IMPACT OF CATALASES AND SUPEROXIDE DISMUTASES ON ANTIBIOTIC-INDUCED OXIDATIVE STRESS

IMPACT OF CATALASES AND SUPEROXIDE DISMUTASES ON ANTIBIOTIC-INDUCED OXIDATIVE STRESS

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CHARLIE JOYCE, B.Sc.

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AUTHOR: Charlie Joyce, B.Sc. (McMaster University)

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Abstract

This thesis is composed of two projects. The first project examined stochastic variation in gene expression in the RpoS regulon of *Escherichia coli*. This variation allows isogenic cells to develop different phenotypes. While this variation is often minimized, variation in stress gene expression may allow sub-populations of stress resistant cells to form within an isogenic population, increasing its fitness level. RpoS is the master stress regulator in *E. coli*, and so a great deal of variation in the expression of RpoS and its regulon may exist. A protocol was partly developed here for measuring GFP expression based on previous *lacZ* reporter protocols. Furthermore, construction of a plasmid-borne chromosomally-integratable *rpoS-mPlum* fusion, allowing determination of the expression of RpoS-dependent *gfp* fusions and *rpoS* itself, has begun with the intermediate plasmid pCJ2. These steps will allow a future student to complete this project.

The second project examined the effect of oxidative stress proteins against antibiotics. It was shown that bactericidal antibiotics produce cell death through an ROS-mediated pathway, regardless of their primary target. As the ROS that are generated are O_2^-, H_2O_2, and ·OH, superoxide dismutases (SOD) and catalases should have an effect on the lethality of these antibiotics. Catalase deletion strains were more sensitive to the majority of bactericidal antibiotics compared to the wild type. Hydroxylamine, a catalase inhibitor, and thiourea, a ·OH scavenger, both reduced this effect, making the wild type and deletion sensitivities more similar. The loss of SodA or SodB did not increase

sensitivity to antibiotics, while a double SOD deletion strain was more sensitive to antibiotics than the wild type. Mutants resistant to bactericidal antibiotics were isolated, and many of these mutants were cross-resistant to paraquat, showing that selection on antibiotics can result in oxidative stress resistance. Therefore ROS scavenging enzymes have an effect on antibiotic sensitivity.

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Part I. Noise in expression of the RpoS regulon

Chapter 1. Introduction

1.1 RpoS, the master stress response regulator

RpoS is a 38 kDa alternative sigma factor of RNA polymerase that regulates the expression of 10% of the *Escherichia coli* genome (Patten *et al.*, 2004). RpoS and its dependent genes are involved in the adaptation to stress conditions, such as hyperosmolarity (Hengge-Aronis *et al.*, 1993;McCann, Kidwell, and Matin, 1991), oxidative stress (Loewen and Triggs, 1984), near-UV light (Tuveson, 1980), ethanol (Farewell, Kvint, and Nystrom, 1998), acidity (Touati, Dassa, and Boquet, 1986), as well as stationary phase of growth (Lange and Hengge-Aronis, 1991), and is also involved in virulence (Fang *et al.*, 1992). RpoS is highly regulated by proteins and molecules that result from or respond to these stresses, and so RpoS is activated in these stress conditions or at the onset of stationary phase. RpoS can thus be considered the master stress response regulator in *E. coli*. Activation of RpoS by one of these stress conditions confers cross-protection to other stresses, e.g. entry into stationary phase also confers protection against oxidative stress (Hengge-Aronis, 2002b), and activation of RpoS induces a switch from growth-oriented to maintenance-oriented metabolism (Hengge-Aronis, 2002a). RpoS is present in many Proteobacterial species, including *Salmonella* spp., *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Yersinia pestis*, *Vibrio cholerae*, and *Legionella pneumophila* (for a review, see (Hengge-Aronis, 2002b;Hengge-Aronis,

2002a)). As it is believed that most bacteria spend the majority of their time in stationary phase, the study of RpoS is of interest to understand environmental bacterial populations, and in addition many of these RpoS-containing species are human pathogens. Interestingly, however, RpoS is not functional in many *E. coli* isolates, including in some pathogenic strains (Dong *et al.*, 2009), possibly due to the energetic cost of expressing it (discussed below).

The *E. coli* K-12 genome encodes seven sigma factors: RpoD, responsible for the expression of core metabolic genes; RpoN, responsible for expression of nitrogen utilization genes; RpoE, involved in response to elevated temperatures and oxidative stress; RpoF (FliA), responsible for flagella synthesis; RpoH, involved in heat shock; FecI, involved in iron transport; and RpoS (reviewed in (Ishihama, 2000)). There is insufficient core RNA polymerase in the cell for all sigma factors, and as a result the sigma factors compete for access to RNA polymerase (Jishage and Ishihama, 1995;Maeda, Fujita, and Ishihama, 2000). In exponential phase (when RpoS is uninduced) RpoD occupies the majority of the RNA polymerase through both a high concentration and high affinity to the polymerase (Jishage and Ishihama, 1995;Maeda, Fujita, and Ishihama, 2000). As the activation of RpoS causes it to occupy a significant fraction of the available core RNA polymerase, this event likely causes competition for core RNA polymerase with RpoD, reducing the expression of RpoD-dependent genes (Ishihama, 2000). RpoS-directed expression diverts a large amount of cellular resources away from growth and, in the absence of an external stress (e.g. induction upon entry into stationary phase), into areas that do not improve the fitness of the organism. Strains with

a wild type RpoS grow poorly on non-preferred carbon sources as compared to *rpoS* mutant strains, possibly as a result of competition for core RNA polymerase (Chen, Patten, and Schellhorn, 2004). Furthermore, wild type cultures will accumulate *rpoS* mutants, and so in prolonged incubation *rpoS* mutants will come to dominate a culture (Zambrano *et al.*, 1993). Thus it is clear from both natural isolates and laboratory environments that heterogeneity in RpoS status in a population is advantageous (discussed further below).

RpoS recognizes promoter sequences that are very similar to those of RpoD, and so there has been a great deal of experimentation to determine the key promoter features of a specifically RpoS-dependent gene. RpoS and RpoD recognize identical consensus -10 and -35 promoter sequences, which are the sequences that the sigma factor binds, and RpoD is able to transcribe RpoS promoter sequences *in vitro* (Ding *et al.*, 1995;Tanaka *et al.*, 1995). However, RpoS has greater tolerance of sequence variation at the -35 sequence and the length of the spacer sequence in between the two sigma factor binding hexamers (Gaal *et al.*, 2001;Typas and Hengge, 2006), RpoS promoters typically have a C at position -13, and have an A/T rich region downstream of the -10 sequence (Becker and Hengge-Aronis, 2001;Ojangu *et al.*, 2000). These differences allow the existence of a regulon that is primarily transcribed by RpoS, separate from RpoD-dependent genes.

1.2 Regulation of RpoS

As it responds to such a wide variety of conditions, RpoS must be heavily regulated to ensure that it expresses its target genes at the appropriate times. This regulation occurs at each of the transcriptional, translational, and post-translational levels

(Figure 1) (Dong, Joyce, and Schellhorn, 2008;Ishihama, 2000;Klauck, Typas, and Hengge, 2007). Transcription of *rpoS* occurs from three RpoD-dependent promoters. Two are upstream of the *nlpD* gene, itself immediately upstream of *rpoS*, and generate a polycistronic *nlpD-rpoS* mRNA. These promoters are not regulated, and contribute a low level of RpoS in the cell independent of growth phase or stress conditions (Lange and Hengge-Aronis, 1994b). The last promoter, within the *nlpD* open reading frame, is heavily regulated and is primarily responsible for any increase in RpoS transcription during stress or stationary phase. That promoter causes the transcription of the *rpoS* gene as well as a long 5' untranslated region which is responsible for much of the translational regulation of RpoS (Lange, Fischer, and Hengge-Aronis, 1995).

Transcription at this promoter is increased by the alarmone ppGpp, which increases as a result of amino acid limitation, polyphosphate, increased by nutrient limitation (Gentry *et al.*, 1993;Hirsch and Elliott, 2002;Kornberg, Rao, and Ault-Riche, 1999;Shiba *et al.*, 1997), and the two-component regulator BarA-UvrY through an unknown mechanism (Mukhopadhyay *et al.*, 2000). Transcription is kept at a basal level in exponential phase by Fis, a histone-like protein that is abundant in exponential phase (Hirsch and Elliott, 2005) as well as through ArcA, an integrator of oxygen availability and energy supply (Mika and Hengge, 2005). cAMP-CRP also represses transcription in exponentially growing cells but activates transcription of *rpoS* in stationary phase cells (Lange and Hengge-Aronis, 1991;Mika and Hengge, 2005). Therefore, the level of *rpoS* transcription is largely determined by the metabolic situation of the cell.

As a result of the long 5' untranslated region, a great deal of translational regulation can and does take place on the *rpoS* mRNA. The 5' UTR itself folds such that the translation initiation region is not accessible to the ribosome (Brown and Elliott, 1997), though upon entry into stationary phase (or through stress conditions) this secondary structure is believed to change into a translatable form (Lange and Hengge-Aronis, 1994b;Muffler, Fischer, and Hengge-Aronis, 1996). The RNA-binding protein Hfq, which facilitates RNA-RNA interactions, is essential for the increase in translation of *rpoS* that occurs during stress or stationary phase (Brown and Elliott, 1996;Muffler, Fischer, and Hengge-Aronis, 1996). DsrA, active in low temperatures, increases *rpoS* translation through Hfq (Sledjeski, Gupta, and Gottesman, 1996), as does RprA, stimulated through cell envelope stress (Majdalani *et al.*, 2001). These RNAs base-pair with the 5'UTR, exposing the translation initiation region, as do (potentially) other unknown small RNAs (Klauck, Typas, and Hengge, 2007). CspC and CspE, both cold-shock proteins, also stabilize *rpoS* mRNA (Phadtare and Inouye, 2001). In addition, DnaK, a heat shock protein, ppGpp, and DksA also increase *rpoS* translation (Klauck, Typas, and Hengge, 2007;Muffler *et al.*, 1997;Rockabrand *et al.*, 1998;Webb *et al.*, 1999). The histone-like protein HU increases translation of *rpoS*, possibly through modulation of the translation initiation region, and is induced in late exponential phase (Balandina *et al.*, 2001). Conversely, the oxyS RNA, activated in oxidative stress, represses *rpoS* translation in a Hfq-dependent manner (Zhang *et al.*, 1998), as does LrhA, a regulator of flagellar synthesis (Peterson *et al.*, 2006). LeuO, a DsrA inhibitor (Klauck, Bohringer, and Hengge-Aronis, 1997), and EIIA-Glc, involved in the

phosphoenolpyruvate:carbohydrate phosphotransferase system (Ueguchi, Misonou, and Mizuno, 2001), represses *rpoS* translation, and H-NS increases degradation of *rpoS* mRNA (Barth *et al.*, 1995;Yamashino, Ueguchi, and Mizuno, 1995). Therefore, high cell density and low temperature both induce RpoS expression at this level, and while the nature of the regulation is unknown, *rpoS* translation also increases in high osmolarity or a pH downshift (Klauck, Typas, and Hengge, 2007).

At the protein level, RpoS is regulated through proteolysis via the protease ClpXP (Schweder *et al.*, 1996). RpoS is targeted to the protease by RssB, which binds to RpoS at K173, exposing the ClpX recognition site (Becker, Klauck, and Hengge-Aronis, 1999;Klauck, Typas, and Hengge, 2007;Pratt and Silhavy, 1996), which is itself possibly activated through phosphorylation during exponential phase (Klauck, Typas, and Hengge, 2007) and whose expression is RpoS-dependent (Pruteanu and Hengge-Aronis, 2002). RssB itself may be regulated by a number of factors, serving as an integration point for a variety of signals to affect RpoS levels (Klauck, Typas, and Hengge, 2007). One such is the protein IraP which inhibits RssB (increasing RpoS levels) under phosphate starvation (Bougdour, Wickner, and Gottesman, 2006). H-NS is also involved in proteolysis through RssB, though the specific mechanism is unclear (Yamashino, Ueguchi, and Mizuno, 1995). In addition, the DnaK chaperone reduces proteolysis of RpoS in carbon starvation (Muffler *et al.*, 1997). Therefore, many stresses may also contribute to RpoS proteolysis.

Finally, the activity of RpoS (i.e. its access to RNA polymerase) is regulated by multiple factors as well. In stationary phase, the Crl protein promotes the formation of

RpoS-core polymerase holoenzyme (Typas *et al.*, 2007), while the regulator Rsd reduces the affinity of RpoD to core polymerase (Jishage *et al.*, 2002), and 6S RNA also binds RpoD and inhibits its expression (Wassarman and Storz, 2000). As there are more sigma factors than there are core RNA polymerase enzymes, any factor that reduces the affinity of RpoD for the core enzyme raises the activity of RpoS. Therefore, in stationary phase, several modulators act to ensure that RpoS can outcompete RpoD for core polymerase enzyme.

1.3 Stochastic noise in gene expression

Even in isogenic cells, variation exists in the overall amount of a particular protein in the cell due to differences in transcription and translation rate, ensuring that the environment of every cell is different. This variation, termed "noise," is defined as the standard deviation divided by the mean of the expression being examined (Elowitz *et al.*, 2002;Ozbudak *et al.*, 2002), and is a stochastic process. Stochastic variation in gene expression was first conclusively demonstrated using two fluorescent proteins (cyan fluorescent protein and yellow fluorescent protein) transcribed from identical promoters in equivalent locations on the chromosome of *E. coli* cells (Elowitz *et al.*, 2002). When visualized by fluorescence microscopy, a spectrum of both brightness and colour was seen in this population of isogenic cells. Different amounts of the two fluorescent proteins were in each cell, which can only be attributed to stochastic noise in expression. Noise has been implicated in the variation in fingerprints between identical twins, the loss of cell cycle synchrony over time in a yeast cell culture (Barkai and Leibler, 2000), and when exposed to UV-light, the different lysis/lysogeny decisions produced by identical λ

phages (Arkin, Ross, and McAdams, 1998). The wide variety of phenotypic examples shows that (i) there could be many more such examples that are simply unknown at this point in time; and (ii) gene expression noise fundamentally affects many processes. Therefore, it is important to understand in what situations noise has an important effect on phenotype, and how noise occurs in cells. This area has only recently come under study due to the development of reporters, such as GFP and its variants, that allow observation of single cells.

GFP, a 238 amino acid, 27 kDa protein isolated from the jellyfish *Aequorea victoria* (Prasher *et al.*, 1992;Shimomura, Johnson, and SAIGA, 1962;Shimomura, Johnson, and Morise, 1974), is a protein that, in the presence of light at either 390 nm (near-UV) or 475 nm (blue), and emits light at 505 nm (green) (Shimomura, Johnson, and Morise, 1974;Stearns, 1995). With the rise of fluorescence microscopy and other detection techniques, the advantages of GFP as an expression reporter, including being fluorescent in a wide variety of organisms, monomeric, highly stable due to its strong β-barrel shape, and non-toxic (for a review, see (Stearns, 1995;Tsien, 1998)), have made it very useful for expression studies. Perhaps its most important feature, however, is its fluorophore, derived from its own amino acid structure (Cody *et al.*, 1993). This removes the need to add a chromogenic substrate for assays, allowing assays of viable cells. In the wild type protein, the three amino acids in the fluorophore are serine, tyrosine, and glycine, which react in an oxygen-dependent manner to produce a dehydrated ring structure (Ormo *et al.*, 1996). Unfortunately, that same fluorophore is also a hindrance to real-time studies, as it requires approximately 2.7 h at room

temperature for half the wild type proteins to produce the ring structure (Heim, Prasher, and Tsien, 1994). Furthermore, wild type GFP is only weakly fluorescent, and thus difficult to detect; it is also non-fluorescent at temperatures above 30 °C. A great deal of optimization work with wild type GFP, primarily through successive rounds of mutagenesis and testing, has produced fluorescent proteins of a wide spectrum of fluorescent colours and with decreased folding time and improved thermal stability (for a review, see (Shaner, Steinbach, and Tsien, 2005)). Fluorescent proteins remain unsuitable for some applications, such as translational fusions where the resulting product is toxic, highly unstable, or sterically inhibits the original protein (e.g. (Thomas and Maule, 2000)), but in general these modifications to GFP have resulted in widespread use of these proteins as reporters of gene expression or protein localization, despite their detection often being more difficult (and less sensitive) than corresponding chemical reporter assays. Furthermore, the extended colour spectrum covered by these mutagenized proteins allow for multiple signals (i.e. multiple genes or multiple proteins) to be detected at once in a single cell, which conventional assays such as β-galactosidase assays cannot do. This in particular makes GFP and other fluorescent proteins desirable expression reporters for this project.

Noise itself is ubiquitous in gene expression: it occurs for all genes. For example, if the probability of RNA polymerase transcribing a target gene within a one minute time interval is 0.05, then after 1 min 5% of cells will have transcribed the gene. After 2 min 0.25% of cells will have transcribed the gene twice, 9.50% of cells will have transcribed the gene once and 90.75% of cells will not have transcribed the gene. After 3 min,

0.0125% of cells will have transcribed the gene three times, 0.7125% twice, 13.5375% once, and 85.7375% of cells will not have transcribed the gene at all. While there is relatively little variance in expression from cell to cell after two or three minutes, after 50 min or 100 min there is significant variation between the cells of the population. So long as the probability of transcription of the gene is not 0 or 1 in any given time interval, then there will always be variation in the expression level from one cell to the next, with differences in both mRNA content and, as a result, protein content. This can result in isogenic cells having divergent levels of a particular protein, ultimately producing different phenotypes. The amount of noise depends (i) inversely on the absolute copy number of the molecules to be interacting and (ii) directly on the volume in which they are to interact. It is estimated that there are approximately 2000 molecules of RNA polymerase in an *E. coli* cell (Ishihama, 2000;Maeda, Fujita, and Ishihama, 2000) and fewer than 2000 sigma factors. Once the volume of the cell, the number of sigma factors, and the low copy number of the target gene within the cell are taken into account, those numbers are low enough for significant noise in gene expression to occur (even when ignoring the need for cofactors, which can increase the occurrence of noise).

1.4 Mechanism and effects of noisy gene expression

Noise in gene expression can be separated into two components, based on their root cause: intrinsic noise and extrinsic noise (Elowitz *et al.*, 2002). Intrinsic noise is essentially any chance variation in the number of interactions between the components necessary for gene expression and is specific to each gene. The noise from the example in the preceding paragraph is thus intrinsic noise. Extrinsic noise is the intrinsic noise of

those components themselves, and is specific to each cell. In another example, two idealized cells with identical levels of RNA polymerase, identical numbers of active transcription factors, and identical copy numbers of the gene of interest will have variation in the expression of that gene of interest as a result of chance fluctuations in microconcentration of these components and chance differences in the success of initiating transcription when all the components meet. This is intrinsic noise. If this gene encodes a transcription factor, then the expression of genes regulated by that transcription factor will be different between cells as a result of the difference in the transcription factor's own expression. This produces extrinsic noise, and is the same for all genes regulated by that transcription factor (though, of course, intrinsic noise is still present – the same factors involved in producing intrinsic noise above also produces it here). Therefore, the origin of noise can be separated into chance variation events acting on the direct precursors of expression and chance events on the precursors of those precursors. These two origins of noise can be isolated experimentally: in the two fluorescent protein system from above, extrinsic variation produces changes in brightness but not colour, while intrinsic variation produces changes in colour and in brightness (Elowitz *et al.*, 2002). This technique has since become a cornerstone of estimating gene expression noise (for example, see (Maamar, Raj, and Dubnau, 2007)).

The level of a given gene's expression influences the noise level of that gene, as found in a study in *B. subtilis* (Ozbudak *et al.*, 2002). The gene for a fluorescent protein was mutated such that several promoters were created, with different transcriptional efficiencies, and several ribosome binding sites were created with different translational

efficiencies, each determined through average fluorescence of the fluorescence protein from that strain. The noise depended primarily on the efficiency of translation, with transcription efficiency (i.e. number of transcripts produced per promoter activation event) being a relatively small factor (Ozbudak *et al.*, 2002). A gene with high transcriptional efficiency (one that is transcribed frequently when its promoter is active) and low translational efficiency (few proteins made per mRNA molecule) will have little noise in its expression, whereas the opposite case (low transcriptional efficiency and high translational efficiency) will have a great deal of noise in its expression (Ozbudak *et al.*, 2002). This is once again due to the effect of absolute copy number. A gene that has a high transcriptional efficiency maximizes the number of mRNA transcripts produced, and an mRNA with a low translational efficiency minimizes the number of proteins per mRNA transcript (and, thus, noise per mRNA transcript). By having a large number of mRNA molecules and producing few proteins per molecule, any noise lessens as a proportion of the overall expression. When it is absolutely essential to have a given product at a particular concentration, genes can be regulated such that the level of noise is minimized.

Other regulatory network patterns can amplify or minimize noise, as well. Regulatory networks that have a positive autofeedback component tend to generate very significant differences in expression level between cells due to variation in promoter activation, itself directly resulting from noise. Slightly higher expression in one cell than another results in a higher promoter activation frequency (binding of transcription factors to the promoter, allowing gene expression to occur) and expression compared to the other

cell, which results in an even greater difference in subsequent expression, and so on, encouraging deviation from the mean expression level. ComK, the protein responsible for competence in *B. subtilis*, positively regulates its own expression, resulting in very noisy expression of the protein (and, consequently, resulting in a small proportion of cells meeting the threshold for induction of competence) (Maamar, Raj, and Dubnau, 2007). Conversely, regulatory networks containing negative feedback tend to minimize noise, as these discourage deviation from the mean expression level.

Yet some genes are regulated in a manner that generates greater noise. For example, the gene encoding adenylate cyclase (*cya*) has both low transcriptional efficiency and high translational efficiency, as does *tetR*, a tetracycline resistance gene, and *malT*, a regulator of the maltose operon (reviewed in (Ozbudak *et al.*, 2002)). As mentioned above, the regulation of *comK* encourages noise in its expression. Why might it be favourable for some genes to have noisy expression? In some cases, the result of this noise is trivial, as in the variation in each human's fingerprints, and so perhaps there is little selective pressure to minimize this noise. In others, such as in core metabolic pathways, genes must be expressed at a particular level to ensure the proper fidelity of the pathway, and so a lack of noise is beneficial. However, noise in some pathways might be beneficial. Noise can ultimately divide a population of isogenic cells into subpopulations, each with high expression of a particular set of genes (for example, the RpoS regulon) thus broadening the range of conditions in which some component of the population can survive, ultimately enhancing the fitness of the population (Raser and O'Shea, 2005). As an added benefit, every cell need not express the full range of genes to the level necessary

for resistance, conserving the energy that would be expended on that expression. In support of this concept, a study in the yeast *Saccharomyces cerevisiae* found that stress genes tend to be expressed in a noisier fashion than genes involved in rRNA processing, proteasome, and ergosterol synthesis (Bar-Even *et al.*, 2006). The noise in these stress genes resulted in increased resistance to particular challenges, such as pH and UV light. In addition, the previously-mentioned induction of competence in *B. subtilis* through noisy gene expression ensures that a small proportion of cells (~15%) is capable of taking up DNA from the environment, which may ultimately provide the genetic content necessary for survival through some future situation (Maamar, Raj, and Dubnau, 2007), while the majority of cells do not have to incur the metabolic disadvantage of producing the proteins of the pathway. In both cases, the overall fitness of the isogenic population is enhanced while minimizing the amount of energy expended in increase that fitness level.

1.5 Project objectives

RpoS, though not essential for growth in optimal conditions, is a critical component for survival during some stresses. RpoS is activated in these conditions and activates a large regulon of genes to ultimately protect against the stress that is present. However, it has become increasingly clear that the expression of non-essential genes is noisy. Assays of pathways for noise in gene expression have been done in *S. cerevisiae* (Bar-Even *et al.*, 2006), showing that stress genes in particular are noisy, though no such studies have been done on an entire bacterial regulon. The global objective of this project is to determine whether the expression of the RpoS regulon is also quite noisy, either at

the level of RpoS itself or at the level of its dependent genes (or both). The first short-term objective of this project is to examine the expression of *rpoS*- and RpoS-dependent *gfp* fusion strains at the cellular level to determine whether their expression is noisy. If there is a substantial amount of noise in RpoS-dependent genes, then the second short-term objective of this project is to examine whether the noise in RpoS is transferred to its regulon (i.e. does noise cause some cells to be extremely well-adapted to all stresses and others to be extremely poorly-adapted to all stresses (noise from RpoS itself transferring to the genes), or does that noise vary from one RpoS-dependent gene to another in a cell (noise primarily from RpoS-dependent genes)). This would be done by constructing a plasmid vector, pCJ1, which contains a chromosomally-integratable *rpoS-mPlum* fusion, allowing simultaneous examination of *rpoS* expression and RpoS-dependent gene expression (when transformed into an RpoS-dependent *gfp* fusion strain). The final short-term objective is to determine whether this noise has a positive or negative effect on cell survival through these stresses by simultaneously examining expression and viability of cells incubated in those stresses. This proposed study would be the first examination of gene expression noise throughout an entire regulon. A flowchart to illustrate the steps of this project is shown as Figure 2.

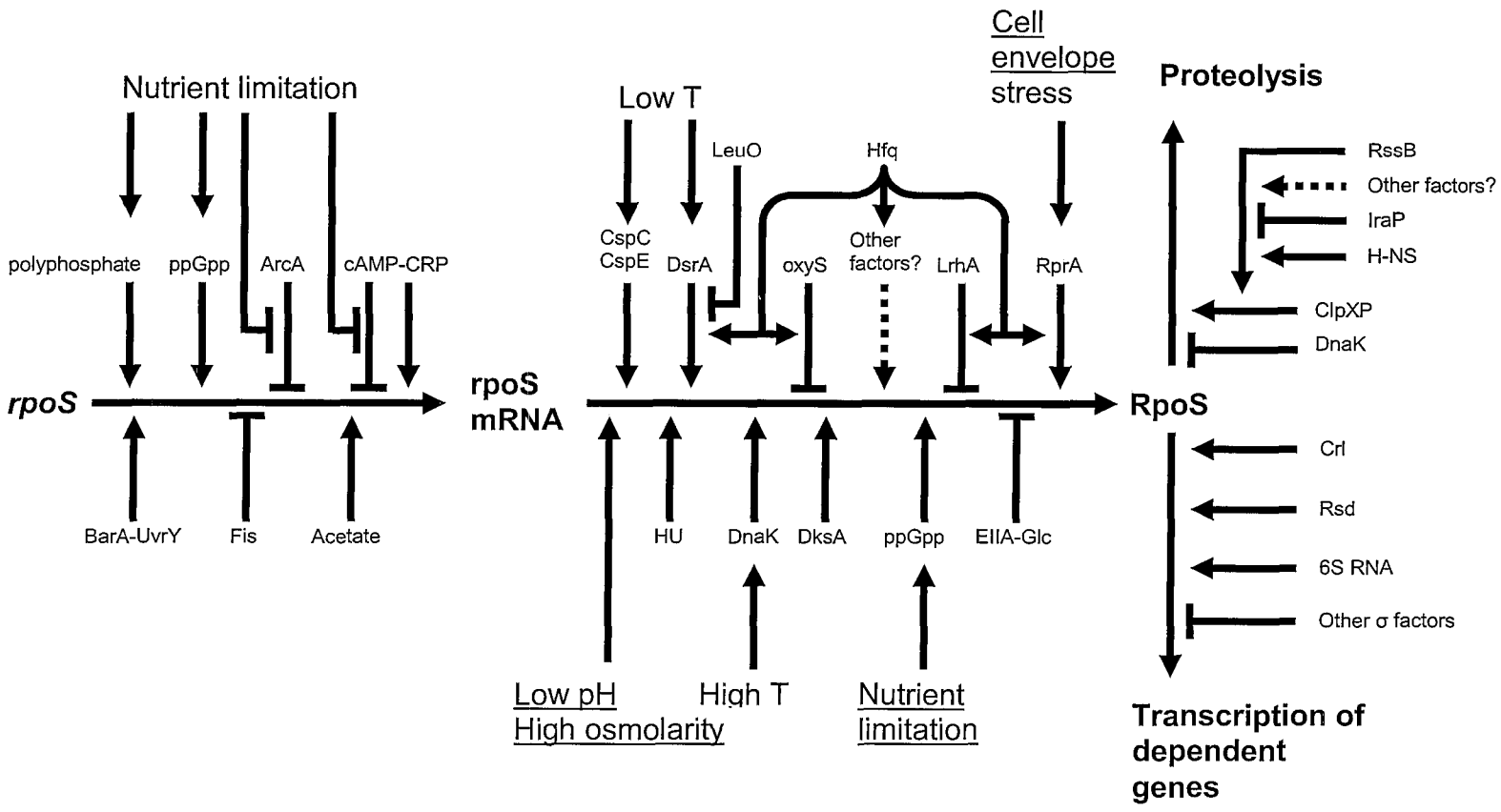


Figure 1. Regulation of RpoS. RNA and proteins that act on RpoS are shown in normal font, while conditions that result in changes in RpoS levels are shown underlined. While unknown factors may be involved at any point, "other factors" signifies areas that are the most likely to have other RNA or proteins involved beyond those currently known.

Table 1. RpoS-dependent *gfp* fusion strains.

		Fold dependence*				Fold dependence*	
		Stationary				Stationary	
Strain	Gene	Phase	RpoS	Strain	Gene	Phase	RpoS
HS1002f	*yjbJ*	12	22	HS1063f	*ugpE*	17	6
HS1006f	*yjbE*	10	22	HS1064f	*yicM*	5	3
HS1008f	*aldB*	18	22	HS1065f	*ygaY*	3	1
HS1010f	*gabP*	22	24	HS1066f	*argH-oxyR*	2	2
HS1011f	*ygaU*	10	4	HS1067f	*ybiO*	5	13
HS1012f	*ydgI*	2	7	HS1070f	*thiA*	6	1
HS1014f	*katE*	6	40	HS1071f	*yhjG*	12	17
HS1019f	*yqhE*	12	8	HS1072f	*argH*	4	2
HS1020f	*mltB*	7	2	HS1073f	*yliI*	8	17
HS1022f	*narY*	8	10	HS1074f	*trkA*	7	5
HS1024f	*ygaF*	16	3	HS1075f	*ugpC*	10	20
HS1026f	*ybaY*	8	15	HS1077f	*yhiV*	48	31
HS1028f	*yjgR*	5	6	HS1078f	*uspB*	7	4
HS1033f	*ydaM*	4	16	HS1079f	*ldcC*	6	7
HS1035f	*ydcS*	3	6	HS1080f	*appB*	42	25
HS1036f	*ydcK*	66	15	HS1081f	*yhiU*	19	39
HS1037f	*gabT*	16	8	HS1082f	*aidB*	16	36
HS1039f	*ygaE*	85	14	HS1084f	*yehX*	16	28
HS1042f	*yhjY*	6	15	HS1090f	*yphA*	11	24
HS1045f	*yhiN*	4	5	HS1091f	*osmY*	17	35
HS1049f	*yeaG*	4	13	HS1092f	*yfcG*	19	37
HS1050f	*phnP*	3	8	HS1094f	*otsA*	19	32
HS1054f	*yebF*	13	41	HS1095f	*ecnB*	10	23
HS1055f	*ybaZ*	15	9	HS1099f	*yhjD*	8	5
HS1056f	*yodC*	23	41	HS1100f	*yjiN*	8	4
HS1057f	*gabD*	53	16	HS143f	*rpoS*	30	N/A
HS1059f	*talA*	32	20	MG1655f	*lacZ*	N/A	N/A
HS1061f	*aroM*	36	15				

Note: Fold dependence was previously determined through β-galactosidase assays prior to construction of these strains (Lange and Hengge-Aronis, 1994a;Vijayakumar *et al.*, 2004). Stationary phase dependence was determined by dividing expression at OD_{600} = 1.5 (stationary phase) by that at OD_{600} = 0.3 (exponential phase). RpoS dependence was determined by dividing wild type expression by that in an *rpoS* mutant strain.

