	-3.55
fliT	b1926	FliD chaperone	-3.51
fliN	b1946	flagella switch component	-2.86
fliM	b1945	flagella switch component; binds CheY-P	-2.66
flgC	b1074	assembly of flagella hook	-2.36
fliF	b1938	flagella M-ring protein	-2.30
ompF	b0929	outer membrane porin protein 1a	-2.03
murE	b0085	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate:meso-diaminopimelate ligase	-2.02
btuB	b3966	receptor for vitamin B12, E colicins, and phage BF23	-2.00
Miscellaneous/unclassified			
ykgG	b0308	function unknown	-3.22
yehT	b2125	function unknown	-2.28
G6388	b0725	phantom gene	-2.23
sthA	b3962	soluble pyridine nucleotide transhydrogenase	-2.21
gatZ	b2095	subunit required for full activity and stabilty of GatY tagatose bisphosphate aldolase	-2.18
ybgE	b0735	function unknown; fourth gene in cydAB operon, expressed in minicells	-2.69
ymdA	b1044	function unknown	-2.45
ykgF	b0307	function unknown	-2.37
ydhO	b1655	function unknown	-2.36

APPENDIX 5. Expression Ratios of Genes With a ≥2-Fold Up Regulation 30-minutes After Addition of 1xMIC Streptomycin.

Gene Name	B Number	Function	Fold Change
Cell processes			
Adaptation			
hslS	b3686	heat-inducible chaperone (HSP20)	3.86
hslU	b3931	heat-inducible ATP-dependent protease HslUV (ATPase subunit, HSP D48.5)	2.23
htpX	b1829	membrane-bound zinc metalloprotease HtpX (Cpx & RpoH regulons)	2.08
Global regulatory functions			
lon	b0439	protease LA; ATP-dependent; DNA binding	2.03
Macromolecule metabolism			
Degradation of DNA			
clpB	b2592	ATP-dependent ClpB protease	2.96
Structural elements			
Ribosomal and stable RNAs			
yfiA	b2597	Protein Y, associated with 30S ribosomal subunit; stabilizes ribosomal 70S complex	2.88

APPENDIX 6A. Expression Ratios of Genes with a ≥2-Fold Up Regulation 30-minutes After Addtion of 2xMIC Streptomycin.

Gene Name	B Number	Function	Fold Change
Cell processes			
Adaptation			
hslS	b3686	heat-inducible chaperone (HSP20)	16.56
htpX	b1829	membrane-bound zinc metalloprotease HtpX (Cpx & RpoH regulons)	5.25
cpxP	b3914	CpxAR-regulated periplasmic protein (feedback inhibition of Cpx signal transduction?)	4.83
hslU	b3931	heat-inducible ATP-dependent protease HslVU (ATPase subunit, HSP D48.5)	2.56
hslR	b3400	abundant heat shock protein that binds RNA & DNA (HSP15)	2.44
Cell division			
ftsJ	b3179	23S rRNA U2552 ribose 2'-O-methyltransferase (SAM-dependent)	2.78
groS	b4142	chaperonin Cpn10; GroESL small subunit	2.54
groL	b4143	chaperonin Cpn60; GroESL large subunit	2.52
Cell killing			
marA	b1531	transcription activator of multiple antibiotic resistance (Mar) locus	2.02
Chaperones			
dnaK	b0014	heat-inducible chaperone ATP-regulated (HSP70); DnaJ co-chaperone	3.88
dnaJ	b0015	heat-inducible DnaK co-chaperone	2.47
hslO	b3401	heat-induced cytoplasmic chaperone (HSP33)	2.15
Transport/binding proteins			
nhaA	b0019	pH-dependent Na+/H+ antiporter 1	2.53
Extrachromosomal			
Colicin-related functions			
pspA	b1304	negative regulatory gene for phage-shock-protein psp operon	4.86
grpE	b2614	nucleotide exchange factor for the DnaKJ chaperone	3.40
pspB	b1305	positive regulatory gene (w/ PspC) for phage-shock-protein operon	3.05
Prophage gene or phage related function			
intF	b0281	CP4-6 prophage	2.08

APPENDIX 6A. Continued.