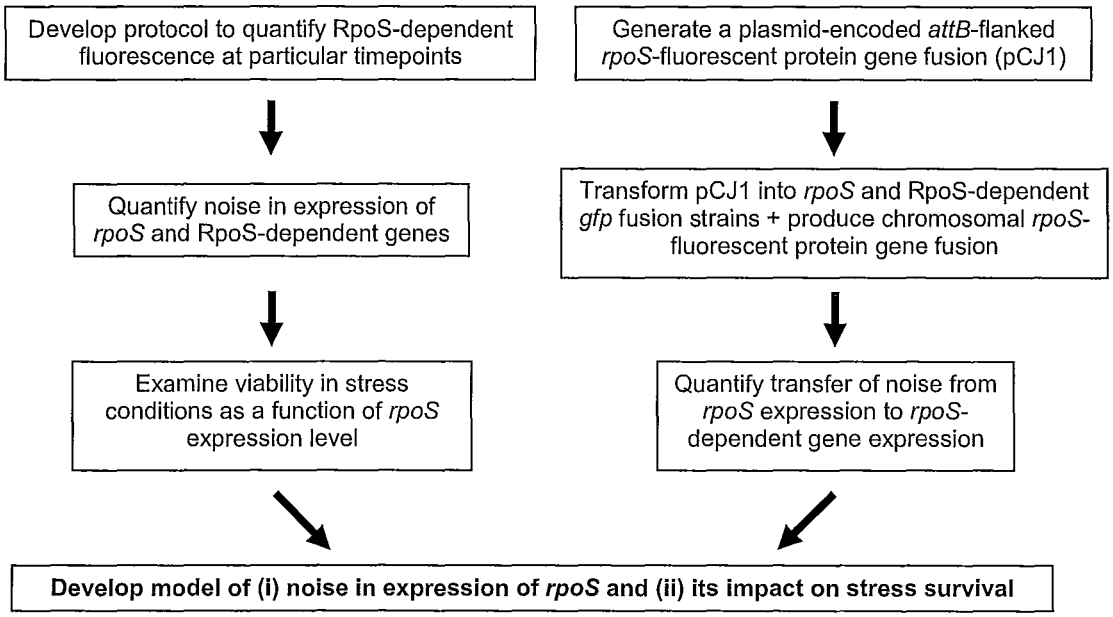


Figure 2. Flowchart of experiments for determination of noise in gene expression. The experiments described below relate to the top two items of the flowchart.

Chapter 2. Materials and methods

2.1 Growth conditions and strain construction

Strains used in this project to date are shown in Table 2. The strains necessary for this project were constructed previously (Joyce, 2006;Schellhorn *et al.*, 1998). This consisted of construction of a collection of RpoS-dependent *gfp* gene fusion strains (48 in total), an *rpoS-gfp* fusion strain, and a *lacZ::gfp* fusion strain (Table 1), and was done using an existing *lacZ* fusion collection already present within the lab (Schellhorn *et al.*, 1998;Vijayakumar *et al.*, 2004) and the pMarMar4 *lacZ::gfp* reporter switching system (Goulian and van der Woude, 2006). All strains were initially grown in triplicate overnight in LB broth + kanamycin (50 μg/ml) and incubated at 37°C and 200 rpm. Cultures were then diluted the following day and incubated at 37°C and 200 rpm, and were maintained in exponential phase for at least 8 generations prior to experimentation. Growth was monitored using either a Shimadzu UV-1201 or a ThermoLabsystems Multiskan Spectrum spectrophotometer. Exponential phase samples were taken at OD_{600} = 0.3 and stationary phase samples were taken at OD_{600} = 1.5 unless otherwise noted.

2.2 Construction of plasmid vector pCJ1

The following restriction enzymes (Fermentas, Burlington, ON) were used, with restriction sequences and cut location in parentheses: Eco81I (CC'TNAGG); SalI (G'TCGAC); NdeI (CA'TATG); HindIII (A'AGCTT); PstI (CTGCA'G); and SacI (GAGCT'C). Primers used to amplify desired DNA fragments are shown in Table 3.

Restriction digestions were done at 37°C for 1 h if the substrate DNA was a plasmid and overnight if the substrate DNA was a linear fragment to maximize the number of digested fragments. Non-specific cutting was not observed for any of the enzymes. Restriction enzyme buffers (Red, Orange, Green, or Tango - Fermentas) were selected for optimal activity of the restriction enzyme. 3 units of shrimp alkaline phosphatase (Fermentas) were added directly to the restriction enzyme reaction where indicated. Once complete, digestions were purified using an MN Nucleospin Extract II kit (Clontech, Mountain View, CA) to remove the restriction buffer. Ligation reactions were done at room temperature overnight with T4 DNA ligase (Fermentas), and were checked on 1% agarose gels. As needed, ligation mixtures were gel-extracted using an MN Nucleospin Extract II kit (Clontech) to isolate the desired ligation fragments. Plasmids were isolated using an MN Nucleospin Plasmid kit (Clontech). The desired plasmid is shown below as Figure 3. The 5' *attB* sequence is 377 nt long, the 3' *attB* sequence is 564 nt long, the *nlpD* sequence is 1404 nt long (including the primary *rpoS* promoter and complete 5' untranslated region), and the *mPlum* gene sequence is 702 nt long. Plasmids were transformed into BW19851, a pir^+ host strain, for storage using either the TSS-dependent method (Chung, Niemela, and Miller, 1989) or the simple and efficient method (Inoue, Nojima, and Okayama, 1990).

2.3 Fluorescence detection

Strains for fluorescence microscopy were grown to either exponential (OD_{600} = 0.3) or stationary phase (OD_{600} = 1.5). These strains were then incubated in chloramphenicol for 3 h to stop protein synthesis and allow the GFP fluorescence to

develop. Samples were taken and washed twice in PBS, then 2 µl was added to a slide for fluorescence microscopy. Microscopy was done using the Leica DMI 6000B widefield deconvolution microscope (Leica, Wetzlar, Germany) in the McMaster Biophotonics Facility (MBF), and GFP was excited at 488 nm and detected at 520 nm. Collected images were analyzed using ImageJ software.

Strains for fluorescence detection in a TECAN Safire plate reader (Mannedorf, Switzerland) were grown overnight in triplicate in LB media as above and subcultured. Cultures were grown in LB to the desired OD_{600}, then samples were taken and washed in PBS three times and 200 µl were added to a 96 well plate. GFP was excited at 488 nm and emission was detected at 520 nm, and OD_{600} was read concurrently. Fluorescence data was normalized to OD_{600}.

Strains for flow cytometry analysis were grown in triplicate overnight and subcultured as above. These were then grown to either exponential or stationary phase, then were incubated in chloramphenicol for 3 h to stop protein synthesis and allow for the GFP fluorescence to develop. Samples were taken, washed in PBS, and diluted to approximately 10^6 CFU/ml. Samples were read by Nicole McFarlane using the Beckman Coulter EPICS Altra flow cytometer (Beckman Coulter, Fullerton, CA) in the MBF. Samples were excited at 488 nm.

Table 2. *E. coli* strains used in this study.

Strain	Genotype	Source
GC4468	*lacU169 rpsL* (K-12)	(Carlioz and Touati, 1986)
HS1033	GC4468 *ydaM-lacZ*	(Vijayakumar *et al.*, 2004)
HS1091	GC4468 *osmY-lacZ*	(Vijayakumar *et al.*, 2004)
HS143f	GC4468 *rpoS-gfp*	(Joyce, 2006)
HS1014f	GC4468 *katE-lacZ*	(Joyce, 2006)
HS1033f	GC4468 *ydaM-lacZ*	(Joyce, 2006)
HS1072f	GC4468 *argH-gfp*	(Joyce, 2006)
HS1091f	GC4468 *osmY-gfp*	(Joyce, 2006)
MG1655	*ilvG-rfb50 rpH1* (K-12)	(Blattner *et al.*, 1997)
MG1655f	MG1655 *lacZ-gfp*	(Joyce, 2006)

Table 3. Primers used in pCJ1 construction.

Primer	Target DNA sequence	Primer sequence (5' to 3')
nlpDF	*nlpD* gene + *rpoS* promoter with 5' SalI site	TTAAGTCGACTTTATTATCGATACCGTGG
nlpDR	*nlpD* gene + *rpoS* promoter with 3' NdeI site	CTGACTCCATATGGTGACTCCTACCGTGAT
plumF	*mPlum* gene with 5' NdeI site	CGTATACATATGGTGAGCAAGGGCGA
plumR	*mPlum* with 3' SalI site	GCCGTCGACTTAATGATGATGATGATGATG
attBF	*attB* with 5' Eco81I site	TATACCTTAGGCCACCATCAAGGGAAA
attBR	*attB* with 3' SacI site	ATATGAGCTCCGGATGCGCCAACC
3attBF	3'*attB* with 5' SalI site	TATAGTCGACCGTTGATCGGGCGGGGTT
5attBR	5'*attB* with 3' SalI site	TATAGTCGACCGCGCCTGGTACCTCTT

Note: restriction enzyme sites are underlined in the primer sequence. All restriction enzyme sequences and sequences upstream of them are not originally present in and do not bind to the DNA to be amplified

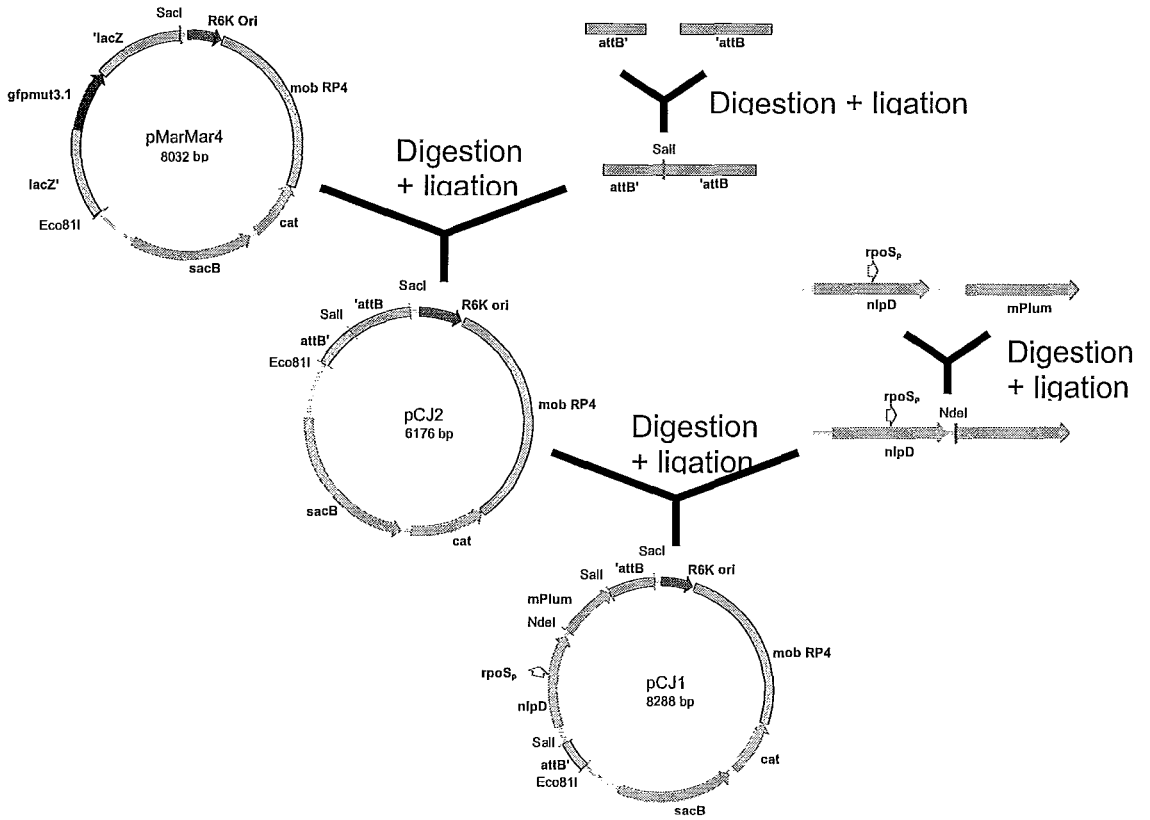


Figure 3. Strategy for the construction of the vector pCJ1. The *attB* fragment, which provides a chromosomal integration site, is to be ligated into restriction-digested pMarMar4, generating the intermediate vector pCJ2. The *nlpD* and *mPlum* sequences, the former including the *rpoS* promoter and the latter encoding the desired fluorescent protein, will then be digested and ligated into restriction-digested pCJ2, producing the desired conjugation vector pCJ1.

Chapter 3. Results

3.1 Construction of plasmid vector pCJ1

The parental plasmid is pMarMar4, with a *pir*-dependent origin of replication, the *cat* gene (Cm^R), and the *sacB* gene, conferring sensitivity to sucrose. This plasmid was selected to transfer the *rpoS- mPlum* gene fusion into any recipient strain's chromosome as it cannot replicate once inside the cell, meaning a recombination event with the chromosome must occur to maintain chloramphenicol resistance. pMarMar4 contains a *lacZ-gfp-lacZ* sequence which was to be removed and replaced with the desired *nlpD-mPlum* fusion flanked by *attB* sequences.

The plasmid can then be conjugated into desired recipient cells, and then either the plasmid must integrate at the *attB* site or be lost, and the integration can be detected through both mPlum fluorescence and resistance to chloramphenicol. Once integrated, the strain will have two equivalent *rpoS* promoters, one driving the expression of *mPlum*, and one driving the expression of either the *rpoS* ORF itself or that of another FP (such as GFP).

The strategy to generate the plasmid pCJ1 involves the cloning of an intermediate plasmid pCJ2. To construct pCJ2, the two desired *attB* fragments were amplified by PCR using primers containing SalI sites, then digested by SalI and ligated together (Figure 4), and a gel extraction was done to isolate the desired product (product II). This ligated *attB* fragment was amplified by PCR, then both pMarMar4 and the *attB* fragment were sequentially digested with Eco81I and ligated (producing a linear fragment containing the entire pMarMar4 plasmid and the *attB* fragment), then digested with SacI and ligated

again, thus removing the *lacZ-gfp-lacZ* fragment of pMarMar4, replacing it with the *attB* fragment, and recircularizing the plasmid. This was then transformed into *E. coli* BW19851 using the simple and efficient method (SEM) (Inoue, Nojima, and Okayama, 1990), along with undigested pMarMar4 into BW19851 in parallel as a control to determine the transformation efficiency. This transformation protocol was used as opposed to the standard TSS-dependent method (Chung, Niemela, and Miller, 1989) used in this laboratory, as the BW19851 strain transformed poorly using the TSS-dependent method (10^2 transformants per μg DNA) and the SEM had a transformation efficiency of 6.7 x 10^5 transformants per μg DNA in *E. coli* BW19851, a 10^3-fold improvement. The ligation mix putatively containing pCJ2 produced 310 transformants in strain BW19851, and sixteen of these were purity streaked and their plasmids were isolated. These transformant plasmids were digested with PstI and HindIII, expected to cut pCJ2 once and twice, respectively, and compared to pMarMar4 cut by the same enzymes, and a sample result is shown as Figure 5. Using the PstI digestions, the parental plasmid pMarMar4 was approximately 8 kb, and the transformant plasmids were approximately 6 kb, consistent with the predicted size of pCJ2. The HindIII restriction profile also matches that predicted for pCJ2, and so these transformant plasmids were considered to be pCJ2.

The next step was to clone the *nlpD-mPlum* fusion into pCJ2, producing pCJ1. The *nlpD* fragment, containing the *rpoS* promoter and *rpoS* 5' untranslated region, were amplified by PCR along with the *mPlum* gene, as done for the *attB* fragments above. These were then digested with NdeI and ligated together (Figure 6), and the desired

product (approximately 2.2 kb – product I) was gel extracted (approximately 10-15 ng). The *rpoS-mPlum* fusion and the pCJ2 plasmid were then cut with SalI, the plasmid was treated with shrimp alkaline phosphatase, and the two were mixed in a ligation reaction overnight. The ligation mixture was then transformed into *E. coli* BW19851 using the SEM method. This process yielded thousands of transformants. Sixteen of these transformants were then purity streaked and grown in LB media, and their plasmids were isolated. These isolated plasmids were then digested with PstI (cutting once) to determine their size. Unfortunately, the transformant plasmid sizes were consistent with pCJ2 (approximately 6 kb) rather than pCJ1 (approximately 8 kb) (Figure 7. Therefore, no successful transformants of pCJ1 were isolated.

3.2 Fluorescence detection

The use of *gfp* fusion strains and fluorescence microscopy allow determination of the expression level of individual cells, in turn allowing estimates for both mean expression and its variation, which is the goal of this project. However, viewing these strains by fluorescent microscopy at the desired time points, exponential phase (OD_{600} = 0.3) and stationary phase (OD_{600} = 1.5), has proven challenging thus far, as they are not significantly fluorescent at either time point. Standard procedure for determining gene expression of the isogenic *lacZ* fusion strains involves stopping protein synthesis with chloramphenicol until testing, and it was hypothesized that these *gfp*-fusion strains could be similarly incubated in chloramphenicol, stopping protein synthesis but allowing GFP maturation. MG1655f (*lacZ-gfp*) and HS1091f (*osmY-gfp*) were grown overnight, sub-cultured, and sampled at each of exponential phase, stationary phase, and after overnight

growth, as is done for the corresponding RpoS-dependent *lacZ* fusion strains in this laboratory, then incubated in chloramphenicol (150 μg/ml) for 3 h. HS1033 (*ydaM-lacZ gfp*$^-$) was grown and sampled similarly and is used as a negative control. Strain MG1655f contains an IPTG-inducible *gfp* fusion, and it was induced with 0.5 mM IPTG 30 min prior to stationary phase sampling. Using this method, only MG1655f is fluorescent at stationary phase (though even this increase is difficult to detect – Figure 8). The cells in these images were then analyzed for fluorescence intensity using the program ImageJ (Table 4). HS1091f, containing a highly-expressed RpoS-dependent *gfp* fusion, was not significantly more fluorescent than the *gfp*$^-$ strain HS1033 in stationary phase, though based on *lacZ* fusion data there should be a great deal of GFP fluorescence in this strain at that time point. Interestingly, that strain did show a significant amount of fluorescence after overnight growth, as did MG1655f.

Flow cytometry, much like fluorescence microscopy, examines each individual cell, and its greater sensitivity to weakly-fluorescent objects was used as a potential alternative to fluorescence microscopy. MG1655f (*lacZ-gfp*) contains a *gfp* fusion that can be expressed much more strongly than the RpoS-dependent fusions, and its fluorescence was used to determine whether flow cytometry can be used to examine GFP fluorescence in *E. coli*. MG1655f was grown in LB with 0.5 mM IPTG, to induce GFP expression, examined, then compared to the non-fluorescent strain HS1091 (Figure 9). Though there was some difficulty examining bacterial cells with the instrument due to their small size, few objects were seen in the buffer (PBS) alone that were of similar size to the bacteria, and so all objects viewed in these experiments are considered to be the

bacteria of interest. There was a great deal of fluorescence in MG1655f (10b), significantly more than that of HS1091 (10a). Therefore, bacteria were detectable and the fluorescence of this GFP is sufficiently strong to be seen through flow cytometry.

The highly-expressed RpoS-dependent *gfp* fusion strain HS1091f (*osmY-gfp*) was then grown in LB media and sampled at exponential phase, stationary phase, and after overnight growth, had growth stopped with chloramphenicol (150 μg/ml), and resuspended in PBS (Figure 10). The *gfp*$^-$ control strain HS1091 was also grown in LB and sampled at stationary phase. The exponential phase sample of HS1091f (11b) was equivalent to that of the *gfp*$^-$ strain (11a), as expected given that the *osmY* promoter is RpoS-dependent, while the stationary phase sample of HS1091f (11c) was only slightly more fluorescent than the *gfp*$^-$ strain. The overnight sample of HS1091f (11d), in contrast, was significantly more fluorescent. In summary, then, there was very little difference observed between exponential phase HS1091f and stationary phase HS1091f, despite the fact that there is a known 17-fold induction in *osmY* expression from exponential phase to stationary phase in LB media (Vijayakumar *et al.*, 2004), while there is clear induction of fluorescence after overnight growth.

Finally, *rpoS* itself is expressed sooner than RpoS-dependent genes. Therefore, though the expression of an RpoS-dependent gene was not truly detectable in stationary phase samples, the expression of *rpoS* itself might be, and was examined using the *rpoS-gfp* fusion strain HS143f (Figure 11). Interestingly, the fluorescence of the stationary phase samples, in which *rpoS* has already been expressed, was significantly higher than that of the exponential phase samples or the *gfp*$^-$ control. Therefore, while expression of

RpoS-dependent genes was not distinctly detectable through flow cytometry, the expression of *rpoS* itself was. Thus there may be a problem with the maturation of the GFP protein, such that either it is not fluorescent so soon after its expression or it is not fluorescent in chloramphenicol.

A different approach was therefore undertaken. That GFP is not immediately fluorescent may indicate that any GFP-based fluorescence is actually the result of expression at a previous time point. A real-time comparison of GFP and OD_{600} values for well-defined strains, then, could establish which time points for GFP expression are equivalent to exponential phase (OD_{600} = 0.3) and stationary phase (OD_{600} = 1.5) β-galactosidase data, and would result in a protocol for determining GFP expression in a robust and reproducible fashion. Fluorimetry was employed for these real-time comparisons, a technology that cannot ultimately be used for the end goals of this project, as fluorimeters cannot determine fluorescence at the cellular level, but useful for this optimization step. In a preliminary experiment to achieve this aim, samples of the RpoS-dependent *gfp* fusion strains HS1014f (*katE-gfp*), HS1033f (*ydaM-gfp*), and HS1091f (*osmY-gfp*) were taken periodically and assayed for both OD_{600} and fluorescence, as was the control strain HS1091 (*osmY-lacZ gfp*$^-$) (Figure 12). This experiment included a highly expressed gene (*osmY*), a moderately expressed gene (*katE*), and a weakly expressed gene (*ydaM*), and significant, sustained fluorescence was only detected from HS1091f. The fluorescence of this strain began to increase at OD_{600} = 1.05 and steadily increased through to overnight growth. Using *lacZ* expression data, expression of *osmY* actually begins at approximately OD_{600} = 0.4 (Schellhorn *et al.*, 1998), so there is an

apparent delay between actual induction and development of fluorescence of approximately 1.5 generations, or approximately 40 min, based on this data.

The above experiment, however, had relatively few time points, and so a follow-up experiment was then done to expand on these results. Strains HS1091f (*osmY-gfp*) and its *gfp*⁻ parental strain HS1091 were inoculated into cultures in triplicate at four twenty minute intervals, then sampled periodically and assayed for fluorescence as above. The advantage of this experiment is that more time points are examined with the same sampling frequency as done previously. This experiment agrees with the previous one in that the induction of GFP fluorescence began at approximately $OD_{600} = 1.1$ (Figure 13), with fluorescence intensity rising steadily through the rest of the experiment. Therefore, this experiment also suggests that there is a delay of approximately 40 min between induction of GFP expression and development of fluorescence.

To obtain more precise results on the time difference between actual GFP expression and GFP fluorescence, follow-up experiments were done as a kinetic assay using a PerkinElmer Envision fluorimeter, capable of both incubation and assaying of cultures. Strains HS1033f (*ydaM-lacZ*), HS1072f (*argH-gfp*), HS1091f (*osmY-gfp*) and MG1655f (*lacZ-gfp*), as well as the *gfp*⁻ strains HS1033 and MG1655 as controls, were grown to $OD_{600} = 0.3$ using standard methods, then incubated in the fluorimeter at 37 °C and 600 rpm and scanned frequently (every 4 min). These experiments were designed to view the development of fluorescence throughout stationary phase in the RpoS-dependent *gfp* fusion strains which can then be compared to previously-published *lacZ* data, giving the ideal time points for sampling of the *gfp* fusion strains. An initial experiment was

done over the course of 5 h (Figure 14), but by the 2 h time point it was found that the generation time was too long (approximately 1 h) to complete the assay properly. Therefore, a second assay was scheduled for 10 h to accommodate this generation time. Unfortunately, the extended incubation did not result in significant detectable growth, possibly as a result of the evaporation of much of the medium by the end of the experiment, as an estimated 50 to 100 µl remained in each well from the original 200 µl (data not shown). Consequently, no induction of fluorescence was visible, and the best estimates of the lag between GFP expression and fluorescence remain from the Tecan SAFIRE fluorimeter experiments.

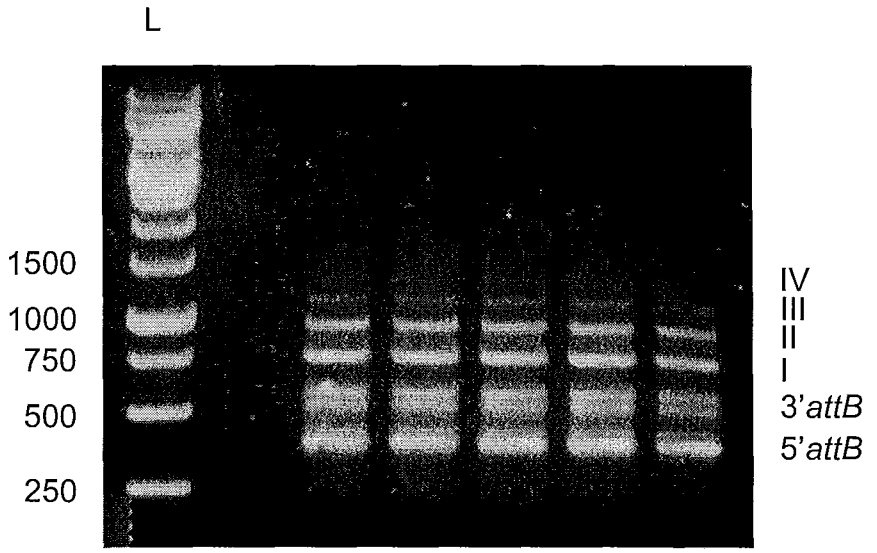


Figure 4. Ligation of 5'*attB* and 3'*attB*. The left lane is the ladder, and all five other lanes contain the same *attB* ligation mixture. At left are the sizes of the ladder fragments, in bp, while at right the original 5'*attB* and 3'*attB* fragments are labeled, as well as the ligation products that were observed. Based on size, product I corresponds to *attB'-attB'* ligation product; product II corresponds to the desired *attB'-'attB* ligation product; product III corresponds to the '*attB-'attB* ligation product; and product IV is a multiple ligation product (more than two fragments).

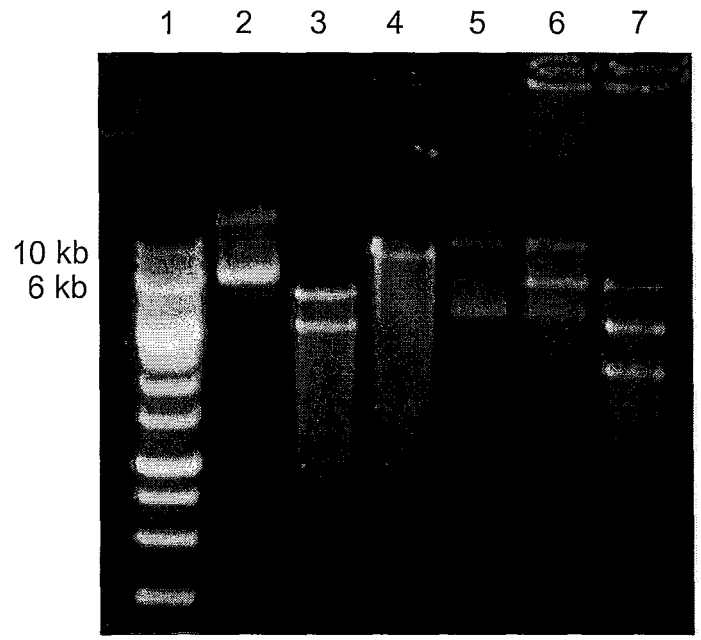


Figure 5. Digestion of putative pCJ2. Lane 1 is the DNA ladder; lanes 2 through 4 are pMarMar4 uncut (2), cut twice with HindIII (3) and cut once with PstI (4); lanes 5 through 7 are putative pCJ2 uncut (5), cut once with PstI (6), and cut twice with HindIII (7). Digestions of the putative pCJ2 were incomplete resulting in some uncut plasmid in lane 6 and some plasmid cut only once in lane 7.

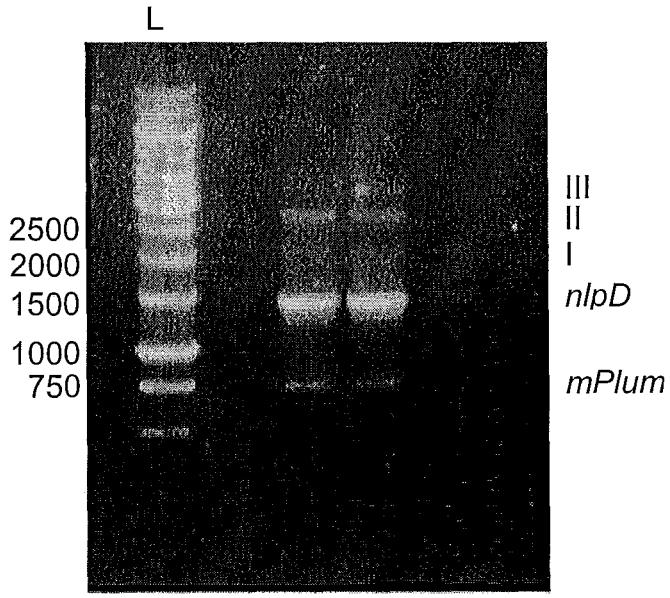


Figure 6. Ligation of *nlpD* and *mPlum*. The left lane is the ladder, and the other two lanes contain the same *nlpD mPlum* ligation mixture. At left are the sizes of the ladder fragments, in bp, while at right the original *nlpD* and *mPlum* fragments are labeled, as well as the ligation products that are visible. Based on size, product I corresponds to the desired *nlpD-mPlum* ligation product; product II corresponds to an *nlpD-nlpD* ligation product; and product III is a multiple ligation product (more than two fragments).

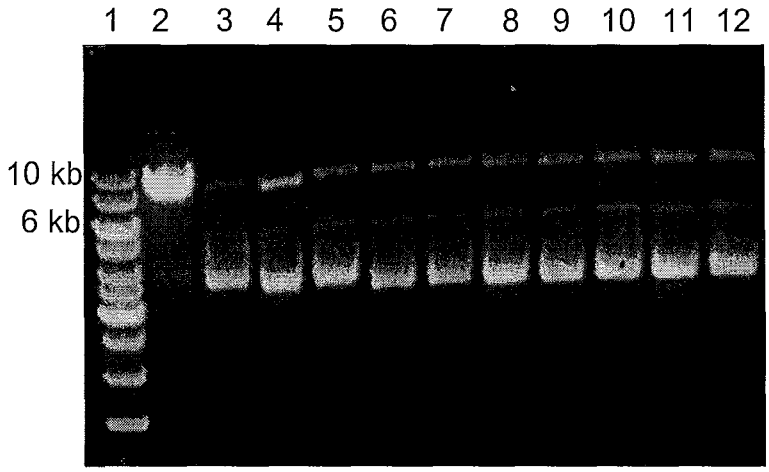


Figure 7. Putative pCJ2 and pCJ1 plasmids isolated from transformants. Plasmids were cut with PstI (cuts once). Lane 1 is the DNA ladder; lane 2 is pMarMar4 (8 kb); lanes 3 through 5 are putative pCJ2 (5.5-6 kb); lanes 6 through 12 are putative pCJ1 (5.5-6 kb). Plasmid digestion of pCJ2 and putative transformant plasmids were incomplete and for these, the middle band represents the cut plasmid.