Gene Name	B Number	Function	Fold Change
Global regulatory functions			
soxS	b4062	soxRS regulon; induces nine-protein sox regulon when superoxide levels increase	6.67
lon	b0439	protease LA; ATP-dependent; DNA binding	2.53
lexA	b4043	repressor of SOS regulon	2.01
Macromolecule metabolism			
Amino acyl tRNA synthesis; tRNA modification			
miaA	b4171	dimethylallyl diphosphate; tRNA hypermodification	3.21
Degradation of DNA			
clpB	b2592	ATP-dependent ClpB protease	4.51
hslV	b3932	heat-inducible ATP-dependent protease HslVU (protease subunit)	2.45
clpP	b0437	protease Ti; proteolytic subunit of ClpXP and ClpAP ATP-dependent proteases	2.25
clpA	b0882	protease Ti; ATPase subunit of ClpAP ATP-dependent protease	2.04
DNA replication			
yccV	b0966	hemimethylated oriC DNA-binding protein, (heat shock protein hspQ)	3.39
Metabolism of small molecules			
Amino acid biosynthesis			
ilvG	b3767	acetohydroxy acid synthase II (AHAS-II); acetolactate synthase II (ALS-II) large subunit	2.39
tusB	b3343	sulfur transfer protein complex subunit	2.21
ilvB	b3671	acetohydroxy acid synthase I (AHAS-I); acetolactate synthase I (ALS-I); large subunit	2.11
Degradation of small molecules			
uxuA	b4322	D-mannonate dehydratase (hydrolase)	2.16
Structural elements			
Inner membrane			
yccA	b0970	membrane-associated protein that binds FtsH(HflB) & HflKC proteins against dissociation at low magnesium concentrations/stationary phase	2.98
Ribosomal and stable RNAs			
yfiA	b2597	Protein Y, associated with 30S ribosomal subunit; stabilizes ribosomal 70S complex	5.00

APPENDIX 6A. Continued.

Gene Name	B Number	Function	Fold Change
Miscellaneous/unclassified			
yhdN	b3293	concerved hypothetical protein	2.88
yfgG	b2504	predicted protein	2.49

APPENDIX 6B. Expression Ratios of Genes With a ≥2-Fold Down Regulation 30-minutes After Addition of 2xMIC Streptomycin.

Gene Name	B Number	Function	Fold Change
Cell processes			
Transport/binding proteins			
malE	b4034	periplasmic maltose-binding protein;substrate recognition for transport & chemotaxis	-4.65
lamB	b4036	maltoporin (maltose high-affinity uptake system); phage lambda receptor	-4.04
malK	b4035	maltose transport complex (ATP-binding subunit)	-2.88
Macromolecule metabolism			
Aerobic respiration			
cyo	b0431	cytochrome o oxidase subunit I; cytochrome bo(3) ubiquinol oxidase subunit I	-2.01
Degradation of small molecules			
malM	b4037	periplasmic protein	-3.85
malP	b3417	maltodextrin phosphorylase	-2.18
melA	b4119	α-galactosidase	-2.06
Structural elements			
Inner membrane			
flgJ	b1081	flagella muramidase	-2.37
flgF	b1077	flagella proximal rod subunit	-2.18
flgI	b1080	flagella P-ring protein	-2.13

APPENDIX 7A. Expression Ratios of Genes with a ≥2-fold Up Regulation 60-minutes After Addition of 4xMIC Streptomycin.

Gene Name	B Number	Gene Description	Fold Change
Cell processes			
Adaptation			
hslS	b3686	heat-inducible chaperone (HSP20)	21.64
htpX	b1829	membrane-bound zinc metalloprotease HtpX (Cpx & RpoH regulons)	7.46
hslR	b3400	abundant heat shock protein that binds RNA & DNA (HSP15)	6.00
cpxP	b3914	CpxAR-regulated periplasmic protein (feedback inhibition of Cpx signal transduction?)	5.47
cspA	b3556	cold shock protein CS7.4 (transcription factor)	5.28
yiiO	b4484	regulator of the Cpx response & possible chaperone involved in resistance to extracytoplasmic stress	4.21
osmB	b1283	OsmB lipoprotein	2.35
betB	b0312	betaine aldehyde dehydrogenase	2.34
Cell division			
ftsJ	b3179	23S rRNA U2552 ribose 2'-O-methyltransferase (SAM-dependent)	3.46
sdiA	b1916	suppresses inhibitory effect of MinC/MinD division inhibitor, positive regulator ftsQAZ	2.42
sulA	b0958	inhibits cell division and ftsZ ring formation	2.36
tig	b0436	trigger factor, protein folding chaperone & peptidyl-prolyl cis-trans isomerase	2.09
Cell killing			
marR	b1530	transcription repressor of multiple antibiotic resistance (Mar) locus	7.33
marA	b1531	transcription activator of multiple antibiotic resistance (Mar) locus	6.65
yefM	b2017	anti-toxin of the YoeB-YefM pair	2.91
tpx	b1324	periplasmic thiol peroxidase, antioxidant, thioredoxin-dependent	2.79
cutC	b1874	copper sensitivity	2.73
yebZ	b1840	function unknown	2.65
dinJ	b0226	RelB antitoxin homolog of the YafQ-DinJ toxin-antitoxin pair induced by DNA damage	2.51
Chromosome replication			
secG	b3175	SecG inner membrane protein; complexes with the SecYE to interact with SecA to export proteins	3.06
Chaperones			
dnaJ	b0015	heat-inducible DnaK co-chaperone	6.74
dnaK	b0014	heat-inducible chaperone ATP-regulated (HSP70); DnaJ co-chaperone	4.38
hslO	b3401	heat-induced cytoplasmic chaperone (HSP33)	3.48
hscB	b2527	dnaJ-like co-chaperone for HscA (HSC20); specific for IscU	3.13

APPENDIX 7A. Continued.

Gene Name	B Number	Function	Fold Change
Motility and chemotaxis			
mqsR	b3022	motility quorum sensing regulator	3.40
Transport/binding proteins			
nhaA	b0019	pH-dependent Na+/H+ antiporter 1	5.49
kgtP	b2587	α-ketoglutarate permease	3.65
pstS	b3728	high-affinity periplasmic phosphate-specific transport protein	3.08
corA	b3816	Mg2+ transport system; mutants resistant to Co2+, Mn2+, and Ni2+, insensitive to Ca2+	2.47
alx	b3088	putative membrane transport or efflux protein	2.31
cmr	b0842	transmembrane multidrug/chloramphenicol efflux transporter, MFS family	2.12
ybeZ	b0660	PhoH paralog	2.12
bglF	b3722	membrane-bound PTS sugar transport EIIABC (β-glucoside phosphotransferase) BglG kinase/dephosphor	2.03
Extrachromosomal			
Colicin-related functions			
pspA	b1304	negative regulatory gene for phage-shock-protein psp operon	15.21
pspB	b1305	positive regulator gene (w/ PspC) for phage-shock-protein operon	8.06
pspC	b1306	positive regulatory gene (w/ PspB) for phage-shock-protein operon	6.40
grpE	b2614	nucleotide exchange factor for the DnaKJ chaperone	4.38
pspG	b4050	phage shock protein G	3.46
rhsD	b0497	function unknown; encoded within repeats that are hotspots for chromosomal duplication formation	2.19
hfq	b4172	HF-I, host factor for RNA phage Q beta replication	2.08
Transposon related			
yi81_1	b0016	IS186/IS421 transposase	3.58
yi81_3	b2394	predicted IS186/IS421 transposase	3.49
insA_1	b0022	IS1 protein InsA	3.22
insB_6	b3445	IS1 protein InsB	2.65
insB_2	b0264	IS1 protein InsB	2.57
insB_1	b0021	IS1 protein InsB	2.55
insB_4	b0983	IS1 protein InsB	2.54
insA_3	b0275	IS1 protein InsA	2.52

APPENDIX 7A. Continued.

Gene Name	B Number	Function	Fold Change
insA_2	b0265	IS1 protein InsA	2.40
insB_5	b1893	IS1 protein InsB	2.30
insA_5	b1894	IS1 protein InsA	2.25
insA_6	b3444	IS1 protein InsA	2.24
insB_3	b0274	IS1 protein InsB	2.03
Prophage gene or phage related function			
yffS	b2450	CPZ-55 prophage predicted protein	2.99
intF	b0281	CP4-6 prophage	2.79
ymfT	b1146	e14 prophage; predicted DNA-binding transcriptional regulator	2.07
Global regulatory functions			
soxS	b4062	soxRS regulon; induces nine-protein sox regulon when superoxide levels increase	6.40
lon	b0439	protease LA; ATP-dependent; DNA-binding	4.07
lexA	b4043	repressor for SOS regulon	3.12
fur	b0683	repressor of ferric iron uptake; zinc metalloprotein; inhibited by nitric oxide	2.95
fnr	b1334	transcription factor for anaerobic growth; [4FE-4S] clusters	2.81
sixA	b2340	phosphohistidine phosphatase affecting His-Asp phosphorelay of ArcB	2.74
rpoH	b3461	RNA polymerase, s32 subunit (heat shock transcription)	2.54
nsrR	b4178	Nsr transcriptional repressor	2.43
Macromolecular metabolism			
Amino acyl tRNA synthesis; tRNA modification			
deaD	b3162	RNA helicase translation factor W2; facilitaties translation of mRNAs w/ 5' secondary structures	11.21
trmD	b2607	tRNA m(1)G37 methyltransferase	5.33
recN	b2616	recombination and repair; lexA regulon	3.72
infA	b0884	protein chain initiation factor IF1	3.44
tpr	b1229	putative protamine-like protein encoded within tyrT transcript	3.11
holE	b1842	DNA polymerase III, theta subunit	3.07
hns	b1237	DNA-binding global regulator H-NS; preferentially binds bent DNA	2.88
ruvA	b1861	Holliday junction recognition; lexA regulon	2.81

APPENDIX 7A. Continued.