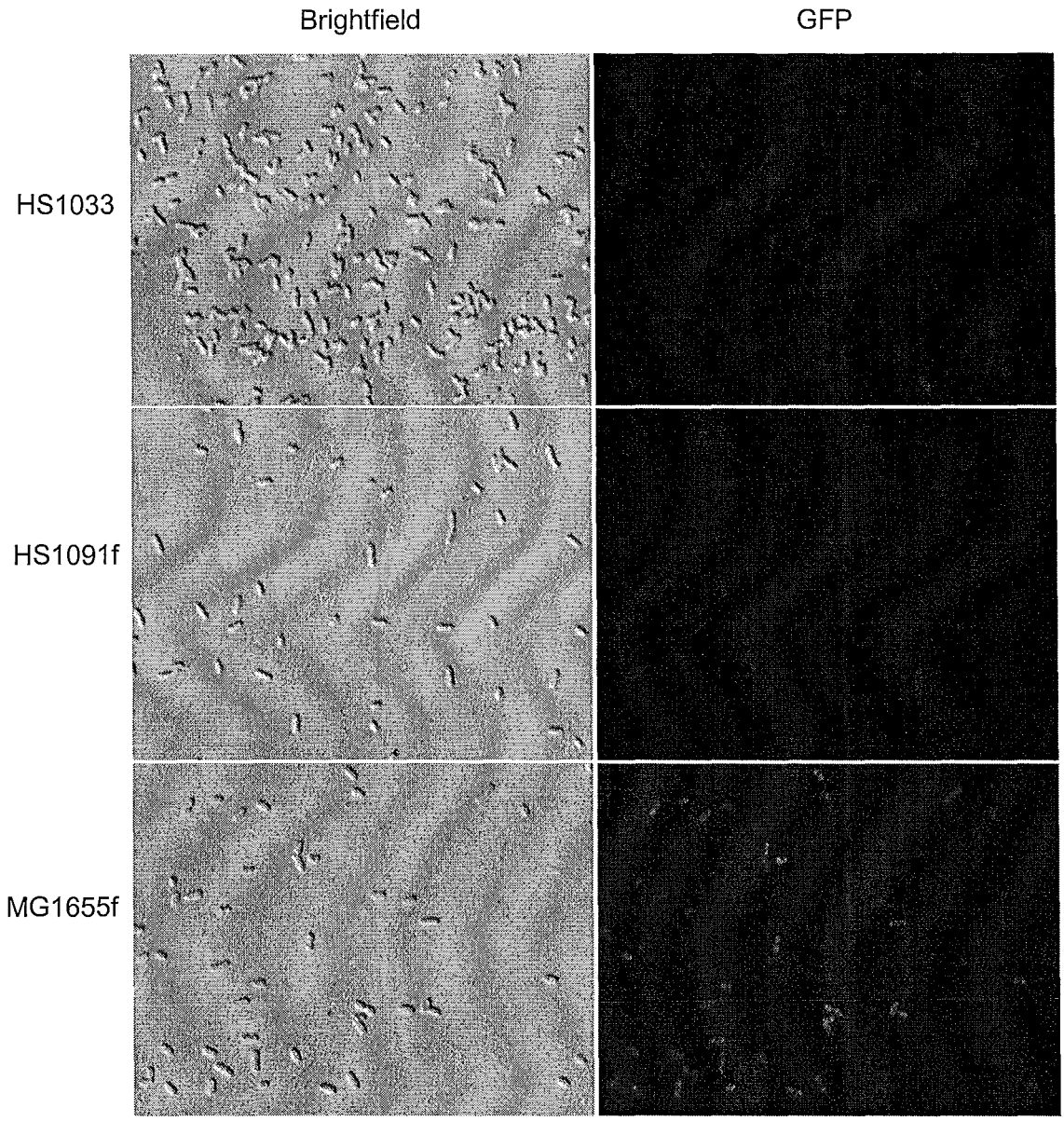


Figure 8. Sample brightfield and GFP images using fluorescence microscopy. The HS1033 (gfp-) strain and the HS1091f (osmY-gfp) strain both were essentially non-fluorescent, while some fluorescence is detectable from the MG1655f (*lacZ-gfp*) strain. Shown are stationary phase samples.

Table 4. Fluorescence level of cells of selected strains by fluorescence microscopy.

		Exponential Phase			Stationary Phase			Overnight		
Strain	Genotype	N	Area	GFP	N	Area	GFP	N	Area	GFP
HS1033	*gfp-*	61	4.43	733.0±29.9	123	1.90	179.9±4.3	231	1.14	202.5±4.9
HS1091f	*osmY-gfp*	8	5.74	1290.9±275.8*	92	2.23	199.3±6.5	649	1.21	344.5±5.4*
MG1655f	*lacZ-gfp*	24	4.87	659.2±28.7*	31	2.37	424.7±24.3*	522	1.05	382.5±6.3*

Note: Area values are given in μm^2. N is the number of cells that were identified as cells by the ImageJ macro in four representative images – cells may have been excluded from analysis if their length:width ratio was abnormal, were in contact with many other cells, or abnormally small or large. GFP values are given as an integrated density (mean fluorescence intensity x area) and are presented as a mean value ± SE. * indicates a value that is significantly different from the *gfp*⁻ strain HS1033 in the same growth phase ($P = 0.05$). Exponential phase cells were commonly observed to have greater background fluorescence, as observed here.

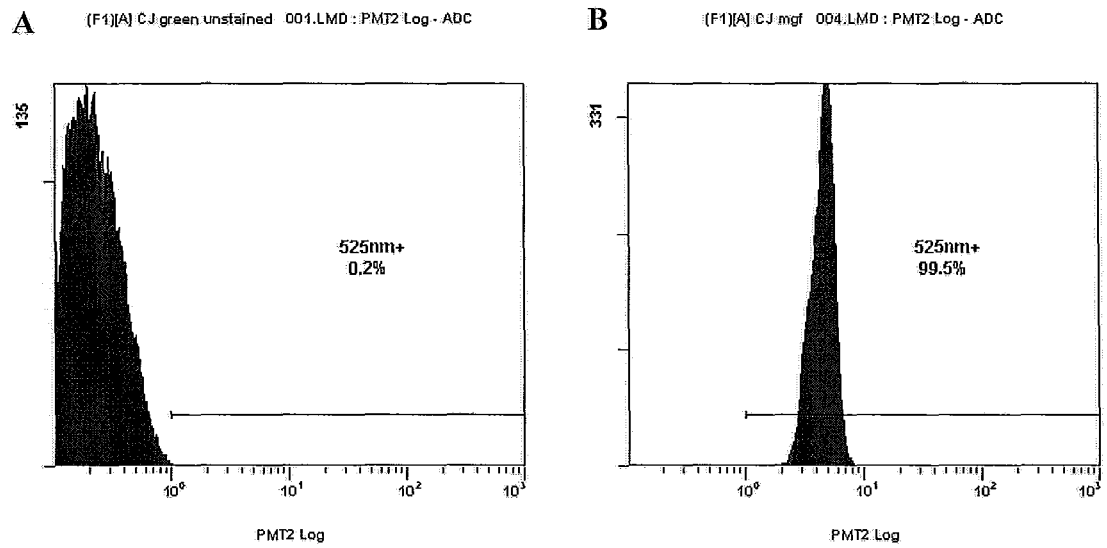


Figure 9. GFP fluorescence of a *gfp*⁻ strain (A) and MG1655f (*lacZ-gfp*) (B) as detected by flow cytometry. The X-axis indicates fluorescence intensity, while the Y-axis indicates the number of cells at that intensity. The horizontal bar within each graph is a reference for the maximum intensity seen in the non-fluorescent control sample, and the percentage is the number of cells more fluorescent than that threshold. Cell size was monitored independently to ensure that the size and internal scatter of light is consistent with other bacterial samples.

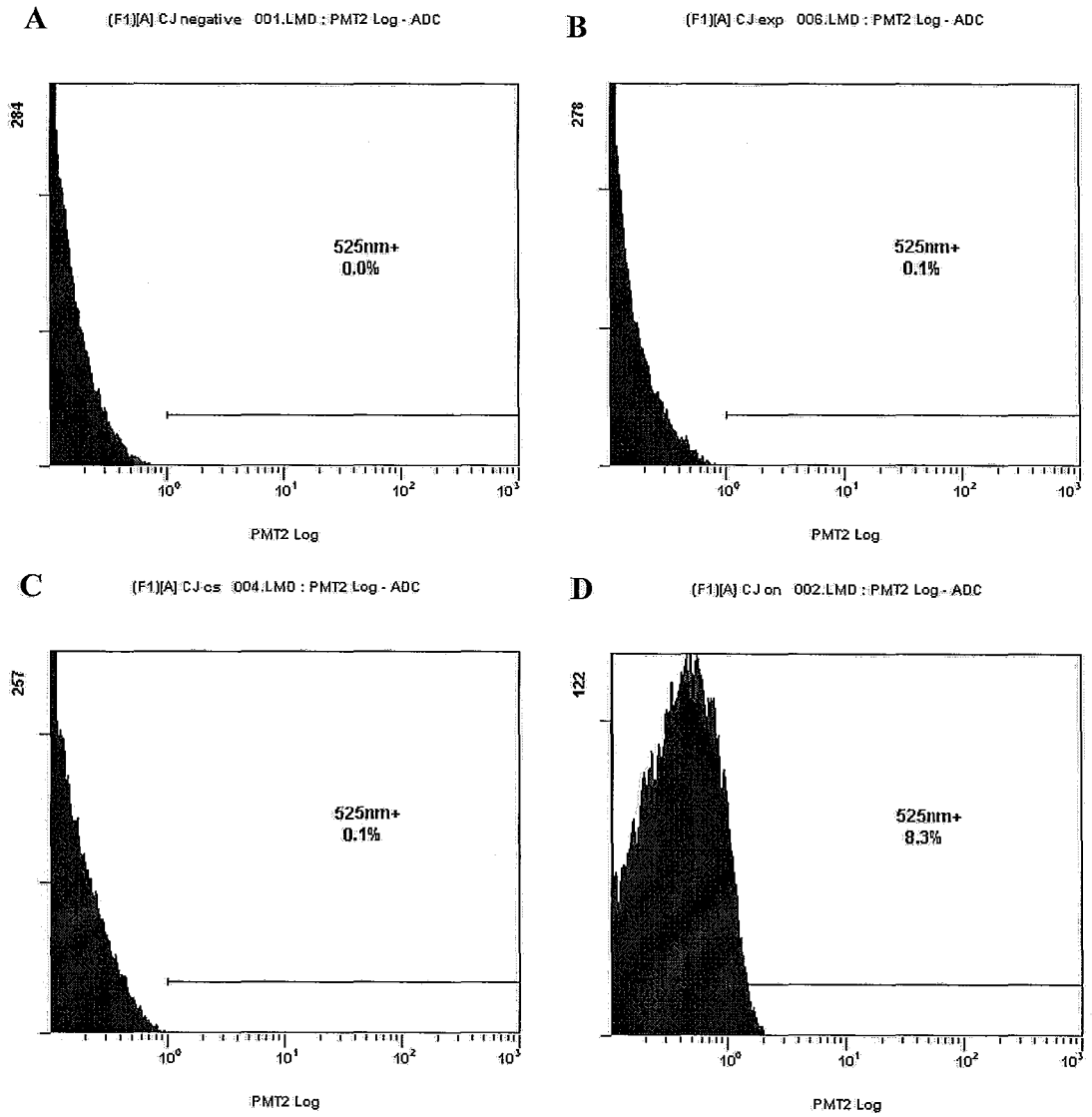


Figure 10. Fluorescence of a *gfp*⁻ strain (A) and HS1091f (*osmY-gfp*) (B-D), as detected by flow cytometry. Shown are exponential phase (B), stationary phase (A, C), and overnight (D) samples. All axes and graph features are identical with the previous figure. Cell size was monitored to ensure that it is consistent with other bacterial samples.

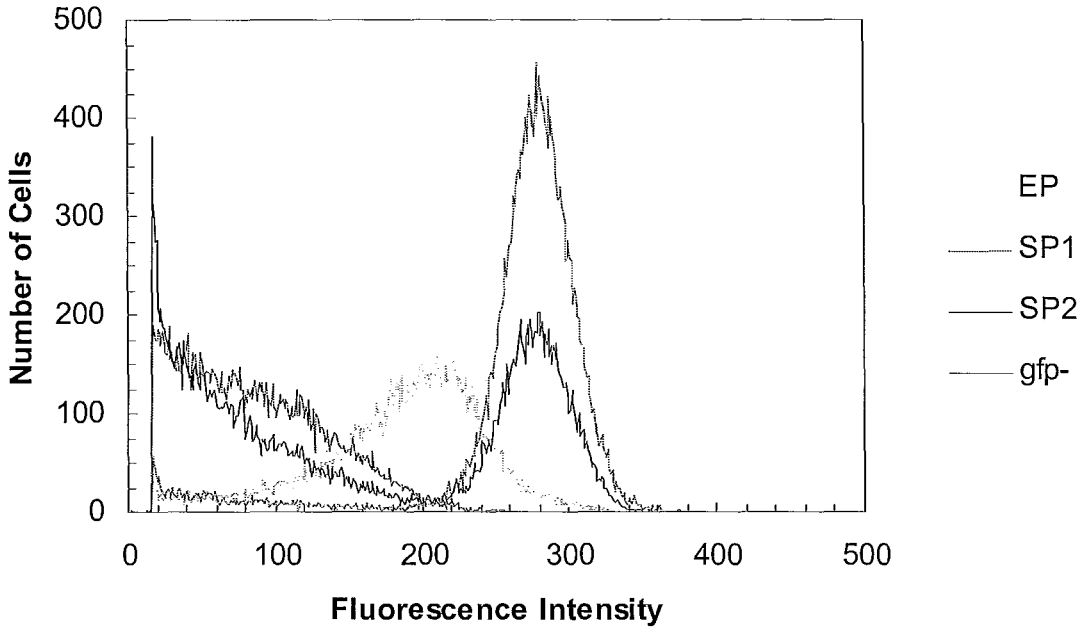


Figure 11. Fluorescence of the *rpoS-gfp* strain HS143f, as detected by flow cytometry. The *gfp*$^-$ strain HS1033 (Neg) and strain HS143f in exponential (EP) and stationary (SP1 and SP2) phase are shown. Two replicates are shown for the stationary phase samples.

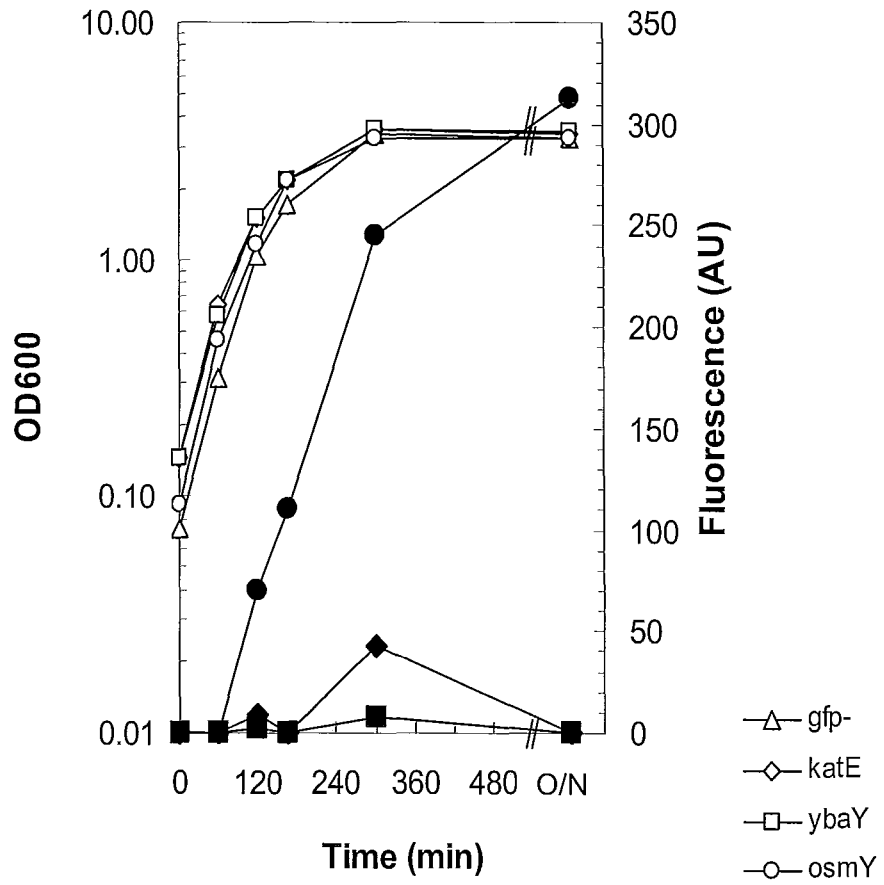


Figure 12. Development of GFP fluorescence over time. The genes listed in the legend are those whose promoter *gfp* is fused to. Growth is shown by the open markers, and fluorescence by the filled markers. Each point is the mean of three replicates. OD_{600} values were measured on a Shimadzu 1021 spectrophotometer, while fluorescence values were read on a Tecan SAFIRE reader and are presented in arbitrary units (raw fluorescence per OD_{600} unit).

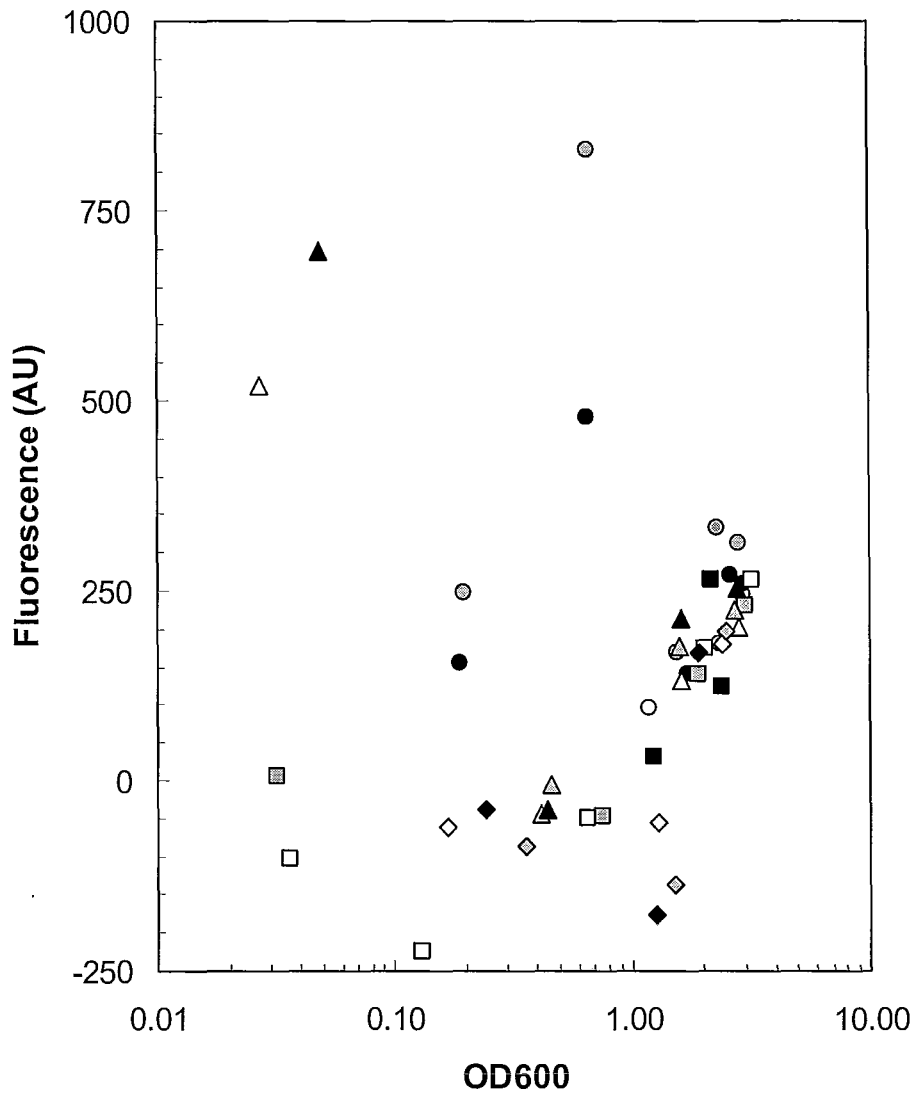


Figure 13. GFP expression in HS1091f as a function of growth phase. Circles indicate cultures inoculated initially, squares indicate cultures inoculated 20 min later, triangles indicate cultures inoculated 40 min later, and diamonds indicate cultures inoculated 60 min later than the initial cultures. White, grey, and black indicate the three replicates of each time point. Fluorescence began to increase at approximately $OD_{600} = 1.1$ and increased rapidly from that point forwards.

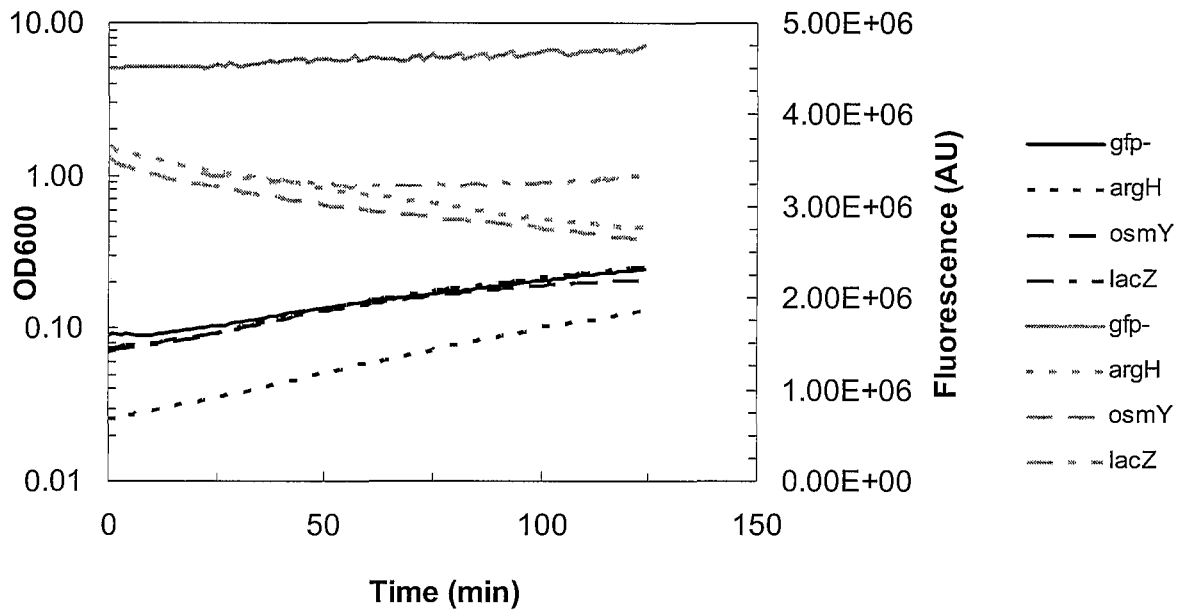


Figure 14. GFP kinetic assay using the PerkinElmer Envision plate reader. Grey lines indicate fluorescence data, while black lines indicate OD_{600} data. Genes listed in the legend refer to the gene that *gfp* was fused to. Fluorescence is reported in arbitrary units (AU; raw fluorescence / OD_{600}).

Chapter 4. Discussion

4.1 Construction of plasmid vector pCJ1

The desired vector pCJ1 has not yet been constructed due to the difficulties described above. The intermediate vector pCJ2 has been produced from the parent pMarMar4 plasmid, and the successful construction of this intermediate suggests that construction of pCJ1 should follow quickly by another student. A significant problem during construction was the transformation into the *pir*$^{+}$ host strain BW19851, as it did not transform well by standard methods in the lab. It readily transformed using the SEM method (Inoue, Nojima, and Okayama, 1990), however, and so this difficulty has been eliminated in the construction of pCJ1.

The pCJ2 plasmid has potential beyond its use in this project, as it can serve as a general, *attB*-specific chromosomal recombination vector. Any DNA sequence could be cloned in between the *attB* fragments, and conjugation into a *pir*$^{-}$ strain (such as nearly all common laboratory strains of *E. coli*) would result in a chromosomally-integrated copy of the DNA sequence of interest. Furthermore, while mPlum was the fluorescent protein chosen for this project due to the presence of proper microscope filter sets at the MBF and its separation from the absorption/emission spectrum of GFP, other fluorescence proteins may also be effective in place of mPlum. The flow cytometer at the MBF does not excite mPlum, and so another fluorescent protein may be more useful in this case. However, once the initial plasmid vector is created with mPlum, switching fluorescent proteins within the vector would be relatively simple, merely a matter of digesting out

mPlum and ligating in the sequence for the other fluorescent protein. Therefore, there is potential for the plasmid pCJ2 other than that specifically mentioned in this project.

After transformation of *E. coli* BW19851 with the pCJ1 ligation mixture, there was a difficulty in isolating the desired plasmid pCJ1 from the transformants, with all transformants containing the empty vector pCJ2. The pCJ1 plasmid is constructed by cutting pCJ2 once with SalI to insert the *nlpD-mPlum* fusion. It was expected that some transformants would contain the empty vector pCJ2 as, if the restriction enzyme is not 100% efficient, uncut pCJ2 plasmids remain in the mixture and confound any subsequent transformation. Gel extracting the SalI-cut pCJ2 plasmid, prior to ligation with the *nlpD-mPlum* ligated fragment, might remove a great deal of the uncut pCJ2 from the reaction mixture, improving the proportion of pCJ1 in a subsequent transformation. A longer restriction digestion, possibly overnight digestion, may also reduce the amount of uncut pCJ2 remaining. Either of these approaches may solve the current difficulty in constructing pCJ1 for a future student.

4.2 Fluorescence detection

Two routes were pursued for detecting the fluorescence from the RpoS-dependent GFP fusion strains, with neither successful. First, no useful images were obtained using fluorescence microscopy, and there are two possible explanations for this. One possibility is that the overall GFP signal is too diffuse. The GFP protein is distributed throughout the cell, and so small or moderate changes in expression of GFP from cell to cell may produce essentially identical cells, due to this difference being distributed throughout the cell's volume. Therefore, if the GFP were tagged to a cell component,

such as the plasma membrane, modest changes in fluorescence may then be detectable. Inserting a localization sequence to the membrane could be done in the original conjugation vector pMarMar4 (containing the *gfp* gene already), then conjugated into the recipient strains to produce a new set of membrane-localized RpoS-dependent *gfp* fusion strains, localizing the GFP and concentrating the fluorescence signal at one part of the cell. However, it is also possible that this tagging process may interfere with the proper maturation of GFP.

The second possibility is that the GFP might not be fully mature at the time of sampling. Strains that express GFP sooner than the RpoS-dependent *gfp* fusion strains, such as MG1655f (*lacZ-gfp*) and HS143f (*rpoS-gfp*) are clearly fluorescent using the techniques attempted here, with GFP expression induced in exponential phase for both strains, rather than the late exponential phase / early stationary phase of RpoS-dependent genes. This time difference may be critical for proper determination of GFP expression. In support of this, overnight samples of RpoS-dependent *gfp* fusion strains were significantly more fluorescent than the gfp^- control samples, unlike at other growth phases, and in overnight samples GFP expression began as much as 20 h prior to sampling.

Initial experiments show that there is a lag of approximately 40 min between GFP expression data and the corresponding *lacZ* data, supporting the idea that GFP maturation is the difficulty being experienced. Unfortunately, a more detailed study using real-time incubation and detection of 96-well plates of cultures in a fluorimeter was unsuccessful, as the generation time was very long and the samples lost a majority of their initial

volume, changing the incubation conditions of the cultures. The latter difficulty could potentially be addressed though leaving a lid on the 96-well plate, though condensation may become a problem in this case. However, given the slow generation time of cultures incubated in the plate reader, any results may not be indicative of typical growth in rich media (with a generation time of 22-25 min). This is a particular concern if the slow generation time is the result of low oxygen concentration, as GFP maturation is an oxygen-dependent process (Heim, Prasher, and Tsien, 1994). A shorter assay could also be a solution, perhaps beginning in early stationary phase when a great deal of GFP should already have been expressed, reducing the need for growth while incubating within the fluorimeter and rendering the slow generation time irrelevant. If the slow generation time is the result of low oxygen concentration, though, this could interfere with GFP as its maturation is an oxygen-dependent process (Heim, Prasher, and Tsien, 1994). Therefore, a kinetic assay within a fluorimeter does not seem to be a viable route for optimizing the quantification of GFP fluorescence.

Flow cytometry was examined as an alternative to fluorescence microscopy, in that it can also detect GFP expression in individual cells, though with increased sensitivity. MG1655f (*lacZ-gfp*) was clearly fluorescent via flow cytometry, as were stationary phase samples of HS143f (*rpoS-gfp*). This was encouraging, as this system is capable of accurately detecting GFP fluorescence and so it holds promise for achieving the objectives of this project. RpoS is expressed prior to the expression of its dependent genes, at approximately $OD_{600} = 0.3$ (Lange and Hengge-Aronis, 1994a), and as GFP

from the *rpoS* fusion is visible but not from its dependent genes, that difference in expression time may be sufficient for GFP to mature.

This technique, however, showed little fluorescence in the RpoS-dependent *gfp*-fusion strain HS1091f (*osmY-gfp*). Given that *osmY* is one of the most highly expressed genes in the RpoS regulon, it may prove very difficult to detect any RpoS-dependent genes at all in stationary phase samples. However, overnight samples of that same strain did show significant amounts of fluorescence, and so at the very least it is possible to detect GFP expression in these strains. As an alternative to using stationary phase samples (OD_{600} = 1.5), gene expression noise in RpoS-dependent genes could be examined through overnight cultures instead. At the very least, this could allow a preliminary examination of noise in RpoS-dependent genes while the stationary phase samples are still being optimized.

It is also possible that a better FP than GFP could be used for this process. By using a different, brighter, and/or faster-maturing fluorescent protein, the expression of the RpoS-dependent gene might be clearly detectable. Several bright fluorescent proteins exist, such as Cerulean Blue and Venus Yellow, that mature quickly, and either of these are excitable using the flow cytometer. Therefore, perhaps a different FP might better achieve the goals of this project.

4.3 Future experiments

The long-term objective of this project, the quantification of noise in the expression of the RpoS regulon, was not achieved. Therefore, future experiments to achieve this are as follows:

(i) Fluorescence microscopy to confirm that the time points indicated by the fluorescence kinetic assay (approximately 40 min after the desired OD_{600}) correspond to the RpoS-dependent expression data using *lacZ*. This would confirm the results above and establish conclusively a protocol by which to examine the *gfp*-fusion strains.

(ii) Fluorimetry of each of the RpoS-dependent *gfp*-fusion strains to confirm that the mean expression of *gfp*-fusions corresponds to mean expression of *lacZ*-fusion strains. To this point, it has not been firmly established that the *gfp*-fusion strains produce equivalent expression levels to the *lacZ*-fusion strains, which should be the case as they are fused to the same promoters. Such a comparison would validate the use of the strains in further experiments.

(iii) Fluorescence microscopy or flow cytometry to determine RpoS-dependent *gfp*-fusion expression on a cell-by-cell basis. This experiment would directly examine the question of noise in RpoS-dependent gene expression through the cell-by-cell examination, answering the principal component of the long-term objective above.

(iv) Completion of the plasmid pCJ1 and transformation into the recipient RpoS-dependent *gfp*-fusion strains and into the *rpoS-gfp* fusion strain (HS143f).

(v) Measurement of the noise in *rpoS* expression and in RpoS-dependent *gfp*-fusion expression in the RpoS-dependent *gfp*-fusion strains containing pCJ1. This (combined with experiment (iv)) would allow examination of noise in both *rpoS* expression and RpoS-dependent *gfp* expression in the same cell, providing insight into whether noise in the RpoS regulon is directly proportional to that in *rpoS* expression itself or whether other factors are involved.

(vi) Measurement of expression of each *rpoS*-fusion in the HS143f (pCJ1) strain to examine intrinsic/extrinsic noise in *rpoS* expression. This strain would have two equivalent *rpoS*-fusions, and examining both of these together, in individual cells, allows the determination of where the noise in *rpoS* expression comes from (Elowitz *et al.*, 2002).

These experiments, in combination, would properly address the objectives of this project.

Part II. Antibiotic-induced oxidative stress

Chapter 1. Introduction

1.1 Antibiotics

Antibiotics are compounds that are either bactericidal, in which they kill >99.9% of bacteria within 18 to 24 h of application, or bacteriostatic, inhibiting bacterial growth but not killing sufficiently to be bactericidal (Pankey and Sabath, 2004). Antibiotics have brought many life-threatening infectious diseases under control and allowed both the advancement of surgical procedures and control of post-operative infections, making them a cornerstone of modern medicine. Antibiotics are also quite prevalent in nature, often produced by bacterial species themselves to provide an advantage over its rival species for an ecological niche. The majority of current antibiotic classes were originally discovered and isolated from bacteria themselves, most commonly from *Streptomyces* species, with the antibiotics themselves often synthetically modified to increase either the efficiency of the antibiotic or the spectrum (discussed below).

Antibiotics have particular targets or cellular processes which they inhibit within the bacterium. These include cell wall synthesis, transcription, translation, DNA replication, nucleotide synthesis, and membrane integrity. These in particular are good targets as they are either not present in humans (e.g. cell wall) or are widely divergent in humans (e.g. eukaryotic ribosomes are different from prokaryotic ribosomes), and can thus inhibit bacteria without adversely affecting the human host. While the antibiotic

classes below are designed to either inhibit growth or kill bacteria, a new process being investigated as an antibiotic target is virulence, which if inhibited would leave the bacterium alive but unable to cause disease. In all cases, the antibiotic must access its target and inhibit it in order to be effective. Resistance occurs when at least one of those two objectives is interfered with, and as the bacterium originally producing the antibiotic must itself be resistant to it, resistance mechanisms can often be found in bacteria. Resistance can either be intrinsic, in which the bacterium is naturally immune to the effects of the antibiotic, or acquired, in which the bacterium acquires either a mutation or an entire gene that interferes with one of the objectives above. Intrinsic resistance varies depending on the class of antibiotic, and the diversity of bacteria with this intrinsic resistance determines whether an antibiotic is narrow spectrum (e.g. glycopeptides, which only affect Gram positive bacteria) or broad spectrum (as with tetracyclines).

There are numerous classes of antibiotics, both bactericidal and bacteriostatic. The classes relevant to this project are briefly outlined below, including their mechanism and means of resistance that have developed.

1.2 Bactericidal antibiotics

Aminoglycosides were discovered in the 1940s, and include the common antibiotics streptomycin, kanamycin, neomycin, and gentamicin. Many aminoglycosides were isolated from *Streptomyces* spp., while others (such as gentamicin) were isolated from *Micromonospora* spp. Aminoglycosides are bactericidal, binding to the 30S subunit of the ribosome and causing both mistranslation of mRNA and arrested translation, depending on whether the ribosome is in the elongation phase of translation when the

aminoglycoside binds or initiation, respectively. Each aminoglycoside produces a different proportion of misreading and arrest; for example, kanamycin tends to produce more misreading than streptomycin does (Lando, Cousin, and Privat, 1973). The current model for the inhibition mechanism for streptomycin (and likely all aminoglycosides) (reviewed in (Davis, 1987;Vakulenko and Mobashery, 2003)) involves an initial slow uptake of streptomycin from the environment, as streptomycin is only slightly permeable across the plasma membrane, causing misreading of mRNA by the ribosome as streptomycin binds to translating ribosomes at the A site, responsible for ensuring high fidelity translation (Lodmell and Dahlberg, 1997). As some ribosomes are translating membrane proteins at any given time, incorrect membrane proteins are produced and incorporated into the membrane, damaging its integrity. This produces the rapid secondary influx of streptomycin which has been observed (Davis, 1987), which then accumulates significantly to arrest all ribosomes and cause cell death, likely through either the arrest of all ribosomes or the loss of membrane integrity itself.

Several resistance mechanisms against aminoglycosides are known. Resistance often arises through enzymatic modification of the antibiotic itself, though efflux pump-mediated resistance has also been isolated in a variety of organisms (reviewed in (Vakulenko and Mobashery, 2003)). These can be readily differentiated based on the intracellular concentration of the aminoglycoside. Furthermore, resistance to streptomycin in particular seems to occur through the modification of the ribosome (Vakulenko and Mobashery, 2003). Unlike other classes of antibiotics, however,

resistance to aminoglycosides typically does not confer cross-protection to other antibiotic classes (Peterson, 2005).

β-lactams, originally discovered from the mold *Penicillium notatum* and the first antibiotic class to be put into medical use in the 1940s, are a diverse group of bactericidal antibiotics with widespread use in medicine. These include penicillin derivatives (such as ampicillin), cephalosporins, and carbapenems, and target transpeptidases, also known as penicillin-binding proteins, which cross-link the peptidoglycan layers of bacterial cell walls (reviewed in (Tipper, 1985)). The resulting build-up of cell wall precursors within the cell causes triggers cell wall hydrolases, which combined with the weakened cell wall itself cause the cell wall to break and the cell (dependent on the rigidity of the cell wall to prevent bursting) to lyse, making the antibiotic bactericidal. Their heavy clinical usage has resulted in many bacterial species being resistant to early forms of these antibiotics, typically through chromosomally- or plasmid-encoded β-lactamase enzymes. Reduced membrane permeability is also a significant route of resistance for Gram-negative bacteria, particularly against cephalosporins (Peterson, 2005). β-lactamases hydrolyze the β-lactam ring found in these antibiotics, freeing the β-lactam from the transpeptidase and allowing the enzyme to resume its function (Tipper, 1985). In addition, the use of these antibiotics often allows the development of post-treatment hospital-acquired infections such as vancomycin-resistant *Enterococci* and *Clostridium difficile*-associated diarrhea (Anand *et al.*, 1994;Carmeli, Eliopoulos, and Samore, 2002).

Two methods have been developed to circumvent bacterial resistance to β-lactams: the development of novel β-lactam antibiotics, and the addition of β-lactamase inhibitors. Novel β-lactams, particularly cephalosporins, are continuously being produced and refined for a broader spectrum of activity and less bacterial resistance. β-lactamase inhibitors, in contrast, do not attack the cell's processes directly but rather inhibit the bacterial resistance enzymes (reviewed in (Bush, 1988)). This strategy bears a great deal of promise to reduce resistance in clinical isolates of bacteria (discussed further below).

Quinolones, originally based on nalidixic acid, inhibit DNA gyrase and topoisomerase IV after these enzymes create a double strand break in DNA but prior to ligating that break, ultimately causing lethal DNA damage (reviewed in (Hawkey, 2003)). *In vivo*, nalidixic acid itself is a relatively narrow spectrum antibiotic due to its quick removal from the bloodstream through the urinary tract, only adversely affecting those Gram negative bacteria that are involved in urinary tract infections, but these antibiotics have undergone successive rounds of improvement, increasing their activity and longevity *in vivo*, making them effective against both Gram positives and Gram negatives in a variety of infections (reviewed in (Ball, 2000)). This class of antibiotics has two subgroups: naphthyridones (nalidixic acid derivatives) and fluoroquinolones. Fluoroquinolones, including the antibiotic norfloxacin, are among the most important antibiotics for treating serious bacterial infections (Peterson, 2005).

Unfortunately, the high usage of quinolones, particularly fluoroquinolones, has resulted in the spread of resistance within bacteria. Resistance typically occurs through a reduction in membrane permeability by a large increase in multi-drug efflux pumps

(reviewed in (Peterson, 2005)), and this often results in resistance to a variety of other antibiotic classes as well. This is particularly exacerbated when fluoroquinolones are used in conjunction with β-lactams – β-lactams select for the outer membrane porin OprD and fluoroquinolones select the efflux system MexEF-OprN. The coregulation of these proteins results in resistance to a variety of antibiotics when β-lactams and fluoroquinolones are used in conjunction (Livermore, 2002). Fluoroquinolone use also commonly results in hospital-acquired infections, much like β-lactams from above (Gerding, 2004).

Other important bactericidal antibiotic classes (which are not used for this project but mentioned in this thesis) include **ansamycins** and **lipopeptides antibiotics**. **Ansamycins**, including the antibiotic rifampicin, directly interacts with the β subunit of RNA polymerase and possibly also the σ factor, inhibiting chain elongation and, as a result, transcription in general within the cell (reviewed in (Goldberg and Friedman, 1971;Villain-Guillot *et al.*, 2007)). **Lipopeptide antibiotics**, including the clinically-used antibiotic daptomycin, cause membrane depolarization, resulting in rapid cell death (reviewed in (Steenbergen *et al.*, 2005)). The lipophilic tail of the antibiotic inserts itself into the membrane and then oligomerizes with other antibiotic molecules, forming a channel and eliminating the membrane potential of the cell.

1.3 Bacteriostatic antibiotics

Originally isolated from *Streptomyces venezuelae* in 1949, **chloramphenicol** is a bacteriostatic antibiotic that is active against both Gram positive and Gram negative organisms. While the exact mechanism of its action is unknown, it inhibits the 50S

subunit of the ribosome by interfering with peptidyl transferase (reviewed in (Yunis, 1988)). Though efflux pump mutations that confer resistance are known, as are ribosome modifications, the primary means of resistance is through the enzyme chloramphenicol acetyl transferase, which acetylates the primary alcohol of chloramphenicol and renders it inactive (reviewed in (Yunis, 1988)). However, chloramphenicol is not used clinically save as a topical agent, as its ingestion results in a fatal blood disorder known as aplastic anemia in approximately 1 in 25000 to 40000 cases of use irrespective of dose or duration of medication (Wallerstein *et al.*, 1969;Yunis, 1988).

Tetracyclines are broad spectrum antibiotics based on the antibiotic tetracycline that are heavily used in both medicine and agriculture (for a review, see (Schnappinger and Hillen, 1996)). They are bacteriostatic and reversibly bind the ribosome, inhibiting protein synthesis. There is a high-affinity binding site on the 30S subunit, involving the S7 protein, which is believed to be where the antibiotic binds, but numerous other sites have been found which may also be important as well (Epe and Woolley, 1984;Goldman *et al.*, 1983). This may interfere with aminoacyl-tRNA binding, which would inhibit protein synthesis (the resulting phenotype of tetracycline application), but this has not been conclusively shown (Schnappinger and Hillen, 1996). Resistance to tetracycline has been seen through each of efflux pumps (tetracycline-specific and multi-drug), ribosomal modification, and tetracycline-modifying enzymes (reviewed in (Schnappinger and Hillen, 1996)).

Tetrahydrofolate synthesis inhibitors, including trimethoprim and sulfonamides, inhibit the production of tetrahydrofolate from dihydrofolate. Tetrahydrofolate is a key component of the biosynthesis of thymidine, and thus application of the antibiotic results in depletion of the available nucleotides, arresting growth (reviewed in (Schweitzer, Dicker, and Bertino, 1990)). Unlike the other antibiotics, trimethoprim acts on a bacterial enzyme, dihydrofolate reductase, with a direct human homolog; however, the binding of trimethoprim to the human dihydrofolate reductase is four orders of magnitude weaker than binding to the bacterial version, making the antibiotic safe for clinical use (Appleman *et al.*, 1988;Schweitzer, Dicker, and Bertino, 1990). Sulfonamides affect the synthesis of dihydropteroic acid by the enzyme didyropteroate synthetase, an earlier step in the pathway, which humans obtain through diet and do not endogenously synthesize (reviewed in (Skold, 2000)). Trimethoprim and sulfonamides are often used in conjunction, thus maximizing the effect of the antibiotics. Trimethoprim resistance in *E. coli* often arises through overexpression or modification of the bacterial dihydrofolate reductase enzyme, though in other organisms modification of membrane permeability may also play a role (reviewed in (Huovinen, 1987)).

There are two other bacteriostatic antibiotic classes which are not used in this project but mentioned in this thesis, and these are listed below. **Oxazolidinones**, including the antibiotic linezolid, were first discovered in 1987 (Slee *et al.*, 1987) and inhibit the initiation of translation by binding to the 23S portion of the 50S subunit of the ribosome (reviewed in (Vara Prasad, 2007)). These antibiotics are largely only effective against Gram positive bacteria due to the presence of an efflux pump in Gram negatives

(Schumacher *et al.*, 2007), and resistance in Gram positives typically arises through modification of the ribosome itself (Roberts, 2008). **Aminocyclitols**, such as the antibiotic spectinomycin, form a branch of the aminoglycoside family of antibiotics and inhibit translation of mRNA by binding to the 30S ribosomal subunit (Davies and Nomura, 1972).

1.4 Current state of antibiotic development

The fraction of bacterial isolates resistant to a particular antibiotic increases every year that it is in use, and of greater concern is the ever-increasing fraction of bacterial isolates that are immune to multiple antibiotic classes. A 2007 survey of European hospitals found that only 47% of invasive *E. coli* isolates showed wild type sensitivity to each of aminopenicillins, fluoroquinolones, third generation cephalosporins and aminoglycosides, while 19.3% of invasive *E. coli* isolates were resistant to at least two of these classes (European Antimicrobial Resistance Surveillance System, 2008). Similarly ominous findings have been documented for a variety of other pathogens. Despite this, little research is being done to discover novel classes of antibiotics, with only two novel antibiotic classes in clinical use developed in the last thirty years: oxazolidinones and lipopeptide antibiotics (Pucci, 2006). Due to the economic advantage of developing drugs treating chronic problems as opposed to short-term infections, few novel antibiotics are in the process of development (Projan and Shlaes, 2004). Focus has therefore been placed on maximizing the utility of current antibiotics. One approach has been to examine behavioural changes with respect to antibiotic prescribing, including limiting antibiotic prescriptions to cases where they are absolutely necessary and rotating

antibiotic use to allow sensitivity to the disused antibiotic to be restored (for examples, see (Lesprit and Brun-Buisson, 2008;Rybak, 2007)). An alternative biochemical route is to introduce a second compound with the antibiotic that increases its potency. One approach has been to target β-lactamases with inhibitors in conjunction with a β-lactam antibiotic (reviewed in (Bush, 1988)), and such inhibitors include clavulanic acid, sulbactam, and tazobactam. While β-lactamases inhibitors have no antibiotic activity of their own, their administration with a β-lactam antibiotic forms a highly potent drug – often reducing the viability of the bacteria 1000-fold (Goldstein, Kitzis, and Acar, 1979) – while little resistance to the inhibitors has developed as yet (White *et al.*, 2004). The potential for other similar combinations, an antibiotic and a bacterial defense inhibitor (multi-drug pump inhibitors, for example), has great potential and forms part of the rationale for this project (discussed below).

1.5 Oxidative Stress

Dioxygen molecules (O_2) can cause a great deal of damage to all cells, as its high electronegativity makes it adept at taking electrons (e^-) away from biomolecules. While the reduction of O_2 is essential for aerobic organisms, inadvertent partial reduction of O_2 generates dangerous by-products, known as reactive oxygen species (ROS). O_2 is a stable diradical molecule, and so it will accept single e^- reductions. Thus, between oxidized O_2 and reduced H_2O are the partially-reduced and ROS superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (·OH). These ROS are unstable and will accept e^- from biological molecules, damaging them, at a greater rate than O_2 itself. The particular rate of damage of biomolecules is dependent on the particular ROS. The

charge on O_2^- partly inhibits both its ability to gain e^- and its ability to cross the plasma membrane, while the strong O-O bond of H_2O_2 imposes a large activation energy on gaining e^-, and so these two molecules have constraints placed on their reactivity (Imlay, 2003). This is not the case with ·OH, and its damage of biomolecules is essentially limited solely by its diffusion rate (Imlay, 2003), resulting in a very short half-life of 10^{-9} s (Pryor, 1986). Its half-life is too short for enzymes to efficiently scavenge the molecule, and so cells instead employ repair mechanisms and scavengers of other ROS to minimize the extreme toxicity of ·OH.

Experiments with respiratory vesicles from *E. coli* show that both O_2^- and H_2O_2 are produced endogenously at the plasma membrane through the electron transport chain (Gonzalez-Flecha and Demple, 1995;Imlay and Fridovich, 1991). Respiratory dehydrogenase enzymes, which oxidize NADH, use flavins such as $FADH_2$ as cofactors which accept hydride (H^-) from NADH and transfer that to Fe-S clusters or quinines. However, *in vitro* flavins will transfer e^- non-specifically to O_2 (Messner and Imlay, 2002), transferring either one e^- and producing O_2^- (typically the minor product) or transferring two e^- and producing H_2O_2 (typically the major product) (Massey, 1994). Such byproducts are considered the primary source of O_2^- in the cell, as no enzyme in *E. coli* is known to generate O_2^- as a stoichiometric product (Imlay, 2003). This is also the primary source of H_2O_2 in the cell, though superoxide dismutases (SOD) will produce H_2O_2 from O_2^- (see below). The unpaired electrons in both O_2 and O_2^- will also readily react with transition metals, though O_2^- reacts at a significantly greater rate, both creating other dangerous molecules (O_2^- and H_2O_2, respectively) and damaging any biomolecule

associated with the transition metal (Imlay, 2003). Thus metalloproteins with exposed transition metals also have a role, albeit a lesser one, in endogenous production of ROS.

Once O_2^- and H_2O_2 are generated, these ROS damage cellular components. Discovered through the use of superoxide dismutase (SOD) mutants (discussed below), O_2^- tends to damage [4Fe-4S] clusters of metalloproteins, such as respiratory dehydratases aconitase B and fumarases A and B (Imlay, 2003), by extracting the exposed Fe^{2+} atom from these clusters, which is also the catalytic atom of the enzyme (Flint, Tuminello, and Emptage, 1993). Interestingly, despite their proximity to ROS production, respiratory proteins containing [4Fe-4S] clusters are typically not affected – dehydratases are the primary target of O_2^- and this is believed to be due to the respiratory proteins having their clusters shielded, preventing Fe^{2+} extraction (Fridovich, 1995). O_2^- will also oxidize aromatic and sulfur-containing amino acids, triose sugars, and DNA, resulting in defective proteins, unusable trioses and a higher mutation rate (reviewed in (Fridovich, 1995)). H_2O_2 damages both these [4Fe-4S] clusters (though to a lesser extent) and oxidizes cysteine residues of proteins, potentially creating cysteine bridges and altering the conformation and activity of proteins (Winterbourn and Metodiewa, 1999).

While the damage to these cellular components is detrimental in itself, the liberation of Fe^{2+} also allows the production of ·OH. This ROS is produced from the Fenton reaction, shown below:

$$H_2O_2 + Fe^{2+} \rightarrow OH^- + FeO^{2+} + H^+ \rightarrow OH^- + \cdot OH + Fe^{3+}$$

Thus, the production of H_2O_2 and O_2^- ultimately allows ·OH production, resulting in non-specific damage to DNA, proteins, and lipids through oxidation of any oxidizable

substrate of the biomolecule. It is the production of ·OH that is ultimately the most toxic part of the ROS pathway in cells.

In light of the toxicity of oxygen and its essential nature for respiration, cells have established several defenses against the effects of ROS. SOD and catalases are enzymes which detoxify O_2^- and H_2O_2, respectively, while NADH and NADPH, glutathione, and both glutaredoxins and thioredoxins maintain a reducing environment within the cell. Furthermore, iron-trafficking and DNA and protein repair pathways are also involved in protection against oxidative stress. These are each more fully described in turn.

1.6 Superoxide dismutases

SOD are enzymes that catalyze the following dismutation (or disproportionation) reaction:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

There are three classes of these enzymes, divided by the transition metal(s) they use to catalyze the reaction: Mn-SOD, Fe-SOD, and Cu/Zn-SOD. Each enzyme operates by successive oxidation-reduction steps, with the first O_2^- molecule reduced to H_2O_2 by the catalytic metal ion and the second O_2^- molecule oxidized to O_2 by the same metal ion, restoring the original oxidation state of the metal (reviewed in (Fridovich, 1995)). The Mn-SOD and Fe-SOD are believed to share a common origin, as the two enzymes have similar amino acid sequences, while the Cu/Zn-SOD likely arose as an independent class of SOD and is present in both Gram positive and Gram negative organisms (reviewed in (Grace, 1990)). These enzymes are also widely distributed throughout aerobic organisms (Grace, 1990), and *E. coli* contains one of enzyme of each class.

The Mn-SOD in *E. coli*, encoded by the gene *sodA*, is part of the SoxRS regulon (for a review of SoxRS, see (Demple, 1996;Touati, 2000)), and is inducible by elevated O_2^- levels through SoxRS. SoxR acts as a sensor of elevated O_2^- through its exposed [2Fe-2S] cluster. In its reduced state, SoxR is inactive, but O_2^- oxidizes the [2Fe-2S] cluster of the protein (Ding, Hidalgo, and Demple, 1996), which as dicussed above is the typical mechanism of O_2^- for damaging proteins. This activates SoxR and triggers *soxS* transcription (Ding, Hidalgo, and Demple, 1996), and SoxS in turn activates transcription of the SoxRS regulon, including Mn-SOD, to remove the O_2^- (Ding, Hidalgo, and Demple, 1996;Nunoshiba *et al.*, 1993). This regulon remains induced until both SoxS terminates its own activation though a negative feedback loop (Nunoshiba *et al.*, 1993) and the [2Fe-2S] cluster of SoxR is repaired. Expression of *sodA* is repressed by both Arc (Tardat and Touati, 1991) and Fur (Schrum and Hassan, 1994) in anaerobic conditions (when presumably detoxification of ROS is unnecessary).

The Fe-SOD in *E. coli*, encoded by *sodB*, is induced by Fur (Dubrac and Touati, 2000;Dubrac and Touati, 2002), a 17 kDa regulator that binds DNA in a Fe^{2+}-dependent manner (Bagg and Neilands, 1987;de Lorenzo *et al.*, 1987). Though the expression of *sodB* is constitutive at a low level, β-galactosidase assays with wild type and *fur* mutants show that Fur increases the transcription of *sodB* 7-fold (Dubrac and Touati, 2000). Fur acts post-transcriptionally as well, binding to an RNase endonucleolytic site and inhibiting degradation (Dubrac and Touati, 2002). Fur regulates iron homeostasis and is itself activated *in vitro* when bound by Fe^{2+} (Bagg and Neilands, 1987). Accordingly, as free Fe^{2+} in the cell is harmful through the production of ·OH, in its active state (high

levels of intracellular Fe^{2+}) Fur represses iron uptake genes as well as many general metabolism genes (Bagg and Neilands, 1987;Escolar, Perez-Martin, and de Lorenzo, 1999) to protect the cell from the harmful effects of Fe^{2+}-produced ROS. Furthermore, Fur itself is activated at the transcriptional level by both OxyR and SoxRS as a means of reducing Fe^{2+} levels in the cell during oxidative stress to minimize the effect of ROS (Zheng *et al.*, 1999), and therefore Fe-SOD is also indirectly regulated by both OxyR and SoxRS. However, as the regulation of *sodB* is Fe^{2+}-dependent and not strictly O_2-dependent, it is expressed in anaerobic conditions unlike *sodA* (Kargalioglu and Imlay, 1994). This is likely to protect the cell in the event of a return to aerobiosis, as the lag phase of *sodB* mutants is 2 h longer than the wild type in such circumstances (Kargalioglu and Imlay, 1994).

The Cu/Zn-SOD in *E. coli*, encoded by *sodC*, was the last SOD discovered and, unlike the other SOD enzymes, it is periplasmic and primarily expressed in stationary phase (20-fold induction over exponential phase) in an RpoS-dependent manner (Benov and Fridovich, 1994;Gort, Ferber, and Imlay, 1999). There is evidence for significant O_2^- generation within the periplasm of *E. coli* (3μM/s) (Korshunov and Imlay, 2006), and *sodC* mutants are hypersensitive to H_2O_2 (Gort, Ferber, and Imlay, 1999). Therefore, Cu/Zn-SOD may play an important role in protecting the exterior of the cell from endogenously-produced ROS and, potentially, exogenously-produced ROS. However, due to its contribution to overall SOD activity within the cell being low (Fridovich, 1995) and its periplasmic location, SodC is not further considered in this project.

1.7 Catalases

Catalases catalyze the following disproportionation reaction that eliminates H_2O_2:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

The first H_2O_2 molecule is oxidized by the catalytic iron of the catalase, producing H_2O and $Fe^{4+}=O$, then subsequently reduced by the second H_2O_2 molecule, producing H_2O and O_2, restoring the iron to its original oxidation state (reviewed in (Chelikani, Fita, and Loewen, 2004)). Note that this mechanism is analogous to that of SOD enzymes with O_2^-. Mn catalases have a similar mechanism, with the Fe atom replaced by two Mn atoms (reviewed in (Wu, Penner-Hahn, and Pecoraro, 2004)). As the production of H_2O_2 is ubiquitous in aerobic organisms, catalases are widely distributed throughout the domains of life, and nearly all aerobic organisms produce at least one catalase (*E. coli*, for example, encodes two).

Catalases are divided into three families: monofunctional heme catalases, catalase-peroxidases, and Mn-containing catalases (reviewed in (Chelikani, Fita, and Loewen, 2004)). The monofunctional catalases, the largest family, are present throughout the domains of life, while the other two families are confined to either bacteria and archaea or bacteria and fungi (Chelikani, Fita, and Loewen, 2004). While catalase phylogeny is not known for all bacterial species, the monofunctional heme catalase HPII of *E. coli* is similar to that of other proteobacteria such as *Sinorhiozbium meliloti*, *Pseudomonas putida*, *Salmonella* spp., and *Helicobacter pylori*, as well as the Gram positives *Bacillus subtilis* and *Staphylococcus aureus* (Chelikani, Fita, and Loewen, 2004). Furthermore, the catalase-peroxidase HPI of *E. coli* is homologous to those of

Mycobacteria, *Streptomyces,* and *Salmonella* species (Chelikani, Fita, and Loewen, 2004). Both monofunctional heme catalases and catalase-peroxidases catalyze the above reaction using a heme group.

As mentioned above, *E. coli* has two catalases, HPI (a catalase-peroxidase) and HPII (a monofunctional heme catalase). HPI, encoded by *katG*, is regulated transcriptionally by OxyR, a protein that has two cysteine residues on its surface that act as a sensor of the oxidation state of the cell. A high concentration of H_2O_2 will turn the normally reducing environment of the cell into an oxidizing one and oxidize these cysteine residues (199C and 208C), forming an intramolecular bond and switching the protein from its resting inactive state into its active state (Aslund *et al.*, 1999;Zheng, Aslund, and Storz, 1998). Upon reduction of the cysteine residues (typically by the GSH-glutathioredoxin system (Aslund *et al.*, 1999)), OxyR returns to its resting state. As H_2O_2 oxidizes cysteine residues, this regulation of OxyR is analogous to SoxR described above. Active OxyR induces the expression of numerous genes, including *katG*, causing the scavenging of H_2O_2 from the cell. Thus, HPI expression is inducible by H_2O_2. Interestingly, HPI expression also has OxyR-independent stationary-phase dependence (Mukhopadhyay and Schellhorn, 1994;Visick and Clarke, 1997), which may be the result of regulation by RpoS (discussed above), though there is no consensus in the literature on this point (Ivanova *et al.*, 1994;Mukhopadhyay and Schellhorn, 1994;Visick and Clarke, 1997). HPI first drew attention when it was discovered that KatG (an HPI homolog) activates the drug isoniazid in *Mycobacterium tuberculosis*, and that isoniazid resistance occurs through inactivation of KatG (Zhang *et al.*, 1992).

HPII, encoded by the *katE* gene, is regulated by the alternative sigma factor RpoS, the regulator that responds to stress conditions such as oxidative stress, osmotic shock, ethanol, and near-UV light, as well as stationary phase starvation (discussed above). RpoS activates the transcription of *katE* by a factor of 7-fold in exponential phase and 40-fold in stationary phase in rich media, as found through β-galactosidase fusions (Vijayakumar *et al.*, 2004).

1.8 Other defenses against oxidative stress

A further enzyme, **alkyl hydroperoxide reductase** (Ahp), scavenges H_2O_2 and was found in *E. coli katE katG* mutants that could still scavenge H_2O_2 (Seaver and Imlay, 2001). Ahp is an NADH-dependent peroxidase that reduces a variety of organic hydroperoxides and H_2O_2 (Jacobson *et al.*, 1989), and is responsible for constitutively maintaining a low level of H_2O_2 in the cell (Seaver and Imlay, 2001). However, despite being regulated by OxyR like HPI catalase, Ahp provides very little scavenging of H_2O_2 during oxidative stress (Seaver and Imlay, 2001), with HPI and HPII catalases primarily responsible for this task.

As reviewed above, iron plays a key role in both the proper functioning of key enzymes (both catalases and SodB require iron) and the production of harmful ROS. Therefore, it is tightly regulated in the cell, both in uptake at the membrane by specific receptors and sequestration once in the cytoplasm by ferritin and bacterioferritin (reviewed in (Escolar, Perez-Martin, and de Lorenzo, 1999)). **Fur** is a key regulatory protein for both these processes. While the activation of Fur increases the level of Fe-SOD, reducing any oxidative stress, Fur also inhibits expression of Fe transporters, thus

reducing the amount of Fe entering the cell. As Fur is activated by OxyR and SoxRS, cells have the ability to reduce the concentration of Fe^{2+} entering the cell during oxidative stress (Zheng *et al.*, 1999). Therefore iron regulation also has a role in the response to ROS.

ROS oxidize biological molecules, and so proteins must be repaired after oxidation damage caused by ROS. Therefore, multiple systems exist in the cytoplasm whose role is to (i) maintain a reducing environment by scavenging oxidizing molecules; and (ii) reduce biomolecules, restoring their proper function. The **GSH-glutaredoxin** system transfers e^- from NADH to, successively, glutathione reductase, glutathione, and then one of three glutaredoxins, which then transfers the e^- to the oxidized protein, reducing its cysteine residues (reviewed in (Fernandes and Holmgren, 2004)). OxyR is a regulator of both glutathione reductase and glutaredoxin 1, which in turn reduce OxyR to its inactive state, forming an autonegative control loop (Carmel-Harel and Storz, 2000;Christman *et al.*, 1985), as discussed above. The **thioredoxin** system, similar to the glutaredoxin system, involves thioredoxin reductase, which is reduced by NADH and in turn reduces thioredoxin, and both thioredoxin reductase and thioredoxin reduce proteins directly (reviewed in (Arner and Holmgren, 2000)). As proteins are oxidized by ROS during oxidative stress, these pathways are important in the maintenance of a proper cellular environment and repair of the damage that is caused by this stress.