Gene Name	B Number	Function	Fold Change
miaA	b4171	dimethylallyl diphosphate: tRNA hypermodification	2.79
lpxC	b0096	lipid A biosynthesis;UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine deacetylase;zinc metalloamidas	2.38
tgt	b0406	tRNA-guanine transglycosylase; queuosine biosynthesis; zinc metalloprotein	2.38
rbfA	b3167	ribosome binding factor required for efficient processing of 16s rRNA; cold-shock adaptation protein	2.05
Degradation of DNA			
clpB	b2592	ATP-dependent ClpB protease	3.35
hslV	b3932	heat-inducible ATP-dependent protease HslVU (protease subunit)	2.98
ftsH	b3178	essential inner membrane ATP-dependent protease	2.65
hflX	b4173	putative GTPase in hflA operon	2.45
clpP	b0437	protease Ti; proteolytic subunit of ClpXP and ClpAP ATP-dependent proteases	2.25
DNA Replication			
yccV	b0966	hemimethylated oriC DNA-binding protein (heat shock protein hspQ)	6.28
Metabolism of small molecules			
Amino acid biosynthesis			
iscR	b2531	transcriptional repressor for isc operon, contains Fe-S cluster, binds RNA in vitro	15.52
iscS	b2530	cysteine desulfurase used in synthesis of Fe-S cluster; tRNA 4-thiouridine sulfurtransferase	11.07
iscU	b2529	Fe-S cluster formation scaffold protein	5.29
iscA	b2528	alternative scaffold protein for Fe-S cluster assembly	4.72
tusB	b3343	sulfur transfer protein complex subunit	3.42
ilvG	b3767	acetohydroxy acid synthase II (AHAS-II); acetolactate synthase II (ALS-II) large subunit	2.49
hisG	b2019	ATP phosphoribosyltransferase	2.49
asnA	b3744	asparagine synthase A	2.29
dapE	b2472	N-succinyl-diaminopimelate deacylase	2.13
ilvB	b3671	acetohydroxy acid synthase I (AHAS-I); acetolactate synthase I (ALS-I) large subunit	2.13
ilvG_2		acetohydroxy acid synthase II (AHAS-II); acetolactate synthase II (ALS-II) large subunit	2.08
asnC	b3743	regulatory gene for asnA, asnC, gidA	2.01
Acyl carrior protein biosynthesis			
lipB	b0630	lipoyl-protein ligase; lipoyl-[ACP]:protein N-lipoyltransferase	2.53

APPENDIX 7A. Continued.

Gene Name	B Number	Function	Fold Change
Aerobic respiration			
cyoA	b0432	cytochrome o oxidase subunit II; cytochrome bo(3) ubiquinol oxidase subunit II	4.46
sdhC	b0721	cytochrome b556; succinate dehydrogenase membrane anchor subunit	3.84
adhC	b0356	alcohol-acetaldehyde dehydrogenase	3.83
sdhD	b0722	succinate dehydrogenase (SQR) hydrophobic subunit	2.66
sdhB	b0724	succinate dehydrogenase (SQR) iron-sulfur protein	2.59
cyoB	b0431	cytochrome o oxidase subunit I; cytochrome bo(3) ubiquinol oxidase subunit I	2.54
acnB	b0118	aconitase B; 2-methylaconitate hydratase; apo-enzyme binds mRNA for negative translational autoregulatic	2.50
aldH	b1300	putative aldehyde dehydrogenase; (by homology)	2.44
mdh	b3236	NAD-dependent malate dehydrogenase	2.06
Central intermediary metabolism			
mqo	b2210	malate:quinone oxidoreductase	2.91
speE	b0121	spermidine synthase	2.72
maa	b0459	maltose O-acetyltransferase	2.52
gntY	b3414	protein involved in utilization of DNA as a carbon source/gluconate metabolism	2.30
Degradation of small molecules			
fadB	b3846	alpha subunit of the fatty acid oxidation multienzyme complex	2.85
glcC	b2980	regulatory gene for glc operon	2.50
uxuR	b4324	regulatory gene for uxuBA operon; the glucuronate branch of hexuronate pathway	2.47
uxuA	b4322	D-Mannonate dehydratase (hydrolase)	2.27
atpI	b3739	membrane-bound ATP synthase subunit, F1-F0-type proton-ATPase	2.24
exuR	b3094	negative regulatory gene for exu regulon (exu, uxu, uxa)	2.11
Purine ribonucleotide biosynthesis			
ndk	b2518	nucleoside diphosphate kinase	3.24
gmk	b3648	guanylate kinase	2.60
trxC	b2582	reduced tioredoxin 2 & oxidized thioredoxin 2	2.01

APPENDIX 7A. Continued.

Gene Name	B Number	Function	Fold Change
Structural elements			
Inner membrane			
crl	b0240	regulatory protein for curli (cryptic csgA)	3.65
fxsA	b4140	suppresses F exclusion of phage T7	3.21
yccA	b0970	membrane-associated protein that binds to FtsH(HflB) & HflKC proteins against dissociation at low magnesium concentrations/stationary phase	2.66
Ribosomal and stable RNAs			
rplS	b2606	50S ribosomal subunit protein L19	3.89
rpsO	b3165	30S ribosomal subunit protein S15	3.83
rpsP	b2609	30S ribosomal subunit protein S16; endonuclease	3.71
rpsB	b0169	30S ribosomal subunit protein S2; binds Zn(II)	3.09
rplK	b3983	50S ribosomal subunit protein L11; kasugamycin sensitivity	3.08
rpsN	b3307	30S ribosomal subunit protein S14	3.05
rpsR	b4202	30S ribosomal subunit protein S18	2.99
rpmI	b1717	50S ribosomal subunit protein A (L35)	2.87
rplA	b3984	50S ribosomal subunit protein L1	2.85
rimM	b2608	21-kDa ribosome maturation protein, essential for 16S RNA processing	2.84
rplJ	b3985	50S ribosomal subunit protein L10; streptomycin resistance	2.69
rplN	b3310	50S ribosomal subunit protein L14	2.61
rpmE	b3936	50S ribosomal subunit protein L31	2.36
rpsJ	b3321	30S ribosomal subunit protein S10	2.10
rplE	b3308	50S ribosomal subunit protein L5	2.07
rpsF	b4200	30S ribosomal subunit protein S6; suppressor of dnaG-Ts	2.00
Miscellaneous/unclassified			
yhbE	b3184	concerved inner membrane protein	6.00
yigI	b3820	concerved hypothetical protein	5.59
yejG	b2181	predicted protein	5.37
ybaJ	b0461	predicted protein	5.32
ygaV	b2667	predicted DNA binding trancriptional regulator	4.99

APPENDIX 7A. Continued.

Gene Name	B Number	Function	Fold Change
yhcN	b3238	conserved hypothetical protein	4.45
ydbA	b1405	function unknow; interrupted by IS2D and IS30C	4.37
frmR	b0357	regulation protein of unknown function	3.58
ydgK	b1626	conserved inner membrane protein	3.57
yfeK	b2419	predicted protein	3.54
yhdN	b3293	concerved hypothetical protein	3.51
yhbC	b3170	concerved protein	3.46
ynaE	b1375	hypothetical protein	3.41
ygeQ	b2863	predicted protein	3.05
yqjA	b3095	concerved inner membrane protein	2.85
pgaD	b1021	predicted inner membrane protein	2.78
yrfG	b3399	purine nucleotidase open reading frame	2.76
ygiT	b3021	predicted DNA-binding transcriptional regulator	2.67
ydjF	b1770	predicted DNA-binding transcriptional regulator	2.67
yjiV	b4343	hypothetical protein	2.64
ymcE	b0991	function unknown	2.64
yadS	b0157	conserved innermembrane protein	2.60
ytfK	b4217	conserved protein	2.56
yffB	b2471	conserved protein	2.54
qmcA	b0489	putative protease	2.53
ybfE	b0685	lexA regulated, function unknown	2.51
yncC	b1450	predicted DNA-binding transcriptional regulator	2.45
yiiX	b3937	conserved hypothetical protein of NlpC/P60 peptidase superfamily	2.36
ygaU	b2665	predicted protein	2.33
yibQ	b3614	putative nucleoside (IDP) diphosphate	2.33
ytfJ	b4216	concerved protein	2.29
yfbV	b2295	concerved inner membrane protein	2.25
yadC	b0135	predicted fimbrial-like adhesion protein	2.24
ycgK	b1178	predicted protein	2.19
yfgG	b2504	predicted protein	2.19

APPENDIX 7A. Continued.

Gene Name	B Number	Function	Fold Change
ydcY	b1446	hypothetical protein	2.19
rutR	b1013	predicted DNA-binding transcriptional regulator	2.15
ycbW	b0946	predicted protein	2.12
ydhM	b1649	predicted DNA-binding transcriptional regulator	2.05
ybeA	b0636	concerved protein	2.04
yohN	b2107	predicted protein	2.02

APPENDIX 7B. Expression Ratios of Genes with a ≥2-fold Down Regulation 60-minutes After Addition of 4xMIC Streptomycin.