ROS also cause DNA damage through oxidation, which activates the **SOS repair pathway** (Kohanski *et al.*, 2007). Activation of the SOS repair pathway of DNA by RecA and LexA causes the transcription of numerous genes whose products repair the

DNA, allowing normal transcription and replication to continue (reviewed in (Courcelle and Hanawalt, 2003)). Therefore, though there is no interaction between LexA or RecA with any of the ROS regulators, such as OxyR and SoxR, this repair pathway may also be of some importance to the protection of the cell from harmful ROS.

1.9 Connection between antibiotics and oxidative stress

It was recently shown that bactericidal antibiotics act via an ROS-dependent mechanism, ultimately producing ·OH (Dwyer *et al.*, 2007;Kohanski *et al.*, 2007). This production of ROS is the result of a spike in cellular respiration (from an unknown cause) upon exposure to the antibiotic, producing a surplus of O_2^-. The resulting O_2^--mediated destruction of Fe-S clusters allows Fe^{2+} to accumulate and, with already-present H_2O_2, initiate Fenton-mediated ·OH production, damaging and killing the cell (Dwyer *et al.*, 2007;Kohanski *et al.*, 2007). The toxicity of bactericidal antibiotics thus have a common cellular pathway through the ROS by-products of respiration, and so it is reasonable to hypothesize that the enzymes protecting the cell from respiratory by-products may also protect the cell from these drugs by competing for and eliminating the O_2^- and H_2O_2 essential to short-term toxicity. Predictions based on this model form the basis of my project (described below).

Since this initial publication in 2007, further study has been done in this area. Two of these examined the relationship between MazEF, a toxin-antitoxin system of *E. coli*, and the action of antibiotics. Each of spectinomycin, chloramphenicol, trimethoprim (all bacteriostatic), rifampicin and nalidixic acid (both bactericidal) induced cell death through MazEF in *E. coli* strain MC4100 in minimal media in the short-term

(10 min to 1 h) (Kolodkin-Gal *et al.*, 2008). Interestingly, overexpression of catalase (*katE*) or SOD (*sodA*) or addition of exogenous catalase prevented this cell death in rifampicin, spectinomycin, and chloramphenicol but not trimethoprim or nalidixic acid, while anaerobiosis (and thus presumably a lack of ROS) prevented cell death due to rifampicin and spectinomycin but not trimethoprim (Kolodkin-Gal *et al.*, 2008). Similar results could be obtained for MG1655 when a factor (EDF) that activates *mazEF* was added (this factor is not necessary for MC4100) (Kolodkin-Gal *et al.*, 2008). Lethality through *mazEF* only occurs in exponential phase *E. coli* (Hazan, Sat, and Engelberg-Kulka, 2004), and *mazEF*-dependent lethality is repressed by RpoS in stationary phase cultures for each of these antibiotics (Kolodkin-Gal and Engelberg-Kulka, 2009). Overexpression of *katE* could only restore viability in rifampicin, spectinomycin, and chloramphenicol, so the effect in trimethoprim and nalidixic acid is independent of RpoS regulation of *katE* expression (Kolodkin-Gal and Engelberg-Kulka, 2009). In summary of the two papers, the authors propose a new model: antibiotics may be either *mazEF*-dependent or independent and ROS-dependent and independent. ROS-independent, *mazEF*-independent antibiotics are bacteriostatic, while all other cases are bactericidal (Kolodkin-Gal *et al.*, 2008;Kolodkin-Gal and Engelberg-Kulka, 2009). Interestingly, many of the antibiotics tested are bacteriostatic, despite producing significant levels of cell death in MC4100 within a maximum of one hour, and bacteriostatic antibiotics were not expected to act through ROS generation (Kohanski *et al.*, 2007).

In addition, new information on the action of aminoglycosides has clarified the original proposed model. CpxA and CpxR (a two-component system) and ArcA all

increase the ROS generated from bactericidal antibiotics, though the direct connection between the proteins themselves and the NADH-dependent ROS generation is unknown, while proteins involved in degrading mistranslated proteins, such as DegP and HflCK, reduce the amount of ROS generated (Kohanski *et al.*, 2008). From these results, they conclude that some mistranslated proteins (which aminoglycosides end up causing) enter the inner membrane and periplasm, which is sensed by CpxA and relayed to CpxR, causing ArcA to be activated, stimulating the TCA cycle and generating a surplus of O_2^- (Kohanski *et al.*, 2008). This pathway then feeds into the model previously proposed, though this addition to the model remains to be tested in other antibiotics besides gentamicin. Interestingly, the authors also found that oxidative stress regulons (both OxyR and SoxRS) are activated briefly upon the addition of aminoglycosides, and that MG1655 Δ*katE* Δ*katG* and MG1655 Δ*sodA* Δ*sodB* have reduced viability in aminoglycosides for 2 h after exposure (viability is then similar afterwards) (Kohanski *et al.*, 2008).

1.10 Project objectives

Recently, bactericidal antibiotics have been shown to induce cell death through an ROS-mediated pathway described above (Dwyer *et al.*, 2007;Kohanski *et al.*, 2007). This proposed model suggests that both O_2^- and H_2O_2 (among other factors) have critical roles in the lethality of bactericidal antibiotics. Therefore, these antibiotics should activate SoxRS, OxyR, and RpoS, and the activities of each of HPI, HPII, SodA, SodB, and SodC would be increased. Conversely, deletion mutants of these catalases and superoxide dismutases or cells with inhibitors introduced may have increased sensitivity

to bactericidal antibiotics. I hypothesize that catalases and superoxide dismutases both have a role in protecting the cell against antibiotics, and the global objective of this project is to determine whether this is indeed the case.

There are three distinct components to this project. The first two have equivalent experiments: one component examining catalases, and one examining superoxide dismutases. A flowchart is shown as Figure 15, illustrating the immediate objectives that will be done to examine whether there is indeed an effect. While this flowchart is specific for catalases, the SOD flowchart would be identical – they are proposed to have similar roles, and have similar tests to examine that. The final component of this project is selecting on a combination of bactericidal antibiotics, then testing those spontaneous mutants. Once the experiments are completed, an overall model from these two separate lines of experimentation can be developed to determine the role of ROS scavenging enzymes against bactericidal antibiotics.

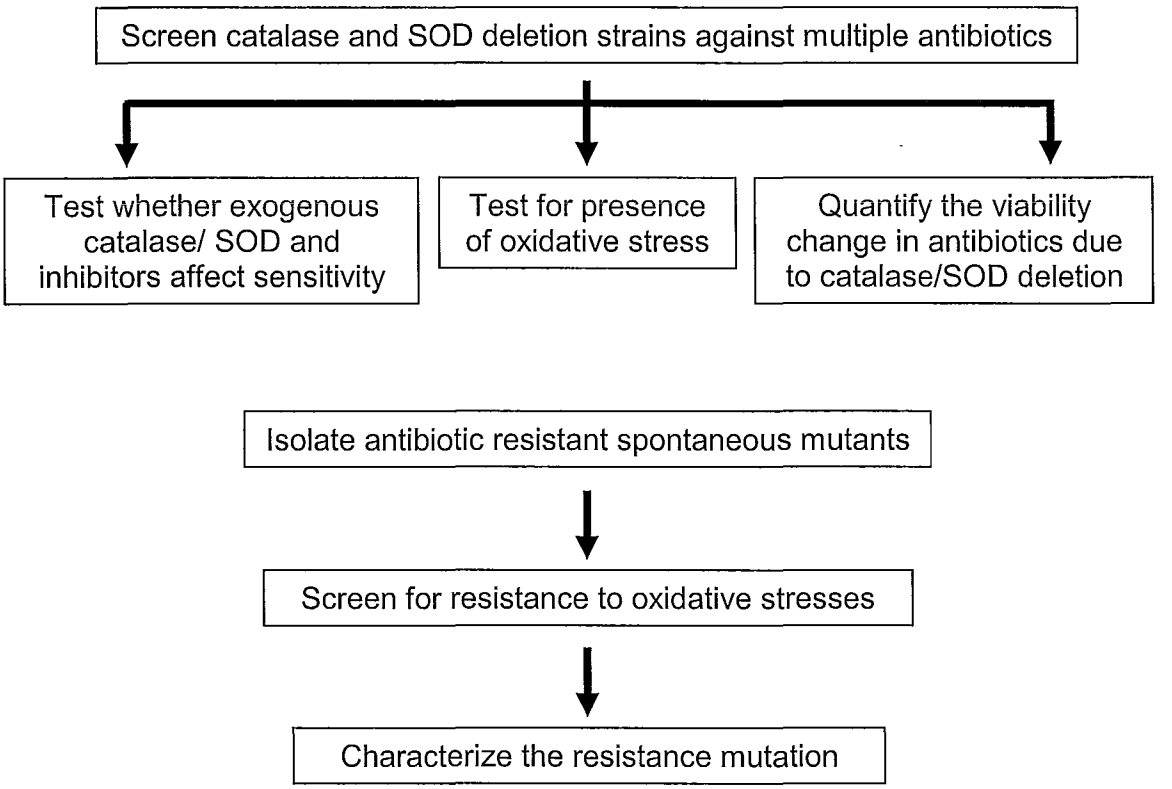


Figure 15. Flowchart of experiments to test for the importance of catalase and SOD activity against antibiotics. The top half of the flowchart shows experiments involving catalase and SOD deletion strains, while the bottom half shows experiments involving antibiotic-resistant spontaneous mutants.

Chapter 2. Materials and methods

2.1 General growth conditions and antibiotics

All LB, M9-glucose (0.2%), and M9 glycerol (0.4%) broth and LB/agar, M9-glucose/agar and M9-glycerol/agar plates were prepared as described by Miller (Miller, 1992). Unless specified otherwise, strains were grown at 37°C and 200 rpm, and plates were incubated overnight (LB) or for 40 h (M9 minimal media) at 37°C. All strains used in experiments were grown overnight in the media to be used for the experiment, were subcultured the following day into fresh media, and were maintained in exponential phase for at least eight generations prior to experimentation. Growth was monitored using OD_{600} read on a Shimadzu UV-1201 or a ThermoLabsystems Multiskan Spectrum spectrophotometer, and where necessary generation times were determined using the equation G = time interval / (3.3 x log (final OD_{600}/initial OD_{600})). Antibiotics were used in media at the specified concentrations. Bactericidal antibiotics used in this study are Ampicillin (Amp), Kanamycin (Kan), Nalidixic acid (Nal), Norfloxacin (Nor), and Streptomycin (Str), while bacteriostatic antibiotics are Tetracycline (Tet), Chloramphenicol (Chl), and Trimethoprim (Tmp). Unless noted otherwise below, all chemicals were obtained from Sigma (St. Louis, MO).

2.2 Strain construction

Strains used in this study are listed in Table 5 and, apart from wild type MG1655, were constructed using the PCR precise deletion method of Datskenko and Wanner

(Datsenko and Wanner, 2000). Strains were not constructed as part of this project – all strains were obtained either from the Coli Genetic Stock Center (Yale University, New Haven, CT – *E. coli* MG1655) or were previously constructed in this laboratory. Catalase and *rpoS* deletion strains were confirmed phenotypically prior to use through a gas evolution test with 30% H_2O_2. SOD deletion strains were confirmed phenotypically during use through measurement of generation time.

2.3 Antibiotic disc sensitivity assays

Desired strains were grown in triplicate to $OD_{600} = 0.8$ in either LB, M9/glucose (0.2%) or M9/glycerol (0.4%) media, were diluted 1:10 in 55°C soft agar (0.6%) media and immediately plated onto agar plates. Any additional compounds (bovine exogenous catalase, thiourea, and hydroxylamine) were added to the soft agar media immediately prior to addition of the culture. Concentrations or amounts given in the results section for these agents refer to that within the soft agar media. Once the soft agar was dry (1 h), antibiotic discs (VWR, West Chester, PA) were applied using a Sensi-disc dispenser (BD, Franklin Lakes, NJ). Discs used were: ampicillin (10 μg); kanamycin (30 μg); nalidixic acid (30 μg); norfloxacin (10μg); streptomycin (10 μg); tetracycline (30 μg); chloramphenicol (30 μg); and trimethoprim (5 μg). For catalase overexpression strains, IPTG (0.5 mM) was added to the culture 30 min prior to plating and to the plates as well. Inhibition zone diameters were measured the following day for rich media and after 40 h for minimal media, only measuring the zone of complete inhibition. Wild type MG1655 was included in each experiment for comparison and to control for any day to day variation in zone of inhibition.

2.4 Viable cell counts

Strains were grown in triplicate in LB broth to either OD_{600} = 0.1 (exponential phase) (Dwyer *et al.*, 2007;Kohanski *et al.*, 2007) or 1.5 (stationary phase). Kanamycin (5 μg/ml) or chloramphenicol (15 μg/ml), both above the MIC (3.75 μg/ml and 10 μg/ml, respectively), were then added and incubation continued for 4 h. Samples were taken from these cultures immediately prior to antibiotic addition and 2 and 4 h after addition, were serially diluted in 10 mM $MgCl_2$ and were plated on LB/agar plates. Only plates with 10 to 500 colonies were counted, and the number of colony-forming units (CFU) per ml was calculated.

2.5 3'-p-hydroxyphenyl fluorescein (HPF) assays

HPF is a fluorescent ROS reporter that is specific to hydroxyl radicals (Setsukinai *et al.*, 2003). Strains were grown in triplicate in LB broth to OD_{600} = 0.1 (exponential phase) or 1.5 (stationary phase), then had kanamycin (5 μg/ml) or chloramphenicol (15 μg/ml) added and continued incubating. Samples were taken prior to antibiotic addition and 4 h after, and samples were washed 3x in PBS buffer and had 5 μM HPF (in PBS) added for 30 min, as done previously (Kohanski *et al.*, 2007). Samples were then washed 3x in PBS buffer and HPF fluorescence was determined on a Tecan SAFIRE plate reader (490 nm excitation, 515 nm emission). OD_{600} was also found using this plate reader, and fluorescence data was normalized per OD_{600} unit.

2.6 qPCR

Strains were grown in triplicate in LB broth to OD_{600} = 0.2, then were either exposed to kanamycin (5 μg/ml) or chloramphenicol (15 μg/ml) for 30 min. RNA was then isolated from these cultures using a phenol-chloroform method. Once isolated, RNA was treated with DNase (Fermentas) and tested for the presence of DNA through PCR. RNA was reverse transcribed using MULV reverse transcriptase (New England Biolabs, Ipswich, MA) and then tested again for the presence of DNA via PCR. SYBR green (Applied Biosystems, Carlsbad, CA) was used to quantitatively monitor the qPCR reaction and qPCR itself was using an ABI 7500 Real-Time PCR System (Applied Biosystems). Optimized primers, designed using PerlPrimer software (Marshall, 2004), were 19-24 nt long, had predicted annealing temperatures of 59 to 61°C, and amplified 100–200 bp at the 3′ end of target genes (shown in Table 6). PCR conditions were 10 min at 94°C, followed by 40 cycles of heating at 94°C for 30 s and 60°C for 1 min, and final extension at 72°C for 5 min. Genes examined were (regulator or pathway in parentheses): *katE* (RpoS); *ahpC* (OxyR); *sodA* (SoxRS); *sodB* (Fur); *sfiA* (SOS repair pathway); and *rrsA* (used as a control gene to normalize expression (Kobayashi *et al.*, 2006)).

2.7 Mutant isolation and testing for cross-resistance

Wild type *E. coli* MG1655 overnight cultures were diluted 1:1000 into 10 ml fresh LB broth and grown to saturation (approx. 8 h). Cultures were concentrated 10-fold by centrifugation and each culture was plated onto 6 plates containing ampicillin (20 μg/ml), kanamycin (5 μg/ml), and norfloxacin (100 ng/ml). Plates were incubated at

37°C for 40 h, then putative resistant mutant colonies were purity streaked onto LB/agar plates containing the same antibiotics and incubated as before in order to confirm resistance. Total CFU plated was counted through serial dilutions onto LB media. 14 replicates were used to generate independent mutants.

After purity streaking, putative mutants were grown in triplicate in 200 µl LB media at 37°C for 8 h and replica plated onto each of the conditions given in Table 7. In addition to ampicillin, kanamycin, and norfloxacin, other agents used to screen for cross-resistance included: tetracycline as a representative bacteriostatic antibiotic; trimethoprim as an oxidative stress-dependent bacteriostatic antibiotic whose sensitivity (see results below); paraquat as an O_2^- generator testing SOD activity; and H_2O_2 testing catalase activity. Those mutants showing cross-resistance to oxidative stress were then grown as above for replica plating, and replica plated as a second screen on the conditions given in Table 8. Mutants were also plated onto EMB/agar to screen for contaminants, as EMB media inhibits the growth of Gram positive bacteria and distinguishes Gram negative bacteria based on whether they produce strong acid end products from lactose fermentation (as does *E. coli*). All putative paraquat cross resistant mutants from the first screen were stocked at -80°C.

Table 5. *E. coli* strains used in this study.

Strain	Genotype	Source
MG1655	*ilvG-rfb50 rpH1* (K-12)	(Blattner *et al.*, 1997)
HS2605	MG1655 Δ*katE*	(Li and Schellhorn, 2007)
HS2606	MG1655 Δ*katG*	(Li and Schellhorn, 2007)
HS2607	MG1655 Δ*katE* Δ*katG*	(Li and Schellhorn, 2007)
HS2210	MG1655 Δ*rpoS*	(Patten *et al.*, 2004)
HS2824	MG1655 Δ*oxyR*	Eva Diakun
HS2828	MG1655 Δ*rpoS* Δ*oxyR*	Eva Diakun
HS2906	MG1655 Δ*sodA*	Tao Dong
HS2910	MG1655 Δ*sodB*	Tao Dong
HS2911	MG1655 Δ*sodA* Δ*sodB*	Rebecca Jarvis

Note: Individuals listed are laboratory members responsible for the construction of the strain(s) indicated.

Table 6. Primers used for qPCR.

Primer	Target gene (regulon)	Primer sequence (5' to 3')
ahpCF	*ahpC* (OxyR)	TGAAGATGAAGGTCTGGC
ahpCR	*ahpC* (OxyR)	GTGAGAAGCTACGTACTGT
katEF	*katE* (RpoS)	GAAGTGACTGCGGATGAC
katER	*katE* (RpoS)	CTTCCATCAGGTAGTAGTTGG
sodAF	*sodA* (SoxRS)	CTACTGCTAACCAGGATTCTC
sodAR	*sodA* (SoxRS)	TTCAGGTAGTAAGCATGTTCC
sodBF	*sodB* (Fur)	TATCACTACGGCAAGCAC
sodBR	*sodB* (Fur)	GTTGTTGAATACGCCACC
sfiAF	*sfiA* (SOS)	TGTGGAGTCAATGGTTCG
sfiAR	*sfiA* (SOS)	CGCATCAACAAGTTCAGC
rrsAF	*rrsA* (Control)	CAAGCGTTAATCGGAATTACTG
rrsAR	*rrsA* (Control)	GCTACACCTGGAATTCTACC

Table 7. Conditions used for testing putative antibiotic resistant mutants.

LB/agar	LB/agar + 0.5 mM H_2O_2
LB/agar + 1 mM H_2O_2	LB/agar + 1.5 mM H_2O_2
LB/agar + 10 μg/ml Amp	LB/agar + 30 μg/ml Amp
LB/agar + 5 μg/ml Kan	LB/agar + 15 μg/ml Kan
LB/agar + 100 ng/ml Nor	LB/agar + 300 ng/ml Nor
LB/agar + 20 μg/ml Amp, 5 μg/ml Kan, 100 ng/ml Nor	LB/agar + 30 μg/ml Amp, 7.5 μg/ml Kan, 150 ng/ml Nor
LB/agar + 1 μg/ml Tmp	LB/agar + 3 μg/ml Tmp
LB/agar + 1.5 μg/ml Tet	LB/agar + 4.5 μg/ml Tet
LB/agar + 400 μM paraquat	LB/agar + 800 μM paraquat

Note: the antibiotic concentrations in the left column are (for Amp, Kan, and Nor) at or near the selective concentrations, while those in the right column are beyond this level and were used to determine whether a mutant is resistant to that concentration of the antibiotic.

Table 8. Conditions used for testing putative paraquat resistant mutants.

LB/agar	EMB/agar
LB/agar + 20 μg/ml Amp, 5 μg/ml Kan, 100 ng/ml Nor	
LB/agar + 200 μM paraquat	LB/agar + 400 μM paraquat
LB/agar + 600 μM paraquat	LB/agar + 800 μM paraquat
LB/agar + 1 mM paraquat	LB/agar + 1.5 mM paraquat
LB/agar + 2 mM paraquat	LB/agar + 3 mM paraquat
LB/agar + 4 mM paraquat	

Note: the top three conditions were used as controls, while conditions below the double line are used to determine the paraquat resistance level of the mutants.

Chapter 3. Results

3.1 Sensitivity of catalase deletion strains to antibiotics

To determine whether catalases have a protective role against bactericidal antibiotics, wild type *E. coli* strain MG1655 and isogenic catalase deletion strains HS2605 (Δ*katE*), HS2606 (Δ*katG*), and HS2607 (Δ*katE* Δ*katG*) were exposed to a representative set of both bactericidal and bacteriostatic antibiotics using antibiotic-containing discs. These were used as a means of screening a large number of strains against a large number of antibiotics efficiently. In LB media (Table 9a), all catalase deletion strains were more sensitive to the bactericidal antibiotics ampicillin, kanamycin, norfloxacin, and nalidixic acid and the bacteriostatic antibiotic trimethoprim than the wild type, with the zone of inhibition diameter being 1 to 4 mm greater in the deletion strain than the wild type. HS2607 was no more sensitive to these antibiotics than either HS2605 or HS2606, showing that the loss of catalase activity is not additive – losing both catalases produces the same result as losing one. While HS2605 was more sensitive to chloramphenicol and tetracycline than the wild type, no such systematic sensitivity differences were found for the bactericidal antibiotic streptomycin and the bacteriostatic antibiotics tetracycline and chloramphenicol resulting from loss of catalase.

The regulators OxyR and RpoS regulate *katG* and *katE* expression, respectively (discussed in detail above). To determine whether catalase regulators have the same impact on antibiotic sensitivity as catalases themselves, strains HS2824 (Δ*oxyR*), HS2210 (Δ*rpoS*), and HS2828 (Δ*oxyR* Δ*rpoS*) were tested against these same antibiotics in LB

media (Table 9b). Strains HS2210 and HS2824 were more sensitive to ampicillin, nalidixic acid, norfloxacin, and trimethoprim than the wild type, while there was no significant difference for kanamycin or streptomycin. Loss of OxyR also resulted in increased sensitivity to chloramphenicol and tetracycline, while there was no significant difference in sensitivity to these antibiotics due to loss of RpoS. Unlike the catalases, deletion of both *oxyR* and *rpoS* was additive to some extent, as strain HS2828 was more sensitive to kanamycin than HS2210, HS2824 or the wild type. Furthermore, HS2828 was similarly sensitive to chloramphenicol and tetracycline as HS2824, while there was no difference between HS2828 and the wild type for streptomycin.

In the proposed model linking bactericidal antibiotics and ROS generation (Kohanski *et al.*, 2007), the lethality of bactericidal antibiotics is the result of a surge of NADH consumption and the resulting autoxidation by O_2. *E. coli* strains lacking key TCA cycle enzymes, and thus forced to grow fermentatively, had increased viability in the presence of these antibiotics (Kohanski *et al.*, 2007), showing that the mode of respiration has an important role in bactericidal antibiotic sensitivity. To determine whether catalases also have a role in these same conditions, an assay involving wild type MG1655 and the isogenic catalase deletion strains HS2605, HS2606, and HS2607 as well as the Δ*rpoS* strain HS2210 was done using (i) M9 minimal media supplemented with glucose (0.2%) and (ii) M9 minimal media supplemented with glycerol (0.4%). *E. coli* can grow fermentatively on glucose minimal media while it cannot on glycerol minimal media (*E. coli* does not appreciably ferment glycerol at neutral or basic pH (Dharmadi, Murarka, and Gonzalez, 2006;Lin, 1976)) or in rich LB media. The results of these

assays are in Table 9c and 9d. On glucose, there were few significant differences from wild type sensitivities, with only HS2605 and HS2210 being more sensitive to ampicillin and HS2210 being more sensitive to trimethoprim. The general increase in sensitivity to bactericidal antibiotics, therefore, is abolished in glucose minimal media. However, in glycerol minimal media the catalase deletion strains HS2605, HS2606 and HS2607 were each more sensitive to ampicillin, kanamycin, and norfloxacin than the wild type, while HS2606 was more sensitive to streptomycin and tetracycline and HS2607 was more sensitive to both trimethoprim and tetracycline. Thus there was a restoration in the increased sensitivities to bactericidal antibiotics resulting from loss of catalase activity. Strain HS2210, in contrast, is more sensitive to ampicillin, nalidixic acid, norfloxacin, trimethoprim, and tetracycline, and this may or may not be catalase-related. Note that, in both minimal media, absolute antibiotic sensitivities were elevated compared to those in LB media, with the approximate sensitivity relationship being M9-glycerol > M9-glucose >> LB, though this is not true for nalidixic acid, trimethoprim, or chloramphenicol. In summary, loss of catalase activity resulted in an increase in bactericidal antibiotic sensitivity in both LB and M9-glycerol media but not M9-glucose media.

To confirm that it is the loss of catalase causing the changes in antibiotic sensitivity observed, wild type MG1655 and the isogenic catalase deletion strains HS2605, HS2606, and HS2607 were tested in LB media containing exogenous catalase (Table 10). While each catalase deletion strain was more sensitive to the antibiotics ampicillin, kanamycin, and norfloxacin initially, in general the sensitivity difference with the wild type decreased as the amount of exogenous catalase increased. Interestingly, the

effect was antibiotic dependent – less catalase was necessary to bring norfloxacin sensitivity down to the wild type level than ampicillin, while even 8 units of catalase was insufficient to restore kanamycin sensitivity to the wild type level for strains HS2606 and HS2607. Furthermore, the addition of exogenous catalase had no effect in the wild type, which suggests that increasing the level of catalase beyond that found commonly within the cell does not appreciably increase antibiotic resistance.

As a loss of catalase activity results in increased sensitivity to bactericidal antibiotics, it follows that a catalase inhibitor might similarly increase the sensitivity of wild type *E. coli* to those antibiotics. Both catalases in *E. coli*, HPI and HPII, contain a heme group as the catalyst for converting H_2O_2 into H_2O and O_2. Thus, catalases are sensitive to heme inhibitors, and hydroxylamine (NH_2OH), an H_2O_2 analog, is such an inhibitor and has been used as such previously (e.g. (Sevinc *et al.*, 1999;Switala and Loewen, 2002)). The sensitivity of hydroxylamine-exposed *E. coli* MG1655 and its isogenic catalase deletion strain HS2607 to antibiotics is shown in Table 11. Adding hydroxylamine appeared to have two effects. One was that the wild type strain became more sensitive to ampicillin, to the point where its sensitivity was nearly that of HS2607 at 1 μM NH_2OH. In many other cases, however, the antibiotic sensitivity actually decreased as the concentration of hydroxylamine increased, for both the wild type and HS2607. Interestingly, sensitivity to three antibiotics was significantly different between the two strains without hydroxylamine, and with 1 μM hydroxylamine the only antibiotic sensitivity that was significantly different between strains was that to ampicillin. Therefore the addition of hydroxylamine did reduce the difference in sensitivity between

the wild type and HS2607, as predicted, but it did not result in a predicted increase in antibiotic sensitivity save to ampicillin.

Thiourea is a powerful ·OH scavenger (Wasil *et al.*, 1987), and has previously been used to counteract the production of ·OH by bactericidal antibiotics (Kohanski *et al.*, 2007). Thiourea, therefore, was predicted to have the opposite effect of hydroxylamine, namely to reduce the sensitivity of a catalase-deficient strain to wild type levels (and reduce sensitivity to bactericidal antibiotics in general). Thiourea only reduced MG1655 sensitivity to kanamycin and chloramphenicol (Table 12), while it decreased the sensitivity of HS2607 to all antibiotics tested. However, there were still significant differences in sensitivity between the wild type and catalase deletion strains for ampicillin and (for particular strains) kanamycin, norfloxacin, and chloramphenicol. Therefore, the difference in sensitivity between wild type and catalase-deficient strains was decreased in thiourea, though not completely eliminated.

3.2 Sensitivity of SOD deletion strains to antibiotics

Much like catalases, superoxide dismutases (SOD) are also predicted to have a role in bactericidal antibiotic susceptibility based on the proposed model (Kohanski *et al.*, 2007). A strain with reduced SOD activity is thus predicted to be more sensitive to these antibiotics compared to the isogenic wild type strain. Combinatorial SOD deletion strains of *E. coli* MG1655 were constructed by two other students (Tao Dong and Rebecca Jarvis) and are listed above in Table 5. As with the catalase deletion strains, antibiotic disc sensitivity assays were done to screen several strains against a variety of antibiotics efficiently. Previously, such an assay has been done in LB media by R. Jarvis,

showing that the deletion of one SOD has little effect on antibiotic sensitivity, while deletion of both *sodA* and *sodB* results in increased sensitivity to a variety of antibiotics, not simply to those with a bactericidal mode of action. This result in LB media was confirmed (Table 13a). Neither deletion of *sodA* nor *sodB* individually resulted in widespread changes in antibiotic sensitivity. Interestingly, HS2911 (Δ*sodA* Δ*sodB*) was significantly more sensitive to all antibiotics save to aminoglycosides (kanamycin and streptomycin), to which it was less sensitive than the wild type. This may, however, be the result of a difference in growth rate – HS2911 had twice the generation time of WT in LB media, and bactericidal antibiotics in particular are known to primarily affect actively growing cells (Moellering, Jr., 1983). In any event, there was a correlation between loss of SOD activity and sensitivity to bactericidal antibiotics in rich media, but whether this is the direct result of a change in SOD activity is unclear.

The loss of catalase activity resulted in increased sensitivity only on non-fermentative media (see previous results above). It was possible that the loss of SOD activity resulted in increased antibiotic sensitivity only in particular media as well, and so strains HS2906 (Δ*sodA*), HS2910 (Δ*sodB*), HS2911 (Δ*sodA*Δ*sodB*) and wild type MG1655 were also tested in M9-glucose (0.2%) and M9-glycerol (0.4%). In M9-glucose (Table 13b), loss of SodA activity resulted in increased aminoglycoside sensitivity, while loss of both SodA and SodB activity resulted in decreased sensitivity to ampicillin and nalidixic acid but increased sensitivity to norfloxacin, trimethoprim, and tetracycline. No effects were observed specifically due to loss of SodB alone. No bactericidal antibiotic-specific effect, then, was observed. In M9-glycerol (Table 13c), neither HS2906 nor

HS2910 showed systematic differences in bactericidal (or bacteriostatic) antibiotic sensitivity. Strain HS2911, however, was less sensitive to the aminoglycosides and to nalidixic acid (each bactericidal) and more sensitive to all other antibiotics. Thus, loss of SOD activity did not categorically result in greater bactericidal antibiotic sensitivity in any of the tested media, and loss of all cytoplasmic SOD activity resulted in altered sensitivity to a variety of antibiotics. One possible confounding factor for these results, though, is growth rate – there was a substantial difference in generation time between the wild type and the SOD deletion strains in these media (Table 14), with an increase of approximately 25 to 60 min for HS2906 and HS2910 and approximately 120 min for HS2911 relative to MG1655, consistent with published data (Carlioz and Touati, 1986). Therefore, even those differences that were observed may not be the direct result of loss of SOD activity.