Gene Name	B Number	Function	Fold Change
Cell processes			
Adaptation			
ravA	b3746	regulatory ATPase of AAA+ family	-3.30
Cell division			
murF	b0086	D-alanyl:D-alanine adding enzyme; UDP-N-acetylmuramoyl-tripeptide:D-alanyl-D-alanine ligase	-2.54
Cell killing			
katG	b3942	catalase hydrogen peroxidase I	-4.02
Chemotaxis and mobility			
cheY	b1882	response regulator for chemotactic signal transduction	-8.36
cheW	b1887	signal transduction	-6.23
cheB	b1883	methylesterase	-5.85
tap	b1885	chemotaxis protein IV (aspartate receptor)	-5.29
cheR	b1884	methyltransferase	-4.43
cheA	b1888	autophosphorylating histidine sensor kinase of chemotactic system	-3.98
trg	b1421	methyl-accepting chemotaxis protein III (ribose acceptor); flagellar regulon	-3.15
tar	b1886	methyl-accepting chemotactic signal transducer protein II (aspartate receptor)	-2.46
tsr	b4355	methyl-accepting chemotaxis protein II (serine receptor)	-2.05
yhjH	b3525	suppresses motility defect of hns mutants in multicopy; in FlhDC flagellar regulon	-2.01
Transport/binding proteins			
malE	b4034	periplasmic maltose-binding protein; substrate recognition for transport & chemotaxis	-23.51
gatA	b2094	galactitol-specific enzyme IIA of phosphotransferase system (PTS)	-11.23
lamB	b4036	maltoporin (maltose high-affinity uptake system); phage lambda receptor	-10.74
rbsB	b3751	D-ribose periplasmic binding protein	-8.00
malK	b4035	maltose transport complex (ATP-binding subunit)	-7.58
gatB	b2093	galactitol-specific enzyme IIB of phosphotransferase system (PTS)	-4.66
focA	b0904	probable bidirectional formate transporter 1	-4.57
dcuA	b4138	anaerobic, C4-dicarboxylate transporter	-3.63
fliY	b1920	cystine-binding protein (may regulate FliA (σ28))	-3.44
malF	b4033	maltose transport complex	-3.42
dcuB	b4123	anaerobic, C4-dicarboxylate transporter	-3.35

APPENDIX 7B. Continued

Gene Name	B Number	Function	Fold Change
srlA	b2702	D-glucitol-specific enzyme II of phosphotransferase system (PTS)	-3.22
potD	b1123	spermidine-binding membrane protein; DNA binding protein regulates pot operon expression	-3.05
ygiS	b3020	function unknown	-2.69
ptsH	b2415	protein Hpr of the phosphotransferase system (PTS)	-2.58
atpD	b3732	membrane-bound ATP synthase subunit beta, F1 sector	-2.50
srlB	b2704	D-glucitol (sorbitol)-specific enzyme III of the phosphotransferase system (PTS)	-2.42
manZ	b1819	mannosephosphotransferase enzyme IIB	-2.35
ptsN	b3204	Pts gene in the rpoN operon	-2.19
manY	b1818	mannose phosphotransferase system (PTS), EIIC component	-2.16
mtlA	b3599	mannitol-specific enzyme II of phosphotransferase system (PTS)	-2.14
Extrachromosomal			
Transposon related			
insH_6	b1994	IS5 transposase and trans-activator	-2.30
Global regulators			
adiY	b4116	transcriptional activator of adiA	-4.21
bssR	b0836	regulator of biofilm formation	-3.50
yhbH	b3203	ribosome-associated factor (stationary phase); induced by AI-2 pheromone	-3.36
yphH	b2550	predicted DNA-binding transcriptional regulator (NAGC-like)	-2.62
Macromolecule metabolism			
Amino acyl tRNA synthesis; tRNA modificiation			
glyS	b3559	glycine--tRNA ligase, beta-subunit	-3.10
trpS	b3384	tryptophan--tRNA ligase	-2.91
lysS	b2890	constitutive lysine--tRNA ligase	-2.84
lysU	b4129	lysine--tRNA ligase (inducible)	-2.84
serS	b0893	serine--tRNA ligase	-2.41
selB	b3590	novel elongation factor forselenocysteine incorporation (specific for selenocysteyl tRNA)	-2.25
glyQ	b3560	glycine--tRNA ligase, alpha-subunit	-2.14

APPENDIX 7B. Continued

Gene Name	B Number	Function	Fold Change
selA	b3591	selenocysteine synthase; with SelD, converts serine residue to selenocysteine on tRNA	-2.06
Degradation of DNA			
amyA	b1927	α-amylase	-3.09
hsdR	b4350	host DNA restriction endonuclease R	-2.81
pepE	b4021	α-aspartyl dipeptidase	-2.23
dcp	b1538	dipeptidyl carboxypeptidase II	-2.01
Transcription			
rpoC	b3988	RNA polymerase, beta' subunit; binds Zn(II)	-2.05
Metabolism of small molecules			
2'-Deoxyribonucleotide metabolism			
gcvH	b2904	glycine cleavage; aminomethyl carrier	-6.88
gcvP	b2903	glycine dehydrogenase; decarboxilating	-6.23
aspA	b4139	L-Aspartate ammonia-lyase; L-aspartase	-5.73
gcvT	b2905	aminomethyl transferase (tetrahydrofolate dependent)	-4.58
talB	b0008	transaldolase B	-3.77
agp	b1002	periplasmic glucose-1-phosphatase	-3.35
glmS	b3729	glucosamine-6-phosphate synthase	-3.16
glcB	b2976	malate synthase G	-3.10
nrdA	b2234	ribonucleoside diphosphate reductase subunit B1	-3.10
tktA	b2935	transketolase; binds Zn(II)	-2.84
cpdB	b4213	2',3'-cyclic nucleotide 2'-phosphodiesterase	-2.61
gltB	b3212	glutamate synthase (large subunit)	-2.47
deoD	b4384	purine-nucleoside phosphorylase	-2.33
codA	b0337	cytosine deaminase	-2.28
ppk	b2501	polyphosphate kinase	-2.01
gssA	b2988	glutathionylspermidine synthase/amidase; bifunctional protein	-2.01

APPENDIX 7B. Continued

Gene Name	B Number	Function	Fold Change
Aerobic respiration			
frdA	b4154	fumarate reductase flavoprotein subunit	-8.43
frdB	b4153	fumarate reductase iron-sulfur protein subunit	-7.08
eno	b2779	enolase; phosphoprotein; component of RNA degradosome	-6.33
hybC	b2994	hydrogenase 2 [Ni Fe] large subunit	-6.29
nrfA	b4070	formate-dependent nitrite reduction; tetraheme cytochrome c552	-6.07
nuoE	b2285	NADH-ubiquinone oxidoreductase subunit E, complex I; NADH dehydrogenase I	-4.95
fdnG	b1474	formate dehydrogenase-N (major subunit)	-4.86
aceF	b0115	pyruvate dehydrogenase, dihydrolipoamide acetyltransferase component E2	-4.81
aceE	b0114	pyruvate dehydrogenase, decarboxylase component E1	-4.66
tpiA	b3919	triosephosphate isomerase	-4.65
hybB	b2995	hydrogenase 2 cytochrome b type component	-4.55
napA	b2206	nitrate reductase homolog	-3.91
pgk	b2926	phosphoglycerate kinase	-3.61
yfiD	b2579	glycine radical cofactor that reactivates pyruvate formate lyase after oxidative stress	-3.54
hypB	b2727	guanine-nucleotide-binding protein, required for metallocenter assembly in hydrogenases 1,2,3	-3.37
nuoC	b2286	NADH-ubiquinone oxidoreductase subunit C, complex I; NADH dehydrogenase I	-3.29
nuoG	b2283	NADH-ubiquinone oxidoreductase subunit G, complex I; NADH dehydrogenase I	-3.05
nuoF	b2284	NADH-ubiquinone oxidoreductase subunit F, complex I; NADH dehydrogenase I	-2.99
dmsB	b0895	DMSO reductase subunit B; apparent Fe-S binding domain	-2.85
ackA	b2296	acetate kinase (creBC regulon)	-2.80
fbaA	b2925	fructose-bisphosphate aldolase; binds Zn(II)	-2.76
nrfB	b4071	formate-dependent nitrite reduction; pentaheme cytochrome c formate-dependent	-2.61
hypA	b2726	hydrogenase 3 accessory protein required for activity	-2.32
pykA	b1854	pyruvate kinase II	-2.27
hypC	b2728	hydrogenase 3 chaperone-type protein; required for Hyd-3 metallocenter; binds HycE subunit	-2.23
pgi	b4025	glucosephosphate isomerase	-2.08
hybD	b2993	maturation endoprotease for Ni-containing hydrogenase 2	-2.07
fdnH	b1475	formate dehydrogenase-N Fe-S subunit	-2.02

APPENDIX 7B. Continued

Gene Name	B Number	Function	Fold Change
Amino acid biosynthesis (Alanine)			
glyA	b2551	serine hydroxymethyltransferase (binds Zn)	-3.02
cysK	b2414	cysteine synthase; O-acetylserine sulfhydrylase A	-2.17
Anaerobic respiration			
yhaS	b3114	2-ketobutyrate formate-lyase/pyruvate formate-lyase 4	-12.90
Biosynthesis of cofactors, carriers			
moaB	b0782	chlorate resistance protein B, MPT synthesis	-2.99
pdxH	b1638	pyridoxine-phosphate oxidase; isoniazid resistance	-2.55
moaE	b0785	molybdopterin (MPT) synthase (large subunit); chlorate resistance	-2.27
moaC	b0783	MPT synthesis; chlorate resistance protein C	-2.23
pdxK	b2418	vitamin B6 kinase	-2.05
Central intermediary metabolism			
aphA	b4055	subunit of acid phosphatase/phophotransferase	-2.34
dkgA	b3012	methylglyoxalreductase	-2.07
Degradation of small molecules (amines)			
tdcB	b3117	threonine dehydratase	-15.58
tnaA	b3708	tryptophanase	-11.71
ansB	b2957	L-Asparaginase II	-11.45
treC	b4239	trehalose-6-phosphate hydrolase, osmoprotectant	-10.17
malM	b4037	periplasmic protein	-8.56
gatD	b2091	galactitol-1-phosphate dehydrogenase	-6.78
tdcA	b3118	transcriptional activator of tdc operon	-6.78
malP	b3417	maltodextrin phosphorylase	-4.96
melA	b4119	α-galactosidase	-4.92
pta	b2297	phosphotransacetylase (creBC regulon)	-4.51
maoC	b1387	putative dehydrogenase adjacent to tynA/maoA gene	-4.43
tdcG	b3112	anaerobic pathway, L-serine deaminase, L-serine dehydratase	-4.27
lacZ	b0344	β-D-Galactosidase	-3.39
cadA	b4131	lysine decarboxylase	-2.89
tnaC	b3707	regulatory leader peptide for tna operon	-2.89