3.3 Viable cell counts

The disc sensitivity assays from above showed a difference in antibiotic sensitivity between catalase deletion strains and the wild type that largely followed the bactericidal / bacteriostatic grouping of the antibiotics themselves. To quantify the actual change in viability (which the disc sensitivity experiments cannot do), direct cell counts were done on cultures exposed to antibiotics. The antibiotics kanamycin and chloramphenicol were selected as representative of bactericidal and bacteriostatic antibiotics, respectively. These counts were done on wild type MG1655 and HS2605 (Δ*katE*), HS2606 (Δ*katG*), HS2607 (Δ*katE*Δ*katG*) and HS2210 (Δ*rpoS*) strains in both exponential and stationary phase (Figures 16-19), as the expression of *katE* is growth

phase dependent. The loss of catalase activity in exponential phase cells had no effect in 5 μg/ml kanamycin (Figure 16), as each strain had a very similar reduction in viability over the 4 h of the experiment. A similar experiment was done with stationary phase cultures as well (Figure 17); however, 5 μg/ml kanamycin did not induce a significant loss of viability save in HS2607 and HS2210. The antibiotic treatment was essentially bacteriostatic for the other strains, with some amount of growth occurring through the experiment. In chloramphenicol, a representative bacteriostatic antibiotic, there were no large differences in viability over time between strains regardless of growth phase (Figures 18 and 19). Therefore, viability only decreases in a catalase-null strain and *rpoS* strain in stationary phase – in all other cases no decrease in viability was observed compared to the wild type.

3.4 Detection of oxidative stress

Based on the proposed model discussed above (Kohanski *et al.*, 2007), strains lacking catalase activity and SOD activity should have more ·OH being produced than the wild type when exposed to bactericidal antibiotics. Hydroxyl radicals have an extremely short lifespan ($t_{1/2} \sim 10^{-9}$ s) and as a result cannot directly be detected. Therefore, 3'-(p-hydroxyphenyl) fluorescein (HPF) is used which is oxidized specifically by ·OH into a brightly fluorescent molecule (excitation 490 nm / emission 515 nm) (Setsukinai *et al.*, 2003). HPF, then, is an indirect measure of the ·OH concentration within the cell, and was used to determine the amount of ·OH produced during exposure to a bactericidal (kanamycin) and a bacteriostatic (chloramphenicol) antibiotic.

To determine whether catalase activity level does indeed affect ·OH concentration during bactericidal antibiotic exposure, the wild type strain MG1655 and the catalase deletion strains HS2605 (Δ*katE*), HS2606 (Δ*katG*), and HS2607 (Δ*katE* Δ*katG*) were assayed using HPF. Samples were assayed both prior to antibiotic exposure and after 4 h of exposure (Figure 20). The ·OH concentration was higher in HS2605 in kanamycin, regardless of growth phase, and was higher in chloramphenicol in exponential phase. Strain HS2606 appeared to have elevated levels of ·OH in all phases and antibiotics, though this was not statistically significant. The ·OH concentration in HS2607 was similar to the wild type in each case. Interestingly, the ·OH concentration actually decreased after the application of kanamycin in all strains – counter to the predicted result based on the above model.

Similar experiments were done with the SOD deletion strains HS2906 (Δ*sodA*), HS2910 (Δ*sodB*), and HS2911 (Δ*sodA*Δ*sodB*). In exponential phase cultures (Figure 21a), there is initially more ·OH in HS2911 compared to the wild type, while strains HS2906 and HS2910 showed no significant difference from the wild type. After 4 h of incubation, no strain had significantly different levels of ·OH compared to the wild type in either antibiotic. Again, the amount of ·OH production on the whole decreased over the course of the experiment, as every final culture was less fluorescent than the initial cultures. In stationary phase cultures (Figure 21b), both HS2910 and HS2911 had significantly higher ·OH levels initially than the wild type. After incubation in chloramphenicol, all strains had statistically similar, elevated levels of ·OH. This was also the result after kanamycin incubation, though the SOD deletion strains may have had

slightly higher levels of ·OH. However, even if real, the difference in ·OH concentration between wild type and SOD deletion strains is small, no more than a 40% difference. In contrast, the ·OH concentration was greater in chloramphenicol-treated cultures than kanamycin-treated cultures and, in fact, greater or similar in untreated cultures after 4 h than in kanamycin-treated cultures. Therefore, loss of SOD activity may result in increased ·OH production upon addition of kanamycin, but this increase is slight and there may be other variables affecting the results.

Alternatively, the level of oxidative stress can also be investigated through the activities of the OxyR, RpoS, and SoxRS regulons (each is described above). Thus, qPCR was done to examine these regulons, as well as that of Fur, involved in Fe^{2+} regulation, and of the SOS repair pathway, as the oxidative stress is hypothesized to be causing damage to the cell (including the bacterial chromosome). Oxidative stress, iron regulation, and repair pathways have all been implicated in the response to bactericidal antibiotics (Dwyer *et al.*, 2007;Kohanski *et al.*, 2007). Five representative genes were examined, with their regulon in parentheses: *katE* (RpoS); *ahpC* (OxyR); *sodA* (SoxRS); *sodB* (Fur); and *sfiA* (SOS repair). To normalize the data, *rrsA* was also examined as a control. Wild type MG1655, HS2606 (Δ*katG*) and HS2210 (Δ*rpoS*) were tested in duplicate after exposure to either kanamycin or chloramphenicol (shown in Table 15). There was no observable effect of the chloramphenicol on the expression level of these genes in any of the three strains, save of on *katE* in HS2210, as expected given that *katE* expression is highly RpoS-dependent. Similarly, there was no observable effect of the

kanamycin on any of these genes, though the variation in the kanamycin samples is quite large.

3.5 Isolation of antibiotic-resistant mutants in oxidative stress pathways

The model proposed for the action of bactericidal antibiotics outlines a common pathway (generation of ROS) for these antibiotics to exert their toxicity. It should be possible, then, to select for and isolate mutants in this common pathway through the application of a combination of bactericidal antibiotics. Based on the model, these mutants would be resistant to those antibiotics within the selective mix as well as (i) other bactericidal antibiotics – as they would use a similar pathway; and (ii) oxidative stresses. Selecting and screening for such mutants is the basis for the following experiments.

Ampicillin, kanamycin, and norfloxacin were used for selection purposes, as each has a different primary target: ampicillin, a β-lactam, inhibits cell wall cross-linking; kanamycin, an aminoglycoside, inhibits translation; and norfloxacin, a fluoroquinolone, inhibits DNA replication. No previous reports of resistance mechanisms include each of these antibiotics (discussed further below). Therefore, the only common pathway should be the oxidative stress pathway described above and in (Kohanski *et al.*, 2007). To determine a proper selective concentration of these three antibiotics, the MICs of these antibiotics to MG1655 was determined (Table 16). From this MIC data, concentrations of 20 μg/ml ampicillin, 5 μg/ml kanamycin, and 100 ng/ml norfloxacin were chosen for selective purposes. This was followed by an experiment to determine the number of cells

that can be plated on one plate and still produce mutant colonies, as too few cells produces no mutants and too many produces a uniform lawn of growth, thus presumably suppressing mutant development. 3×10^9 to 5×10^9 CFU per plate generated the desired result of several mutants arose per plate with little to no background growth, and this number of CFU per plate was used below.

Next, the number of CFU necessary for a comprehensive mutational study was calculated. Given that the mutational frequency, per base, in *E. coli* is approximately 5.4×10^{-10} (Drake *et al.*, 1998;Drake, 1999), 1.85×10^9 cells should contain one mutant, on average, at that particular base (and at every base within the chromosome). However, bases do not mutate at the same frequency – it is believed that there is a difference of 10^4 in frequency between the most mutable base and the least mutable base within the *E. coli* genome (Drake *et al.*, 1998). Approximately 10^4-fold coverage of the genome, then, is necessary to ensure that each of these bases has mutated in some cell of the population. In addition, individual base substitutions (e.g. T→C, T→A, and T→G) occur at different frequencies. Apart from the effects of hotspots, there is approximately a 10-fold difference in frequency between C·G→T·A (the most common) and C·G→G·C (the least common) and 3- to 4-fold difference between C·G→T·A and the other substitutions, based on *lacI* data (Hutchinson, 1996). Between this difference, and the fact that three base substitutions are possible at each base, approximately 30-fold coverage of the genome is necessary to ensure that each mutation (of the three possible at each base) has happened. In total, then, approximately 3×10^5-fold coverage of the genome would ensure that every point mutation that could happen within the genome has happened in

some cell of the population, requiring $1.85 \times 10^9 \times 3 \times 10^5$ = approximately 5.5×10^{14} cells, or approximately the number of cells present in 200 L of saturated *E. coli* culture. Here, approximately 0.07% of this number of cells was examined.

14 independent replicates were grown to saturation, then plated onto LB/agar plates containing the selective antibiotics from above. 3.97×10^{11} cells in total were plated, giving 214-fold coverage of the genome. In total, 738 mutants were obtained on the original selection plates (Table 17), and of these, 241 grew after being purity streaked on the same selective media (32.7% of the total). These 241 mutants were screened for resistance to ampicillin, kanamycin and norfloxacin (beyond the concentration on which they were originally selected), as well as paraquat and H_2O_2, indicative of superoxide resistance and hydrogen peroxide resistance, respectively, and many mutants were screened against tetracycline and trimethoprim as well. In this screen test, a mutant was considered resistant if it grew as a full spot (i.e resembling a lawn in that spot) and grew better than the wild type, while a sensitive mutant either grew poorer than the wild type, as independent colonies (likely from secondary mutations), or simply did not grow. While many mutants were resistant to at least one of ampicillin, kanamycin, and norfloxacin beyond the selective concentration (Table 18), many others were also resistant to tetracycline (33.3%), trimethoprim (3.2%), and paraquat (36.1%) beyond wild type levels, none of which were directly used for selection – cross-resistance therefore occurred. No mutants were resistant to H_2O_2 beyond wild type levels.

The paraquat-resistant mutants in particular were examined further as they represent antibiotic-resistant mutants with increased resistance to oxidative stresses.

These were tested against a broader range of paraquat concentrations, along with the wild type, to determine to what extent these mutants are resistant to paraquat. Wild type *E. coli* MG1655 is resistant to paraquat to between 1 and 1.5 mM (as determined through this experiment); therefore, mutants that grew in the presence of 1.5 mM paraquat or more were considered resistant, with the same growth criteria as in the preliminary screen (see Table 19 and Figure 22). 37 of the 87 mutants grew at this concentration of paraquat (42.5%), with 23 (26.4%) resistant to greater than 2 mM paraquat, at least 1.5-fold that of the wild type (assuming a wild type sensitivity to ~1.2 mM) and 3 (3.4%) resistant to 3 mM paraquat, at least 2-fold that of the wild type. Therefore, some of these mutants are significantly resistant to paraquat despite being selected on bactericidal antibiotics, supporting the model that bactericidal antibiotics and oxidative stress resistance are related.

Mutants that are resistant to oxidative stresses should be more resistant to a variety of antibiotics, based on the above model. Therefore, the resistances of these 37 mutants to ampicillin, kanamycin, norfloxacin, tetracycline, and trimethoprim were compared to those of the other 204 mutants. Of the mutants cross-resistant to paraquat, a greater proportion were also cross-resistant to other stresses (save trimethoprim) than is seen in the overall population of mutants (Figure 23). The greatest correlation was between paraquat resistance and norfloxacin resistance, with 78.4% resistance in the paraquat-resistant mutants but only 34.3% resistance in the paraquat-sensitive mutants. In addition, paraquat resistance also correlated with tetracycline resistance (54.5% in paraquat-resistant mutants vs. 28.8% in paraquat-sensitive mutants) despite no selection

occurring on tetracycline. Both ampicillin and kanamycin resistance were also more common in paraquat-resistant mutants. Interestingly, then, paraquat resistance occurs with greater resistance to other antibiotics, as predicted by the model.

Paraquat resistance readily occurs through overexpression of the *emrE* gene, encoding a multi-drug transporter which pumps paraquat, ethidium, tetracycline, tetraphenylphosphonium, erythromycin, sulfadiazine, and other toxic compounds out of the cell along with H^+ (Morimyo *et al.*, 1992;Yerushalmi, Lebendiker, and Schuldiner, 1995). In contrast, the above model for ROS generation through antibiotics (Kohanski *et al.*, 2007) predicts that paraquat resistance resulting from selection on antibiotics should be in a direct oxidative stress pathway, such as in SOD genes. To test whether the increase in paraquat resistance is due to a mutation in *emrE*, the *emrE* gene was sequenced in wild type MG1655 and in five paraquat-resistant mutants. The gene was identical to the wild type in all five mutants (and to the published sequence). Therefore, this change in paraquat-resistance was independent of *emrE*, supporting the model for ROS generation through antibiotics.

Resistance to oxidative stress can also be the result of a reduction in growth rate, as this would reduce the endogenous production of ROS through metabolism. The generation time of the five mutants whose *emrE* gene was sequenced was therefore determined (Table 20), and none had a growth rate comparable to the wild type. Four of the five mutants had generation times approximately twice the length of the wild type, while one mutant had a generation time approximately four times that of the wild type. Therefore, the reduction in growth rate itself may also be a factor in paraquat resistance.

Table 9. Inhibition zone diameters of selected antibiotics in wild type and catalase mutants.

A			Luria-Bertani media (rich media)						
Strain	Genotype	Amp	Kan	Nal	Nor	Str	Tmp	Chl	Tet
MG1655	Wild type	12.8±0.3	10.7±0.2	18.2±0.7	12.8±0.5	9.5±0.2	20.0±0.4	16.2±0.4	18.6±0.4
HS2605	Δ*katE*	16.3±0.3**	11.7±0.2*	21.3±0.5**	16.2±0.4**	9.2±0.3	23.7±0.3**	17.8±0.2*	19.8±0.2*
HS2606	Δ*katG*	15.5±0.3**	12.0±0.0**	20.5±0.5**	16.8±0.4*	9.3±0.2	22.2±0.2**	17.5±1.0	18.7±0.7
HS2607	Δ*katE* Δ*katG*	15.8±0.2**	12.5±0.3*	21.3±0.3**	16.0±0.7*	9.7±0.2	22.3±0.4**	16.5±0.5	18.5±0.3
B			Luria-Bertani media (rich media)						
Strain		Amp	Kan	Nal	Nor	Str	Tmp	Chl	Tet
MG1655	Wild type	11.5±0.3	11.0±0.3	11.3±0.3	13.5±0.3	9.5±0.2	14.5±0.6	12.7±0.2	12.3±0.2
HS2210	Δ*rpoS*	12.5±0.2*	12.2±0.5	13.0±0.4*	17.0±0.4**	9.8±0.2	17.0±0.4**	13.2±0.2	12.3±0.3
HS2824	Δ*oxyR*	12.7±0.2*	12.2±0.7	13.8±0.4**	16.2±0.5*	9.7±0.2	16.2±0.2*	14.2±0.4*	13.5±0.3*
HS2828	Δ*rpoS*Δ*oxyR*	13.2±0.2*	13.3±0.2**	13.5±0.3**	17.8±0.9*	10.0±0.3	17.5±0.2**	14.5±0.4**	13.3±0.3*
C			M9-glucose (0.2%) media (minimal media)						
Strain		Amp	Kan	Nal	Nor	Str	Tmp	Chl	Tet
MG1655	Wild type	21.5±0.3	18.7±0.6	19.2±0.3	26.0±0.7	14.0±0.3	14.5±0.5	16.2±0.8	26.5±0.5
HS2605	Δ*katE*	22.8±0.5	19.7±0.4	20.0±0.5*	26.7±0.7	14.7±0.4	15.0±0.4	16.8±0.7	26.3±0.4
HS2606	Δ*katG*	21.0±0.5	17.7±0.8	18.8±0.3	25.5±1.4	14.2±0.2	15.8±0.7	16.0±0.7	24.8±0.7
HS2607	Δ*katE* Δ*katG*	21.7±0.3	18.0±0.4	19.3±0.3	25.3±0.7	14.0±0.4	15.2±1.1	17.0±0.4	25.0±0.6
HS2210	Δ*rpoS*	23.8±0.7**	18.5±0.7	19.8±0.5	27.0±0.4	14.0±0.3	19.8±0.7**	17.8±0.7	26.7±0.9
D			M9-glycerol (0.4%) media (minimal media)						
Strain		Amp	Kan	Nal	Nor	Str	Tmp	Chl	Tet
MG1655	Wild type	23.5±0.2	21.8±0.6	19.3±0.5	39.7±0.4	16.8±0.6	19.8±1.3	20.7±1.3	30.7±0.4
HS2605	Δ*katE*	25.2±0.2**	24.2±0.5**	20.2±0.3	42.7±0.5**	17.0±0.4	21.5±0.2	21.2±0.3	31.7±0.4
HS2606	Δ*katG*	26.3±0.5**	25.8±0.8**	20.7±0.7	42.8±0.5**	19.5±0.6*	23.0±1.1	19.3±0.6	32.3±0.6*
HS2607	Δ*katE* Δ*katG*	25.8±0.5*	24.2±0.2*	19.7±0.3	42.7±0.4**	16.8±0.3	23.5±0.5*	20.7±0.8	31.8±0.3
HS2210	Δ*rpoS*	26.7±0.4**	22.0±0.4	23.0±0.3**	42.8±0.9**	17.7±0.2	24.0±0.5*	21.7±0.6	34.8±0.5**

Note: Inhibition zone diameters include the disc itself and are given as the mean of two discs of three replicates ± SE.

* = significantly different from the wild type at P = 0.05. ** = significantly different from the wild type at P = 0.01.

Table 10. Effect of exogenous catalase on antibiotic sensitivity.

Strain	Genotype	Catalase (U)	Amp	Kan	Nor	Str	Tmp	Chl
MG1655	Wild type	0	13.8±0.3	11.3±0.3	16.0±0.4	10.0±0.0	21.0±0.7	15.3±0.5
		0.8	13.5±0.5	11.3±0.5	16.0±0.0	10.3±0.3	20.5±0.3	14.8±0.3
		8	14.5±0.5	10.5±0.5	16.5±0.5	10.0±0.0	20.5±0.5	15.0±0.0
HS2605	Δ*katE*	0	16.0±0.0**	15.0±0.6**	18.0±0.0*	10.0±0.0	22.3±1.1	15.0±0.6
		0.8	15.0±0.0	13.7±0.8	16.7±0.7	10.0±0.0	21.3±0.3	15.0±0.0
		8	13.5±0.5	12.0±0.0	16.0±0.0	9.5±0.5	19.5±0.5	14.5±0.5
HS2606	Δ*katG*	0	15.5±0.3*	15.0±0.4**	19.2±0.7	10.2±0.2	22.5±1.0	15.0±0.3
		0.8	15.5±0.3*	15.0±0.4**	16.5±0.3	10.0±0.0	21.0±0.4	15.5±0.3
		8	14.3±0.3	12.7±0.3*	16.2±0.2	9.5±0.2	18.3±0.5	14.7±0.2
HS2607	Δ*katE* Δ*katG*	0	15.7±0.2*	15.5±0.2**	18.7±0.5*	10.2±0.2	22.0±0.0	16.2±0.2
		0.8	15.2±0.3**	12.8±0.3**	16.5±0.2	10.0±0.3	20.5±0.2	15.7±0.2
		8	14.3±0.2	12.8±0.3*	17.0±0.4	10.0±0.3	19.8±0.2	15.5±0.2

Note: inhibition zone diameters include the disc itself and are given as the mean of three replicates ± SE. * = significantly different from the wild type (0 catalase units) at P = 0.05. * = significantly different from the wild type (0 catalase units) at P = 0.01. Amount of each antibiotic per disc is given in the methods.

Table 11. Antibiotic sensitivities in hydroxylamine.

0 nM NH_2OH							
Strain	Genotype	Amp	Nal	Nor	Tmp	Chl	Tet
MG1655	Wild type	14.0±0.0	16.0±0.3	18.9±0.4	19.6±0.4	17.3±0.2	16.1±0.2
HS2607	Δ*katE*Δ*katG*	15.8±0.5**	16.3±0.4	20.9±0.6*	20.5±0.4	17.3±0.2	16.8±0.2*
500 nM NH_2OH							
Strain	Genotype	Amp	Nal	Nor	Tmp	Chl	Tet
MG1655	Wild type	15.5±0.3**	16.3±0.4	18.9±0.4	19.3±0.4	16.8±0.2*	16.3±0.2
HS2607	Δ*katE*Δ*katG*	16.1±0.1	16.3±0.3	19.9±0.4	19.8±0.4	16.3±0.2*	16.4±0.2
1 μM NH_2OH							
Strain	Genotype	Amp	Nal	Nor	Tmp	Chl	Tet
MG1655	Wild type	15.6±0.2**	15.5±0.4	19.1±0.3	19.1±0.2	15.8±0.2**	15.9±0.2
HS2607	Δ*katE*Δ*katG*	16.1±0.1*	15.6±0.2	19.0±0.3	19.5±0.2	16.0±0.2	16.1±0.1

Note: values are the diameter of the zone of inhibition, in mm, given as mean ± SE and are the average of two plates each of three replicates. Amount of each antibiotic per disc is given in the methods. Concentration of NH_2OH is the concentration in the soft agar LB media prior to plating on LB/agar plates. * indicates significantly different from the wild type (MG1655), 0 NH_2OH sample (for other wild type samples) or from the corresponding wild type sample at the same NH_2OH concentration (for HS2607) at $P = 0.05$. ** indicates significantly different at $P = 0.01$.

Table 12. Antibiotic sensitivities in thiourea.

0 mM thiourea								
Strain	Genotype	Amp	Kan	Nal	Nor	Tmp	Chl	Tet
MG1655	Wild type	15.3±0.3	13.3±0.2	16.4±0.2	18.0±0.4	21.6±0.6	16.9±0.2	17.0±0.3
HS2607	Δ*katE*Δ*katG*	18.4±0.3**	15.3±0.3**	17.6±0.5*	20.8±0.4**	23.3±0.5*	18.0±0.2**	18.5±0.4**
150 mM thiourea								
Strain	Strain	Amp	Kan	Nal	Nor	Tmp	Chl	Tet
MG1655	Wild type	14.9±0.2	12.3±0.3**	16.0±0.3	17.6±0.5	21.9±0.4	15.5±0.3**	16.1±0.4
HS2605	Δ*katE*	16.5±0.2**	14.1±0.1**	16.5±0.2	18.3±0.2	22.4±0.3	17.3±0.4**	16.8±0.3
HS2606	Δ*katG*	17.0±0.3**	12.9±0.2	16.3±0.2	19.3±0.3*	22.1±0.2	17.4±0.3**	16.9±0.4
HS2607	Δ*katE*Δ*katG*	17.1±0.2**	13.1±0.5	16.0±0.3	17.6±0.5	21.9±0.4	15.5±0.3	16.1±0.4

Note: Values are the diameter of the zone of inhibition, in mm, given as mean ± SE and are the average of two plates each of three replicates. Amount of each antibiotic per disc is given in the methods. Concentration of thiourea refers to the concentration in the soft agar LB media prior to plating on LB/agar plates. * indicates significantly different from the wild type (MG1655), 0 thiourea sample (for the 150 mM thiourea wild type sample) or from the corresponding wild type sample at the same thiourea concentration at P = 0.05. ** indicates significantly different at P = 0.01.

Table 13. Antibiotic sensitivities of wild type and SOD mutants.

A					Luria-Bertani media				
Strain	Genotype	Amp	Kan	Nal	Nor	Str	Tmp	Chl	Tet
MG1655	Wild type	15.3±0.2	11.5±0.1	13.8±0.1	16.8±0.2	10.8±0.2	15.8±0.1	15.8±0.2	15.8±0.1
HS2906	Δ*sodA*	14.8±0.2	12.0±0.2	13.8±0.2	16.5±0.3	10.0±0.2	15.5±0.1	15.5±0.1	15.5±0.1
HS2910	Δ*sodB*	14.7±0.1	12.0±0.2	13.0±0.2	16.3±0.2	9.8±0.1	15.7±0.1	15.7±0.1	15.7±0.1
HS2911	Δ*sodA*Δ*sodB*	16.7±0.1**	10.3±0.1*	15.7±0.2**	17.7±0.2	8.7±0.1**	17.0±0.2	17.2±0.3	17.0±0.2
B					M9-glucose (0.2%)				
Strain	Genotype	Amp	Kan	Nal	Nor	Str	Tmp	Chl	Tet
MG1655	Wild type	19.7±0.2	16.7±0.2	19.3±0.3	30.2±0.6	13.5±0.3	26.0±0.4	20.7±0.6	26.3±0.3
HS2906	Δ*sodA*	19.3±0.8	18.3±0.4**	19.5±0.3	30.3±0.5	14.8±0.5*	24.8±1.4	20.8±0.5	27.3±0.3
HS2910	Δ*sodB*	19.2±0.3	16.2±0.5	19.8±0.4	29.7±0.8	13.3±0.4	26.5±0.5	20.8±0.6	26.7±0.6
HS2911	Δ*sodA* Δ*sodB*	16.8±0.7**	17.7±0.6	14.8±1.3**	35.8±0.8**	13.4±0.4	27.3±0.5	20.5±0.6	29.0±0.4**
C					M9-glycerol (0.4%)				
Strain	Genotype	Amp	Kan	Nal	Nor	Str	Tmp	Chl	Tet
MG1655	Wild type	22.2±0.5	27.0±0.4	21.7±0.7	40.7±0.3	21.0±0.3	22.7±0.7	18.8±0.5	31.0±0.4
HS2906	Δ*sodA*	21.8±0.4	26.8±0.4	20.2±0.3	39.3±0.3*	21.0±0.4	22.2±0.3	18.8±0.3	31.7±0.2
HS2910	Δ*sodB*	21.0±0.5	26.0±0.4	21.2±0.3	40.3±0.5	20.0±0.4	23.0±1.2	18.5±0.7	31.5±0.4
HS2911	Δ*sodA* Δ*sodB*	24.7±0.6**	23.8±0.9*	19.7±0.5*	45.2±1.4**	18.8±0.6**	37.0±0.9**	25.8±0.7**	39.3±1.1**

Note: Values are the diameter of the zone of inhibition, in mm, given as mean ± SE and are the average of two plates each of three replicates. Amount of each antibiotic per disc is given in the methods. * indicates significantly different from the wild type in the same media at P = 0.05. ** indicates significantly different at P = 0.01.

Table 14. Generation times of SOD mutants in minimal media.

		Generation Time	
Strain	Genotype	M9-glucose	M9-glycerol
MG1655	Wild type	81.4 ± 20.0	99.2 ± 4.7
HS2906	Δ*sodA*	93.8 ± 10.9	120.0 ± 7.7
HS2910	Δ*sodB*	125.8 ± 24.1	117.7 ± 16.8
HS2911	Δ*sodA* Δ*sodB*	134.1 ± 5.3	236.4 ± 118.0

Note: Values are the given as mean ± SE and are the average of three replicates. Each value was calculated from at least three time points.

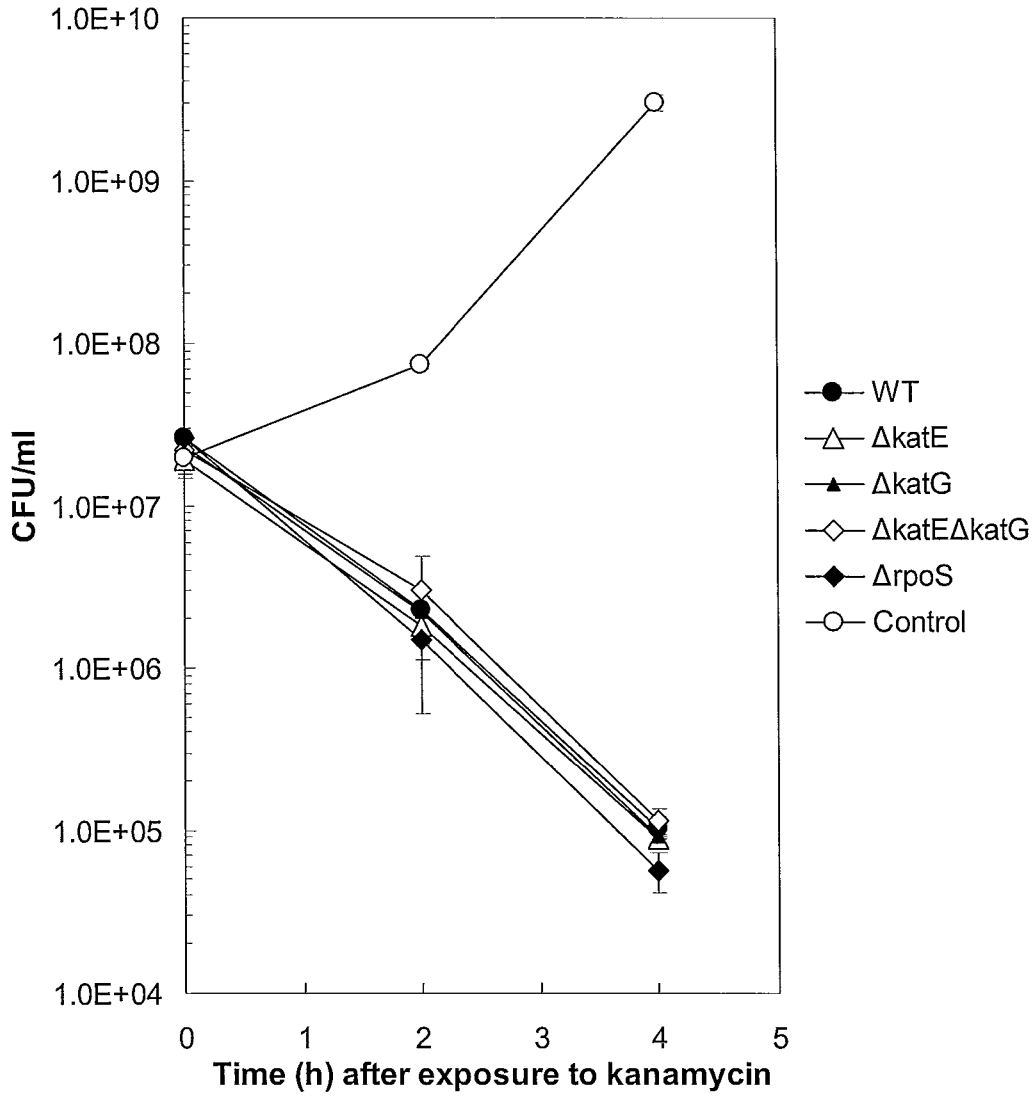


Figure 16. Viability of exponential phase catalase deletion strains in kanamycin. Strains were incubated in 5 μg/ml kanamycin save for the control strain (no antibiotics). Values are the means of three replicates. Error bars represent SE of the values.

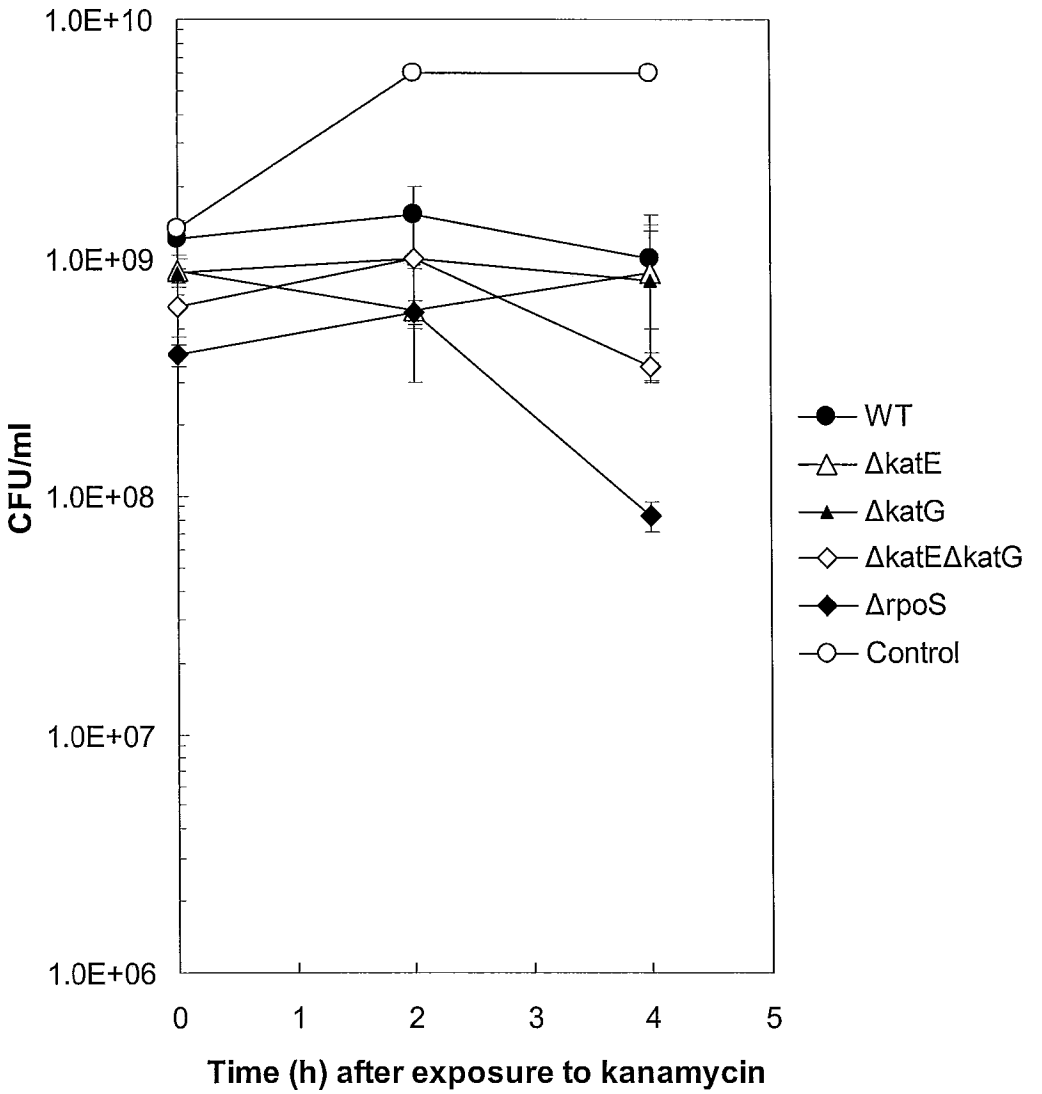


Figure 17. Viability of stationary phase catalase deletion strains in kanamycin. Strains were incubated in 5 μg/ml kanamycin save for the control strain (no antibiotics). Values are the means of three replicates. Error bars represent SE of the values.

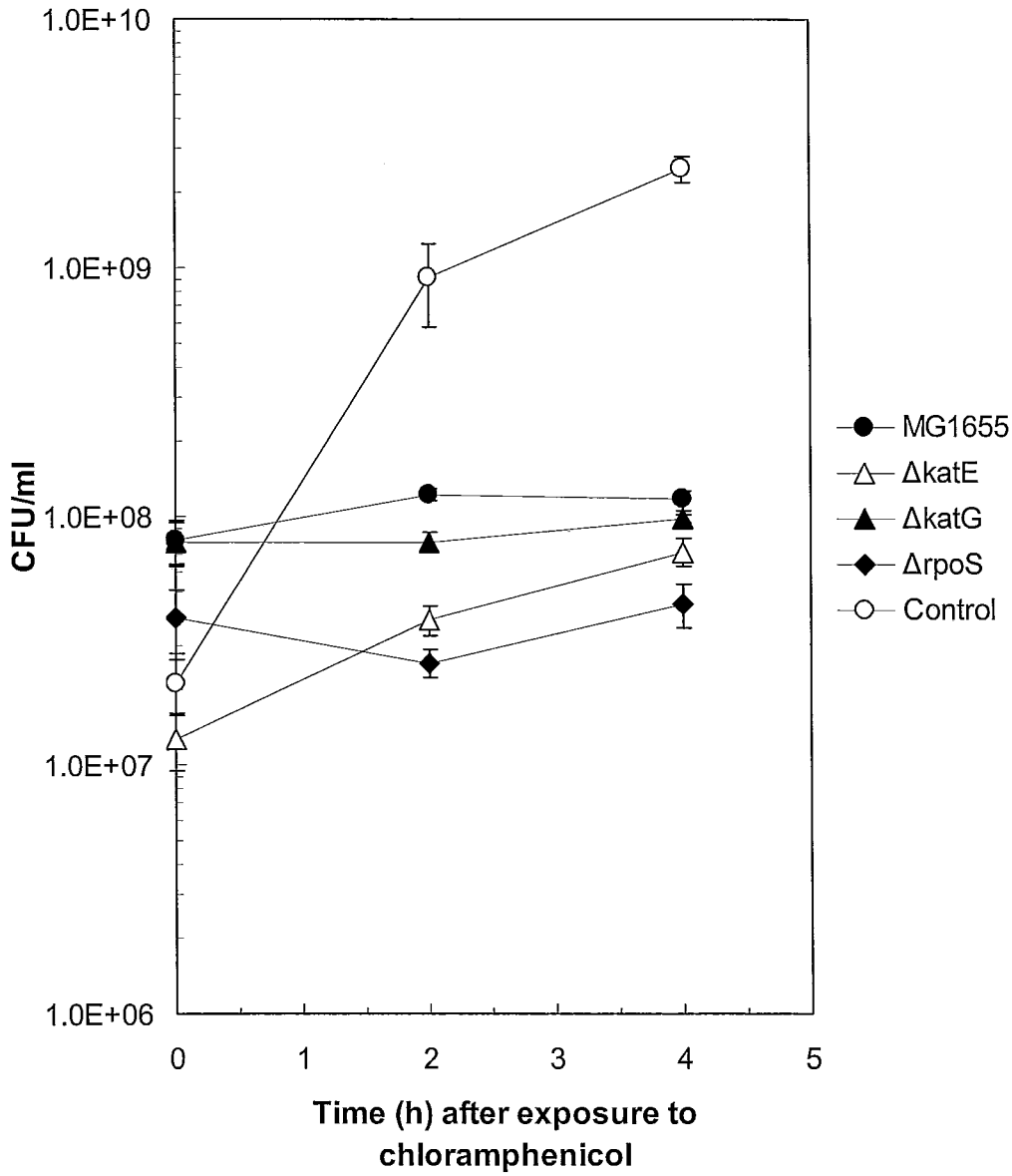


Figure 18. Viability of exponential phase catalase deletion strains in chloramphenicol. Strains were incubated in 15 μg/ml chloramphenicol save for the control strain (no antibiotics). Values are the means of three replicates. Error bars represent SE of the values.

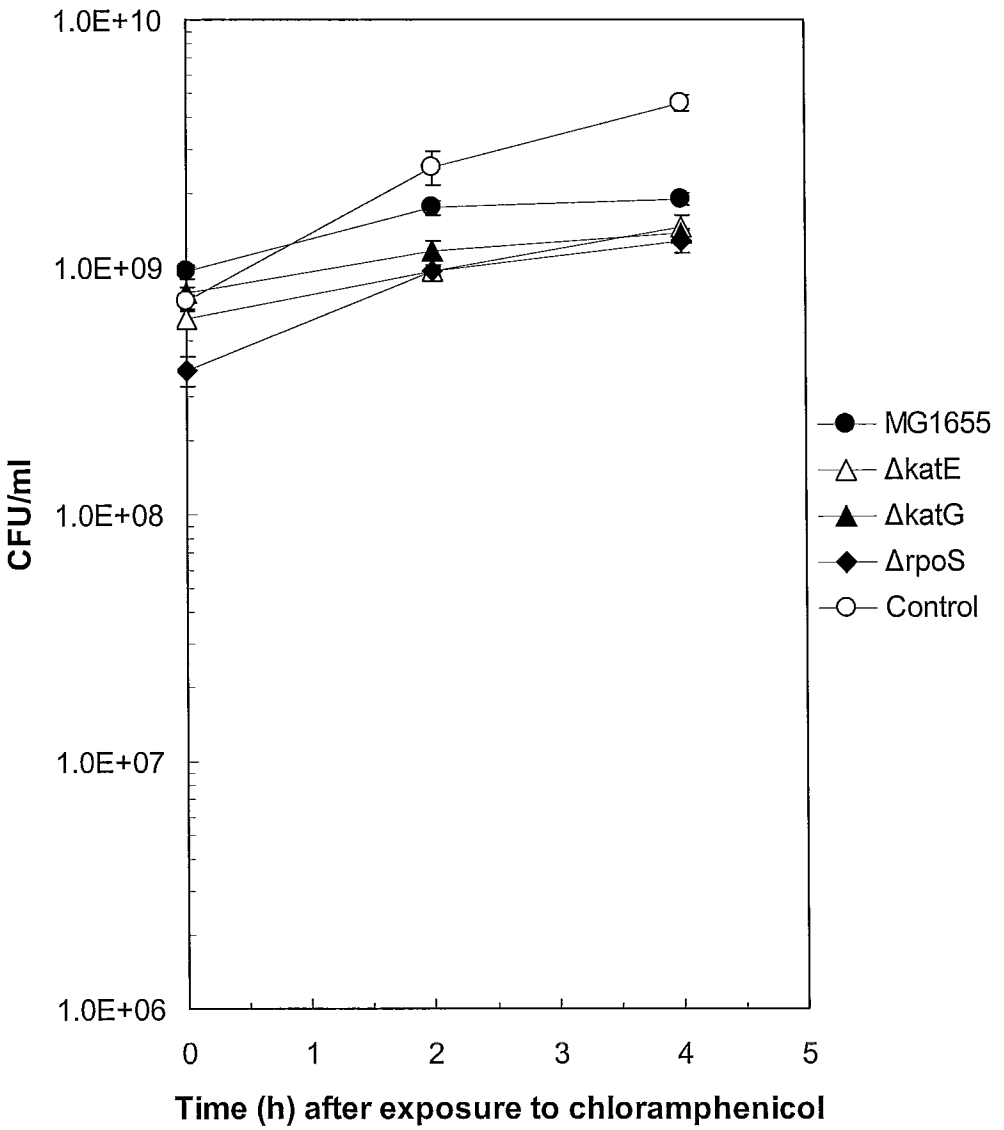


Figure 19. Viability of stationary phase catalase deletion strains in chloramphenicol. Strains were incubated in 15 μg/ml chloramphenicol save for the control strain (no antibiotics). Values are the means of three replicates. Error bars represent SE of the values.

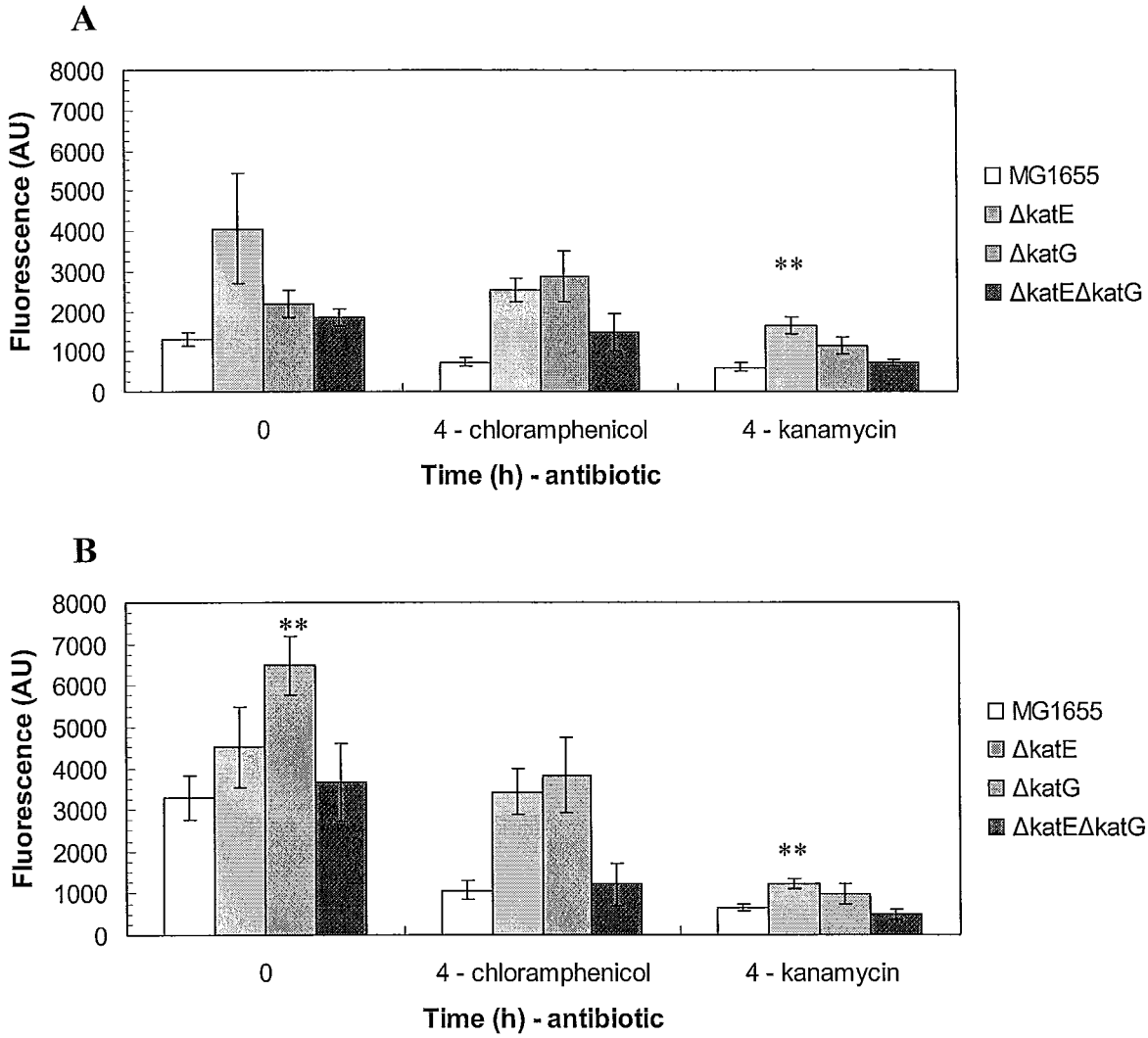


Figure 20. Hydroxyl radical production in *E. coli* catalase deletion strains. All results shown are means ± SE of three replicates, and the X-axis indicates the amount of time incubated in the antibiotic indicated. Fluorescence is given in arbitrary units (AU; raw fluorescence value per OD_{600}). A shows exponential phase samples and B shows stationary phase samples. * indicates significantly different from the WT in the same media at P = 0.05. ** indicates significantly different at P = 0.01.

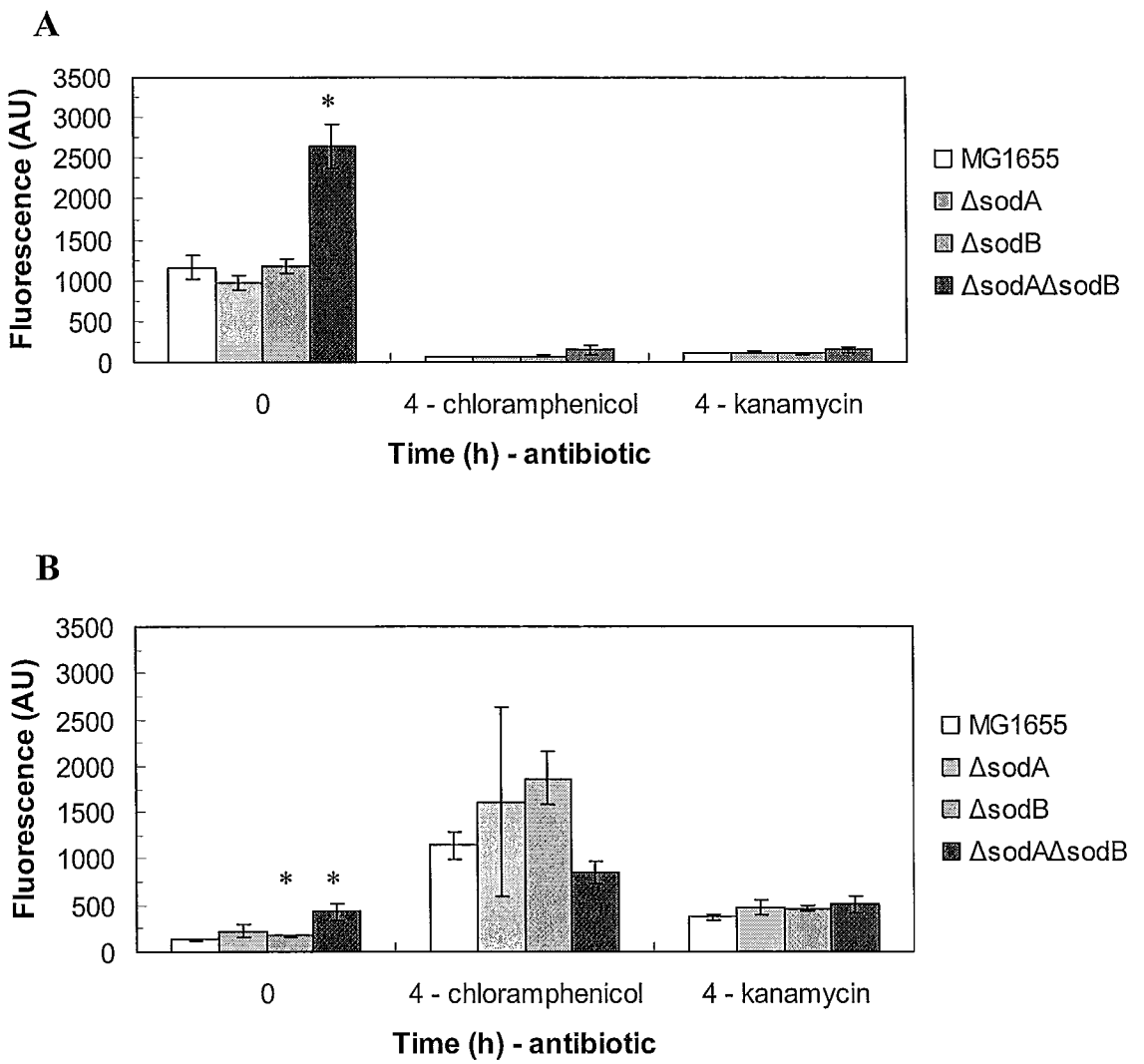


Figure 21. Hydroxyl radical production in *E. coli* SOD deletion strains. All results shown are means ± SE of three replicates, and the X-axis indicates the amount of time incubated in the antibiotic indicated. Fluorescence is given in arbitrary units (AU; raw fluorescence value per OD_{600}). A shows exponential phase samples and B shows stationary phase samples. * indicates significantly different from the WT in the same media at P = 0.05. ** indicates significantly different at P = 0.01.

Table 15. Expression level of selected genes as determined by qPCR.

Strain	Genotype	Antibiotic	RNA level normalized to rrsA (log2)				
			katE	ahpC	sodA	sodB	sfiA
MG1655	Wild type	--	0.802±0.047	0.879±0.059	0.879±0.049	0.873±0.046	0.974±0.052
		Kan	0.725±0.066	0.758±0.069	0.752±0.070	0.784±0.074	0.844±0.082
		Chl	0.865±0.051	0.899±0.065	0.887±0.039	0.855±0.470	0.920±0.507
HS2606	Δ*katG*	--	0.822±0.031	0.890±0.051	0.904±0.037	0.856±0.019	0.967±0.028
		Kan	0.779±0.123	0.835±0.164	0.822±0.131	0.846±0.141	0.939±0.167
		Chl	0.869±0.070	0.928±0.102	0.863±0.037	0.923±0.087	1.007±0.097
HS2210	Δ*rpoS*	--	0.687±0.050	0.738±0.060	0.769±0.071	0.758±0.060	0.835±0.086
		Kan	0.789±0.095	0.801±0.108	0.817±0.111	0.821±0.085	0.887±0.099
		Chl	0.927±0.146	0.947±0.169	0.883±0.143	0.927±0.121	0.976±0.147

Note: For each experiment, N = 2. Values are given as means ± SE.

Table 16. MICs of selection and cross-resistance antibiotics on *E. coli* MG1655.

	MIC	
Antibiotic	Day 1	Day 2
Ampicillin	5.0 μg/ml	7.5 μg/ml
Kanamycin	3.75 μg/ml	3.75 μg/ml
Norfloxacin	150 ng/ml	150 ng/ml
Tetracycline	3.0 μg/ml	3.5 μg/ml

Note: MICs were calculated from 12 biological replicates of *E. coli* MG1655 incubated at 37°C on LB/agar plates. Isolated colonies alone were not counted as growth, though background growth in addition to isolated colonies was counted as growth. MICs were consistent with previously published data (Goswami, Mangoli, and Jawali, 2007;Goswami and Jawali, 2007;Olofsson *et al.*, 2007).

Table 17. Number of antibiotic-resistant mutants isolated from wild type MG1655.

Replicate	CFU/ml	Total CFU plated	# mutants plated	# mutants streaked	# mutants/ CFU
1	4.09E+10	2.94E+10	26	8	2.72E-10
2	2.86E+10	2.06E+10	17	12	5.84E-10
3	2.76E+10	1.98E+10	17	6	3.02E-10
4	2.66E+10	1.92E+10	15	7	3.65E-10
5	2.85E+10	2.05E+10	9	2	9.76E-11
6	2.35E+10	1.69E+10	15	5	2.96E-10
7	2.54E+10	1.83E+10	13	4	2.19E-10
8	2.17E+10	1.56E+10	17	8	5.12E-10
9	4.22E+10	4.30E+10	96	22	5.12E-10
10	3.62E+10	3.69E+10	57	25	6.77E-10
11	3.48E+10	3.55E+10	91	38	1.07E-09
12	4.03E+10	4.11E+10	108	24	5.84E-10
13	3.94E+10	4.01E+10	146	45	1.12E-09
14	3.88E+10	3.95E+10	111	35	8.86E-10
Total		**3.965E+11**	**738**	**241**	**6.08E-10**

Note: CFU/ml values are the mean of duplicates. Volume plated is the total of 6 plates. "# mutants plated" refers to mutants on the original selective plates, while "# mutants streaked" refers to the number of mutants that grew after purity streaking. "# mutants/CFU" was calculated as # mutants streaked per CFU plated. Mutants were selected on 20 μg/ml ampicillin, 5 μg/ml kanamycin, and 100 ng/ml norfloxacin.

Table 18. Summary of resistances of antibiotic-selected *E. coli* MG1655 mutants.

Replicate	Mutants Screened	Selective agent resistance				Cross-resistance			
		Amp^R	Kan^R	Nor^R	Amp^R Kan^R Nor^R	Tet^R	Tmp^R	$H_2O_2{}^R$	$Paraquat^R$
1	8	3	1	0	0	n.t.	n.t.	0	0
2	12	9	5	0	3	n.t.	n.t.	0	6
3	6	4	1	0	1	n.t.	n.t.	0	1
4	7	2	2	0	0	n.t.	n.t.	0	3
5	2	2	0	1	0	n.t.	n.t.	0	1
6	5	4	2	0	0	n.t.	n.t.	0	4
7	4	2	0	0	0	n.t.	n.t.	0	1
8	8	8	0	3	1	n.t.	n.t.	0	2
9	22	13	0	8	0	10	1	0	8
10	25	10	1	8	1	3	0	0	5
11	38	30	4	20	1	12	2	0	8
12	24	13	14	19	2	11	1	0	15
13	45	22	24	13	6	10	1	0	19
14	35	4	8	27	2	17	1	0	14
Total	**241**	**126**	**62**	**99**	**17**	**63**	**6**	**0**	**87**
%	**100.0**	**52.3**	**25.7**	**41.1**	**7.1**	**33.3**	**3.2**	**0.0**	**36.1**

Note: mutants were deemed resistant if a full spot grows on media containing the selective agent. Isolated colonies were not counted as growth. Mutants were resistant to ampicillin if they grew at 30 μg/ml; resistant to kanamycin if they grew at 15 μg/ml; and resistant to norfloxacin if they grew at 300 ng/ml; resistant to tetracycline if they grew at 4.5 μg/ml; and resistant to trimethoprim if they grew at 1 μg/ml. "Amp^R Kan^R Nor^R" refers to this combination of antibiotics used at 1.5 times the selective concentration (Amp 30 μg/ml; Kan 7.5 μg/ml; Nor 150 ng/ml). "n.t." indicates that those mutants were not tested on those antibiotics.

Table 19. Summary of paraquat resistance testing of putative paraquat resistant mutants.

Category	N	% of Total
Number of mutants	87	100.0
Number of mutants resistant to 200 μM	83	95.4
Number of mutants resistant to 400 μM	78	89.7
Number of mutants resistant to 600 μM	76	87.4
Number of mutants resistant to 800 μM	71	81.6
Number of mutants resistant to 1 mM	55	63.2
Number of mutants resistant to 1.5 mM	37	42.5
Number of mutants resistant to 2 mM	23	26.4
Number of mutants resistant to 3 mM	3	3.4
Number of mutants resistant to 4 mM	0	0.0

Note: mutants were deemed resistant if a full spot grows on media containing the selective agent. Isolated colonies were not counted as growth. The double line is the sensitivity of wild type MG1655 to paraquat – the wild type was resistant to anything above that line and sensitive to anything below that line.

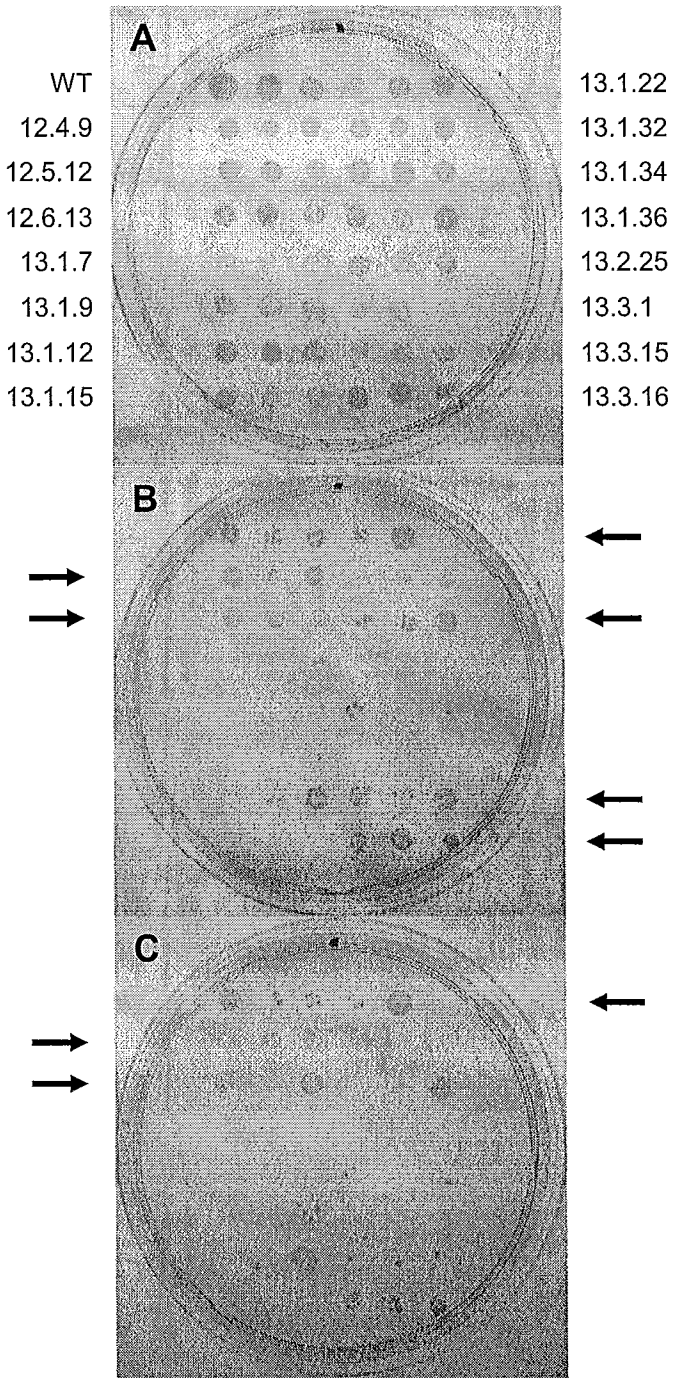


Figure 22. Replica plate images of paraquat cross-resistant mutants. All strains are shown in triplicate. Strains are identified in image A. Arrows show paraquat (PQ) resistant mutants. Plate A: 0 mM PQ; plate B: 1.5 mM PQ; plate C: 2 mM PQ.

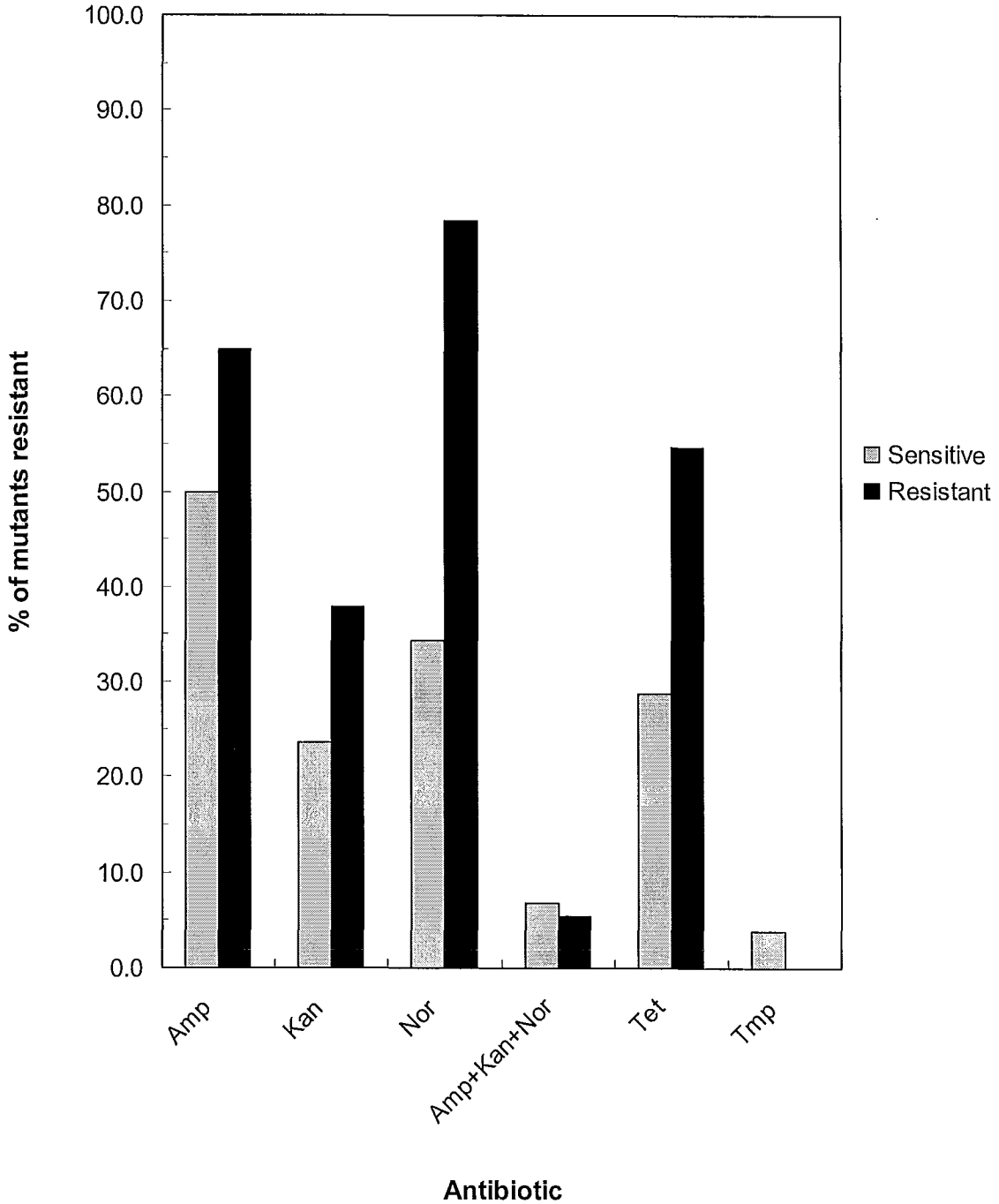


Figure 23. Mutants resistant to both paraquat and other compounds. Amp Kan Nor is the combination of these antibiotics at 1.5-fold the selective concentration. The light bars represent paraquat sensitive and the dark bars paraquat resistant mutants.