APPENDIX 7B. Continued

Gene Name	B Number	Function	Fold Change
rbsK	b3752	ribokinase	-2.87
malT	b3418	positive regulator for mal regulon; phage lambda sensitivity	-2.64
galT	b0758	galactose-1-phosphate uridylyltransferase (Rob regulon)	-2.43
tdcG	b3111	anaerobic pathway, L-serine deaminase, L-serine dehydratase	-2.39
galE	b0759	UDP-galactose 4-epimerase; hexose-1-phosphate uridylyltransferase	-2.26
atpG	b3733	membrane-bound ATP synthase subunit gamma, F1 sector	-2.20
atpH	b3735	membrane-bound ATP synthase subunit delta, F1 sector	-2.05
Purine ribonucleotide biosynthesis			
purA	b4177	adenylosuccinate synthase	-2.97
purB	b1131	adenylosuccinate lyase	-2.30
Structural elements			
Inner membrane			
flgE	b1076	flagella hook subunit	-19.93
flgF	b1077	flagella proximal rod subunit	-15.78
flgG	b1078	flagella distal rod subunit	-14.94
flgC	b1074	assembly of flagella hook	-14.42
flgB	b1073	flagellar basal body rod subunit	-13.19
flgD	b1075	flagellar basal body rod modification	-11.57
flgH	b1079	flagella L-ring protein	-8.97
flgJ	b1081	flagella muramidase	-8.38
fliS	b1925	FliC chaperone	-7.68
fliT	b1926	FliD chaperone	-6.96
fliM	b1945	flagella switch compnent; binds CheY-P	-6.76
fliH	b1940	negative regulator of FliI ATPase	-6.61
flgI	b1080	flagella P-ring protein	-6.37
fliG	b1939	flagella rotor component; binds MotA	-6.19
fliJ	b1942	flagella rod, hook, & filament chaperone	-6.16
fliF	b1938	flagella M-ring protein	-5.61
fliK	b1943	flagella hook length control	-5.34

APPENDIX 7B. Continued

Gene Name	B Number	Function	Fold Change
flgA	b1072	flagella synthesis	-4.77
fliI	b1941	flagella cytoplasmic membrane expore ATPase	-4.21
fliL	b1944	flagella synthesis	-4.13
fliN	b1946	flagella switch component	-3.69
ompC	b2215	outer membrane protein 1b (Ib, c)	-3.45
flgL	b1083	flagella hook associated protein	-3.30
yhiP	b3496	peptide POT transporter	-3.03
flhB	b1880	flagellar biosynthesis protein FlhB	-2.32
btuB	b3966	receptor for vitamin B12, E colicins, and phage BF23	-2.21
fimA	b4314	fimbrin type 1, major structural subunit; phase variation	-2.18
Outer membrane			
ompW	b1256	outer membrane protein; colicin S4 receprtor	-15.76
Miscellaneous/unclassified			
ydhV	b1673	predicted oxidoreductase	-5.80
gatZ	b2095	subunit required for full activity and stabilty of GatY tagatose bisphosphate aldolase	-5.52
ymdA	b1044	function unknown	-4.20
tdcF	b3113	predicted endoribonuclease	-3.80
yhdH	b3253	predicted NAD(P)-binding oxidoreductase	-3.48
chbG	b1733	conserved protein	-2.83
ybgE	b0735	function unknown; fourth gene in cydAB operon, expressed in minicells	-2.65
yjgZ	b4277	predicted KpLE2 phage-like element	-2.59
yecR	b1904	predicted protein	-2.44
yhbJ	b3205	predicted P-loop containing ATPase	-2.41
ykgG	b0308	function unknown	-2.37
yobF	b1824	predicted protein	-2.34
ygjR	b3087	predicted NAD(P)-binding dehydrogenase	-2.32
yhbV	b3159	predicted protein	-2.26
ykgF	b0307	function unknown	-2.25
yhbU	b3158	predicted peptidase	-2.24

APPENDIX 7B. Continued

Gene Name	B Number	Function	Fold Change
yjgF	b4243	conserved protein	-2.23
ydbK	b1378	putative pyruvate synthase	-2.19
tas	b2834	putative NAD(P)-linked reductase that works with starvation associated mutations	-2.08
yehT	b2125	function unknown	-2.06
yjdA	b4109	concerved protein with nucleoside triphosphate hydrolase domain	-2.06
yfcZ	b2343	conserved protein	-2.05
yehU	b2126	putative 2-component sensor protein	-2.04
ymdB	b1045	putative protein	-2.03