Table 20. Generation times of *E. coli* MG1655 and mutants.

Strain	Generation time
MG1655	22.7 ± 0.2
2.1.1	95.7 ± 6.2
9.1.25	36.2 ± 3.7
10.3.1	46.0 ± 6.6
11.5.2	40.0 ± 7.6
14.1.7	53.7 ± 19.2

Note: generation time was calculated in LB broth without antibiotics, and is reported as mean ± SE. Cultures were incubated in triplicate at 37°C and 200 rpm.

Chapter 4. Discussion

4.1 Sensitivity of catalase deletion strains to antibiotics

In general, the disc sensitivity assays support the model that bactericidal antibiotics induce cell death through ROS. This effect was observed on exposure to the bactericidal antibiotics ampicillin, kanamycin, nalidixic acid, and norfloxacin in LB media, though streptomycin did not show such an effect. Also in agreement with the model, this effect was not observed with the bacteriostatic antibiotics tetracycline or chloramphenicol, though it was observed on exposure to trimethoprim. The type of media did affect the importance of catalases against these antibiotics, which is consistent with the model that ROS generation is dependent on the use of NADH in respiration (Kohanski *et al.*, 2007). LB media has few fermentable energy sources (Baev *et al.*, 2006), and so *E. coli* in LB must grow using the TCA cycle and a terminal electron acceptor. Cells grown in M9-glucose minimal media are able to grow fermentatively, thus reducing the use of the TCA cycle and its consequent generation of NADH. The inability to ferment glycerol forces *E. coli* to grow using the TCA cycle in M9-glycerol media (Lin, 1976), and catalase activity was an important factor in sensitivity to ampicillin, kanamycin, and norfloxacin in this medium.

H_2O_2 is diffusible through the plasma membrane of *E. coli*, though not fully permeable as differences in internal and external concentrations of H_2O_2 can be maintained (Seaver and Imlay, 2001). The majority of H_2O_2 in a culture is generated intracellularly, as exponential phase *E.* coli can generate up to 14 μM of H_2O_2 per second

(Seaver and Imlay, 2001). By the proposed model, H_2O_2 is converted into ·OH through free Fe^{2+} in the cell (Kohanski *et al.*, 2007). That exogenous catalase can complement a catalase deletion strain shows that the diffusion of H_2O_2 is sufficient to reduce the intracellular concentration of H_2O_2 by exogenous detoxification. It seems reasonable, then, that catalases within other cells may produce this protective effect as well (though this would require penetrating more layers of plasma membrane), and so a catalase-deficient strain may still be afforded some protection against the toxic ROS generated by antibiotics if it is part of an environment containing cells with high catalase activity. Therefore any potential use of this data in medical applications (such as for catalase inhibitors) may have to take into account the population of cells, not simply the target species. Interestingly, adding exogenous catalase did not reduce sensitivity of the wild type MG1655 strain despite reducing the antibiotic sensitivity of catalase-deficient strains. Furthermore, an undergraduate student (R. Jarvis) tested catalase overexpression strains (taken from (Kitagawa *et al.*, 2005)) for antibiotic sensitivity, and found that, even for antibiotics whose sensitivity was catalase-dependent, overexpressing these catalases did not reduce sensitivity to the antibiotic below the wild type level. This is in contrast to a published work showing that there was indeed an effect in MC4100 (Kolodkin-Gal *et al.*, 2008). The data presented here, then, suggests that wild type MG1655 has an optimal catalase level for responding to antibiotics – neither more catalase activity nor less resulted in increased resistance to these antibiotics. It is possible that the difference between this data and the published data in MC4100 is due to strain-dependent effects (discussed further below).

While these experiments were conducted on catalase deletion strains, a more medically-relevant test would be to conduct these experiments on catalase inhibited strains (as these could be administered with antibiotics), with the goal of generating a system much like the β-lactam/β-lactamase inhibitor combination drug. The catalase inhibitor hydroxylamine was expected to increase wild type antibiotic sensitivity to the level of the catalase deficient strains. The hydroxylamine did, in fact, do so with respect to ampicillin, but while the difference in sensitivity between a wild type and catalase-deficient strain was reduced in hydroxylamine, as predicted, the absolute sensitivity often decreased in general, where it was predicted to rise. Catalase HPII activity is 80% inhibited at 200 nM hydroxylamine *in vitro* (Sevinc *et al.*, 1999) and catalase HPI is 67% inhibited in *Salmonella* at 5 μM (Loewen and Stauffer, 1990). The concentrations listed in the table are in the soft agar only (4 ml) which was added to the surface of the plate (28 ml). If hydroxylamine can diffuse freely between the two agar layers, then the maximum level of hydroxylamine tested was approximately 125 nM (75% inhibition of HPII (Sevinc *et al.*, 1999)), essentially the same level of inhibition for HPII catalase. This inhibition should, then, increase the sensitivity of the wild type strain to the level seen in HS2605 (Δ*katE*), and the fact that this did not happen may be due to the non-specific inhibition of heme groups by hydroxylamine. Heme is a common cofactor in *E. coli* proteins, and inhibition of the entire set of heme-containing proteins may somehow produce an effect that protects against bactericidal antibiotics. These antibiotics tend to affect rapidly growing cells most, and inhibition of these enzymes may reduce the growth rate (this was not tested in hydroxylamine), thus reducing sensitivity to these antibiotics

(reviewed in (Brown, Allison, and Gilbert, 1988;Brown and Gilbert, 1993)). In summary, the effect of hydroxylamine cannot be separated from the other proteins that it inhibits concurrently.

The addition of thiourea did, in fact, reduce the sensitivity of strain HS2607 (Δ*katE*Δ*katG*) to all antibiotics as predicted, while the wild type either had similar or reduced sensitivity to antibiotics (with the greatest reductions, interestingly, in tetracycline, kanamycin, and chloramphenicol, two of which are bacteriostatic). A difference in antibiotic sensitivity remained, however, between the wild type and HS2607, even upon addition of thiourea. Much like for hydroxylamine, the thiourea concentration was calculated based on the soft agar (4 ml), and so the actual concentration of thiourea in the plate (if it can freely diffuse) was approximately 20 mM. The concentration that is known to inhibit the lethality of bactericidal antibiotics is approximately 150 mM (Kohanski *et al.*, 2007). A higher concentration of thiourea, then, might allow the catalase deletion strains' sensitivity to antibiotics to match that of the wild type, but in any event, it is clear that thiourea reduces the effect of bactericidal antibiotics in catalase-deficient strains.

4.2 Sensitivity of SOD deletion strains to antibiotics

SOD have an analogous role as catalases in the proposed model for the action of bactericidal antibiotics (Kohanski *et al.*, 2007), and so it was predicted that SOD activity might have a similar effect, particularly after finding that catalase activity influenced sensitivity to bactericidal antibiotics. Interestingly, single SOD mutants are not significantly more sensitive to bactericidal or bacteriostatic antibiotics than the wild type.

The SOD double mutant HS2911 was less sensitive to aminoglycosides in non-fermentative media (LB and M9/glycerol), and generally more sensitive to an antibiotic than the wild type in minimal media, particularly M9-glycerol, regardless of the mode of action of the antibiotic. In general, therefore, there is no comparable effect seen in SOD-deficient strains as in catalase-deficient strains when exposed to antibiotics. The decreased sensitivity to aminoglycodies in HS2911 may have been the result of a slower growth rate, as discussed above. This cannot be ignored as a potential confounding factor, though if this were the only factor involved strains HS2906 (Δ*sodA*) and HS2910 (Δ*sodB*) would be less sensitive to aminoglycosides than the wild type in minimal media, as their generation times were significantly slower than the wild type. This was not the case.

While not shown here, an experiment (done by R. Jarvis) was conducted to see if the addition of exogenous SOD could complement strain HS2911, restoring it to wild type sensitivity to antibiotics much like exogenous catalase can reduce antibiotic sensitivity. There was no effect from the exogenous addition of SOD on antibiotic sensitivity. This is not altogether surprising, as the SOD activity lost from this strain is cytoplasmic – the periplasmic SOD (SodC) is still present and O_2^- is not freely permeable across the membrane (reviewed in (Gort, Ferber, and Imlay, 1999)). Therefore, if the increased antibiotic sensitivity of strain HS2911 is indeed the direct result of loss of SOD activity, then the exogenous SOD likely could not reach the stressed area (cytoplasm), thus limiting its effectiveness. It has been shown in *E. coli* MC4100 that overexpression of SodA can result in reduced lethality in rifampicin, spectinomycin, and chloramphenicol (Kolodkin-Gal *et al.*, 2008). It is unclear how much of this applies to

MG1655-derived strains, as none of these antibiotics are bactericidal in MG1655 and chloramphenicol sensitivity (the only antibiotic of these tested) was only affected by loss of SOD activity in M9-glycerol media. Overexpression of either SodA or SodB may, though, have an effect where exogenous SOD did not – it would more directly replace the missing activity of the strain. It is also possible that overexpression of SodA or SodB might be beneficial for the cell in the face of antibiotic stress. This was not found for catalases, but it is entirely possible for SOD, and would be consistent with the spontaneous mutation results obtained (see below). Therefore, future overexpression experiments involving SodA and SodB would produce valuable results.

Unlike for catalase regulators, SOD regulator mutants were not made and (consequently) not tested for increased antibiotic sensitivity. It would be interesting to see whether SOD regulator mutants (Fur, SoxRS) would result in similar sensitivities as the direct SOD mutants. In the case of catalase regulators, this was not the case – their deletion led to increased sensitivity to a wide variety of antibiotics, unlike catalases themselves. However, (i) none of the catalase mutants were more resistant to any antibiotics (unlike the SOD mutants); and (ii) many of the SOD mutants themselves had changes in antibiotic sensitivity that did not correlate to whether an antibiotic was bactericidal. It is difficult to predict, then, the effect of the loss of SOD regulators from the catalase results, as there are so many differences between the two, and such an experiment would provide useful data, much like the overexpression study above.

4.3 Viable cell counts

Previously-published viable cell counts show that both exponential phase MG1655 Δ*sodA*Δ*sodB* and MG1655 Δ*katE*Δ*katG* are more sensitive to gentamicin than the wild type up to 4 h after exposure (Kohanski *et al.*, 2008). Here, no difference in viability was found in exponential phase between the wild type and any catalase or *rpoS* deletion strain. The stationary phase results show reduced viability in kanamycin for HS2607 (Δ*katE*Δ*katG*) and HS2210 (Δ*rpoS*) compared to the wild type, as a small amount of lethality occurred in those strains and essentially a bacteriostatic result was obtained from the others tested. This appears to contradict the disc sensitivity assays, which correlate loss of catalase activity with increased kanamycin sensitivity. This may be a concentration-dependent effect, as those cells close to the discs would be exposed to higher concentrations of kanamycin than during the viable cell counts. Furthermore, there may be a significant difference in sensitivity only after greater than the four hours tested here – a previously published report found that MG1655 Δ*sodA*Δ*sodB* and MG1655 Δ*katE*Δ*katG* had similar viability as the wild type after 4 h but did not continue the experiment beyond that point (Kohanski *et al.*, 2008). Either dependency (concentration or time) would reconcile the somewhat contradictory disc sensitivity and viable cell count results. As expected based on previously published results (Kohanski *et al.*, 2007), and in agreement with the disc sensitivity data, there was no difference in viable cell counts between any strain and the wild type in the bacteriostatic antibiotic chloramphenicol in either growth phase.

4.4 Detection of oxidative stress

HS2605 (Δ*katE*) produced more HPF fluorescence (and, by extension, ·OH) than the wild type in kanamycin at each growth phase and in chloramphenicol in exponential phase, while both strains HS2606 (Δ*katG*) and HS2607 (Δ*katE*Δ*katG*) produced statistically similar amounts of ·OH as the wild type in both antibiotics. While the results for HS2605 are expected in kanamycin, as it is more sensitive to kanamycin based on the disc sensitivity experiments, this is not the case with chloramphenicol, and so the increase in ·OH levels is puzzling. Furthermore, HS2606 and HS2607 are also more sensitive to kanamycin than the wild type, yet its ·OH levels are the same – another confusing result. A possible confounding variable in this is the fact that the majority of cells in the culture die during the exposure to kanamycin (as seen in the viability assay above – 99% of cells are dead after 4 h exposure). This could explain why the absolute values of HPF fluorescence tend to decrease from the initial sample (immediately prior to antibiotic addition) through to the final sample. While the fluorescence data is normalized to OD_{600}, this measurement does not distinguish between live and dead cells, and during the viable cell counts the cultures' OD_{600} is continuously increasing despite a decrease in CFU/ml – showing that dead cells constitute the bulk of the OD_{600} reading. In order to obtain accurate results, it may be necessary to normalize the fluorescence to a better indicator of live cell count than an optical density measurement. Furthermore, it is entirely possible that the antibiotic affects the permeability of the HPF (Hassett and Imlay, 2007), which could add a further variable to these results. As an alternative, recent studies have examined protein carbonylation as a method of examining *mazEF*-dependent

production of ROS, as ·OH causes protein carbonylation (Kolodkin-Gal *et al.*, 2008;Kolodkin-Gal and Engelberg-Kulka, 2009). It is possible such an approach could be adapted for this application as well, and it could be a superior route to examine the level of toxic ROS within the cell, though it is not specific for ·OH. Four hours may also be too long after exposure to capture the elevated ·OH production, and so perhaps examining the cultures after 1 h or 2 h would improve the assay as well.

The loss of one or two SOD resulted in little change in ·OH levels, which is consistent with the disc sensitivity assay results. The latter shows little change in antibiotic sensitivity upon partial loss of SOD activity, and so based on the former there should be little change in ·OH production, as was found. In both growth phases, HS2911 (Δ*sodA*Δ*sodB*) had elevated levels of ·OH initially. This is to be expected, as this strain would have greater amounts of O_2^- within the cell, triggering the production of ·OH (as discussed above). Interestingly, however, that significant difference in ·OH level disappears after the application of antibiotics. The criticisms of HPF and potential confounding variables apply here as well as the HPF assays for the catalase deletion strains. In addition, O_2^- is produced as a by-product of respiration, and it is possible that cells under antibiotic stress have slowed metabolic activity, resulting in a significant reduction in O_2^- production. Therefore, the HPF data may not accurately reflect the state of oxidative stress within the cell for either the SOD-deficient or catalase-deficient strains.

A published report showed that many OxyR- and SoxRS-dependent genes are activated upon addition of gentamicin (Kohanski *et al.*, 2008). This was not the case here, as addition of chloramphenicol or kanamycin did not result in changes in any of the

representative genes for the RpoS, OxyR, SoxRS or Fur regulons in wild type MG1655. It is possible that this is a concentration dependent effect – perhaps exposure to a greater amount of kanamycin would result in a detectable change in gene expression. A longer time-course may also provide a similar result. A third replicate would help reduce the error in the measurement and perhaps give a more accurate indication of whether this transcription is occurring. Therefore, this experiment should be repeated in the future.

4.5 Isolation of antibiotic resistant mutants

A selection strategy involving three different bactericidal antibiotics was used in which many antibiotic resistant spontaneous mutants were generated from wild type *E. coli* MG1655. Many of these mutants were also cross-resistant to other antibiotics or paraquat, an O_2^- generating compound. While many of these mutants may be clonal (which would be identifiable by having multiple mutants with similar resistance profiles) mutants were originally produced from 14 independent colonies, and paraquat-resistant mutants were obtained from several of these original colonies, showing that multiple non-clonal mutations did occur. This result was predicted based on the model (Kohanski *et al.*, 2007) that bactericidal antibiotics induce oxidative stress – logically, a mutant resistant to multiple such antibiotics owes its resistance to a mutation in an oxidative stress pathway. Based on the disc sensitivity results, cross-resistance to H_2O_2 would be expected as opposed to paraquat, as loss of SOD has less effect on bactericidal antibiotic sensitivity than loss of catalases. This was not the case. However, a hypothesis that overexpression of SOD might be beneficial against these antibiotics, which was not directly tested here, was presented above. A second hypothesis, that changing the level

of catalase in the cell only results in an increase in sensitivity, was also presented above. Interestingly, these mutational results, isolating paraquat resistant mutants but not H_2O_2 resistant mutants, are consistent with both of these hypotheses.

While paraquat resistance is typically the result of a mutation in the multi-drug transporter EmrE, this was not observed in any of the five mutants whose *emrE* gene was sequenced, suggesting that such a mutation is not a common route for the paraquat cross-resistance observed in the antibiotic-resistant mutants isolated. It is possible that such a combination of antibiotics would select for a multi-drug transporter, as the outer membrane porin OprD is involved in resistance to both β-lactams and fluoroquinolones (Livermore, 2002), while multi-drug transporters are implicated in resistance to aminoglycosides (Vakulenko and Mobashery, 2003). However, no known multi-drug transporter is involved in resistance to each of β-lactams, aminoglycosides, and fluoroquinolones, much less paraquat as well, and so it seems unlikely that a transport protein is responsible for both the antibiotic resistance and paraquat cross-resistance. The observed reduced growth rate of mutants compared to the wild type may also be responsible for the observed paraquat resistance. However, during the screening process, nearly all mutants were observed to grow more slowly than the wild type, yet most mutants were not cross-resistant to paraquat, suggesting that some other factor is at work than simply a growth rate reduction. The above model for the action of bactericidal antibiotics (Kohanski *et al.*, 2007) predicts that oxidative stress pathways would be a common pathway for these, and while unconfirmed, it is the most parsimonious explanation for the set of resistances observed. To conlusively determine where the

mutations are, the mutants' genomes could be sequenced, or alternatively could be mapped using an Hfr strain. The mutation would be predicted to be dominant, as it should represent a gain in O_2^--scavenging function, and could be detected by such mapping.

The paraquat cross-resistant mutants were also more likely to be cross-resistant to one of the selection antibiotics (beyond the actual selective concentration used) – the predicted result as oxidative stress pathways should affect a variety of antibiotics. An upregulation of a pathway that detoxifies paraquat, then, should reduce the overall number of ROS in the cell, potentially reducing lethality to antibiotics. This result supports the previously-proposed model (Kohanski *et al.*, 2007). A result that does not support the model is the increased frequency of tetracycline cross-resistance in paraquat-resistant spontaneous mutants, as tetracycline is a bacteriostatic antibiotic. The EmrE multi-drug transporter pumps both paraquat and tetracycline, but the *emrE* gene was not mutated in any of the five mutants sequenced, and three of these were tetracycline cross-resistant (with one not tested for tetracycline resistance). However, it was shown that even bacteriostatic antibiotics can produce ROS, and that catalases and SOD might not have an effect because the production of ROS is simply too high (Kolodkin-Gal *et al.*, 2008). Therefore, it is possible that tetracycline is ROS-dependent, which would explain the tetracycline cross-resistance in paraquat-resistant mutants.

As sensitivity to bactericidal antibiotics is dependent on catalase activity, it was surprising that no H_2O_2-resistant mutants were found through this selection strategy. However, this may be an artifact of the experimental system. Detoxification of H_2O_2 is

through the breakdown of the molecule itself (unlike detoxification of paraquat, which is the result of both efflux of the compound and scavenging of its generated O_2^-). As the molecule is permeable, detoxification of H_2O_2 is done as a population, not individual cells (Seaver and Imlay, 2001) – the catalase activity of one cell will reduce the concentration of H_2O_2 in the environment as a whole. There are two variables involved in H_2O_2 detoxification, then: the total population size and the combined activity of the proteins involved in detoxifying the H_2O_2. Unfortunately, many of the mutants have different growth rates from both the wild type and each other, and so these mutants were not at the same density when replica plated (and typically at a lower density than the wild type). This could mask the effect of a cross-resistance mutation in an H_2O_2-dependent pathway, and so whether H_2O_2 cross-resistant mutants occurred was not conclusively established.

While the mutants of particular interest were cross-resistant to paraquat, it is of note that some mutants were not cross-resistant to other agents. There may be another protein, possibly an entire pathway, that is involved in spontaneous resistance to these three antibiotics. A transporter protein of *Vibrio parahaemolyticus*, NorM, confers resistance to norfloxacin and kanamycin and is homologous to the hypothetical protein YdhE in *E. coli* (Morita *et al.*, 1998). The MdfA transporter of *E. coli* also slightly increases resistance to both kanamycin and norfloxacin when overexpressed (Edgar and Bibi, 1997), and so either of these could be involved in spontaneous resistance. In neither case, however, is ampicillin resistance, or β-lactam resistance in general, also a component of the resistances of the pumps, though such multi-drug transporters do exist

for β-lactams and fluoroquinolones (Livermore, 2002). The alternative common pathway involved, therefore, remains a mystery.

This discussion, to this point, has treated these mutants as though they only have one mutation, and this is not necessarily true. In order to have some rare mutational events occur, many more cells were screened than necessary to obtain (on average) a single substitution at each base (calculated in full in the results), which potentially allows multiple common single base substitutions to occur in one cell. Indeed, double mutants are typically found far more commonly than would be expected based on probability alone (Drake, 2007). Therefore, some of the phenotypes observed (e.g. cross-resistance between multiple antibiotics and/or oxidative stress inducing agents) may simply be the result of two coincidental mutations. However, assuming only two mutations, and assuming that there are approximately 20 genes whose mutation will affect paraquat resistance (likely an overestimate), and assuming all mutations affect a single gene (thus ignoring intergenic regions and silent mutations), then one in (4000 genes / 20 genes involved in paraquat resistance) 200 double mutants would be expected to affect paraquat resistance as well (0.5%), one in 100 triple mutants (1%), one in 67 quadruple mutants (1.5%), one in 50 quintuple mutants (2%), and so forth. Despite the fact that these figures are likely vast overestimates, the actual rate of cross-resistance to paraquat is much higher than these values (15.4% of mutants were confirmed resistant to paraquat). In addition, based on a mutation rate of 5.4×10^{-10} mutations•bp^{-1}•genome replication^{-1} (Hutchinson, 1996), the total population of cells assayed contained 9.85×10^{8} base substitutions in 3.965×10^{11} cells. Allowing for an elevated rate of doubly mutated cells,

this would still be a very small fraction of the population. Finally, apart from base substitutions, other mutations occur. Single base frameshifts also occur approximately once for every four base substitutions (reviewed in (Hutchinson, 1996)), and so with the number of cells being examined, a great proportion of cells will contain these as well. Large insertions and deletions are also common mutations (Hutchinson, 1996). Many of these mutants may, therefore, not be base substitution mutants but rather insertion/deletion mutants, and this may result in the resistances seen. The vast majority of these cross-resistant mutants, though, should be treated as genuinely resulting from a single mutation.

Of the fourteen cultures used to generate mutants, the six slightly denser cultures generated the majority of the mutants. This is possibly the result of adaptive mutagenesis (reviewed in (Rosenberg, 2001)), whereby intrinsic mutation mechanisms in *E. coli* become more active, at least in a fraction of cells of the culture, during stress or nutrient limitation. This often results in many cells bearing multiple mutations (discussed more above) but also generates more mutants per CFU in the culture, explaining the observed mutants. This may result in an elevated number of multiply mutated cells (Drake, 2007), though based on the calculations above, mutants harboring more than one mutation are still likely a very small fraction of the population.

4.6 Overall conclusions

The recent data published in this area (this study, (Kohanski *et al.*, 2008;Kolodkin-Gal *et al.*, 2008;Kolodkin-Gal and Engelberg-Kulka, 2009)) has generated a great deal of information based on the original model (Kohanski *et al.*, 2007) which proposed that

bactericidal antibiotics induce death through ROS while bacteriostatic antibiotics do not. This is partly supported here by the inverse correlation between catalase activity and bactericidal antibiotic sensitivity (save streptomycin, a bactericidal antibiotic in which catalase activity has no effect, and trimethoprim, a bacteriostatic antibiotic in which the inverse correlation holds). Interestingly, overexpressing these catalases did not reduce antibiotic sensitivity below the wild type level, suggesting that either an increase or decrease in catalase activity has no effect on antibiotics. Oxidative stress regulons (OxyR and SoxRS) are activated when exposed to gentamicin (Kohanski *et al.*, 2008), which is consistent with the data in my previous report showing that loss of OxyR and RpoS activity, both regulating stress regulons, result in significant increases in antibiotic sensitivity, particularly when combined. Interestingly, however, loss of OxyR and RpoS also resulted in increased bacteriostatic antibiotic sensitivity, which is in agreement with recently-published data showing that deletion of *rpoS* in MC4100 resulted in a significant increase in stationary-phase lethality when exposed to rifampicin, spectinomycin, and chloramphenicol (the latter two of which are bacteriostatic) (Kolodkin-Gal and Engelberg-Kulka, 2009). These authors also found that overexpression of *katE* or addition of exogenous catalase can, to a large extent, eliminate the lethality resulting from rifampicin, spectinomycin, and chloramphenicol (Kolodkin-Gal *et al.*, 2008), which is consistent with the results here that exogenous catalase reduces the sensitivity of HS2607 (Δ*katE*Δ*katG*) to chloramphenicol (the other two were not tested), as well as ampicillin and norfloxacin. Therefore, it is clear that catalases are an important component of cellular defenses against bactericidal antibiotics, but interestingly they are

also important against some bacteriostatic antibiotics as well. This suggests that the division between antibiotics producing ROS and not producing ROS is not as simple as a bactericidal/bacteriostatic division as originally proposed (Kohanski *et al.*, 2007), particularly given that that division is somewhat arbitrary in its own right and the antibiotics within each group can vary between strains and organisms.

With respect to SOD, one group found that, as mentioned above, the SoxR regulon is activated in MG1655 upon gentamicin exposure (Kohanski *et al.*, 2008). Another group found that the overexpression of *sodA* in MC4100 can prevent the lethality of rifampicin, spectinomycin, and chloramphenicol (Kolodkin-Gal *et al.*, 2008), supporting the above result. However, the deletion of *sodA* or *sodB* resulted in relatively little effect here, though the deletion of both resulted in increases in antibiotic sensitivity (though, again, not limited to bactericidal antibiotics). Spontaneous mutants to a combination of bactericidal antibiotics were also resistant to paraquat, demonstrating a link between SOD activity and these antibiotics. It is possible, then, that physiological levels of SOD are not relevant to the action of antibiotics but high concentrations of SOD can indeed have an impact. This is consistent with all the observed data.

This data suggests that it may be possible to target catalases for therapeutic purposes. However, many infections are in anaerobic conditions, such as the gastrointestinal tract, and catalases were not involved in the action of antibiotics in fermentative conditions (and likely not at all in anaerobic conditions). However, aerobic infections do occur, and it is these sites and these organisms that these results may be useful for.

4.7 Future directions

There are several small experiments that could be done to expand on the results presented here. The antibiotic sensitivity of SOD regulator mutants could be tested, much like catalase regulators were tested in the previous report. Actual gene expression of catalases and SOD could be quantified during antibiotic stress (using qPCR) to determine whether these genes' expression are activated, as would be expected if these antibiotics are causing oxidative stress. This was briefly touched on here but the results were quite variable and only in duplicate, and so a more complete assay might prove useful. Lastly, a catalase-null cytoplasmic SOD-null MG1655 strain (Δ*katE* Δ*katG* Δ*sodA* Δ*sodB*) has not yet been constructed, and it is predicted to have the greatest sensitivity to these antibiotics. Whether it does, and whether the sensitivity of this strain corresponds to that of HS2607 (Δ*katE* Δ*katG*), HS2911 (Δ*sodA* Δ*sodB*), or is a combination of the two would be informative.

On a larger scale, there is some strain to strain variation in the effects of these antibiotics, potentially in conjunction with *mazEF*. For example, rifampicin is reported to be bactericidal in *E. coli* MC4100 but not in MG1655 as a result of *mazEF* being more active in MC4100. In addition, *mazEF*-dependent lethality is not a particularly significant component of antibiotic action in MG1655, as a particular factor (EDF) must be added to cause it to occur, however it causes significant lethality in response to a variety of antibiotics in MC4100. From a medical perspective, neither the response of MG1655 nor MC4100 are particularly relevant if these results cannot be applied to natural isolates of *E. coli* as well, and this should be examined. Natural isolates of *E. coli*

(taken from the *E. coli* reference collection, a set of strains thought to represent the genetic diversity of *E. coli*) have very different levels of catalase activity (Stieber, 2007), and it is entirely possible that they have very different levels of SOD activity as well. Either of these may affect antibiotic sensitivity (though catalases resulted in a greater overall effect in MG1655), and a screen of a variety of ECOR strains or other natural isolates for both antibiotic sensitivity and both catalase and SOD activity would help determine whether this effect is physiologically relevant. If possible, antibiotic sensitivities of catalase and SOD deletion strains of a selection of natural isolates would also be informative, though genetic manipulations of natural isolates can be difficult and may not be possible in all cases. In particular, as the most direct application of this research might be in potentiating current antibiotics through the combined use of antibiotics and catalase or SOD inhibitors, examining the response of pathogenic *E. coli* strains would be of interest. Many such pathogenic strains have been isolated clinically, and these strains could be screened for whether they have altered sensitivity to ROS. It is interesting that antibiotic resistant, ROS resistant strains have not yet been documented, showing that either ROS resistance is not a significant factor in antibiotic resistance or such resistance has simply never been examined in these isolates. A screen of pathogenic *E. coli* isolates could, therefore, determine whether ROS are a significant factor in determining antibiotic resistance outside of the laboratory.

Finally, ROS generation through antibiotic exposure should be examined in a variety of organisms. This has been shown in *Staphylococcus aureus* (Kohanski *et al.*, 2007), but a variety of other pathogenic organisms might be useful. Organisms closely

related to *E. coli*, such as *Salmonella* sp., are also responsible for significant morbidity and some mortality, and these could also be tested and better compared with *E. coli* to potentially examine the biochemical basis for this ROS production. It is important to understand how significant the effects of ROS are on a variety of strains and to determine whether the data obtained in *E. coli* MG1655 can be applied to other organisms.

References

Anand,A., Bashey,B., Mir,T., and Glatt,A.E. (1994) Epidemiology, clinical manifestations, and outcome of Clostridium difficile-associated diarrhea *Am.J.Gastroenterol.* **89**: 519-523.

Appleman,J.R., Prendergast,N., Delcamp,T.J., Freisheim,J.H., and Blakley,R.L. (1988) Kinetics of the formation and isomerization of methotrexate complexes of recombinant human dihydrofolate reductase *J.Biol.Chem.* **263**: 10304-10313.

Arkin,A., Ross,J., and McAdams,H.H. (1998) Stochastic kinetic analysis of developmental pathway bifurcation in phage lambda-infected Escherichia coli cells *Genetics* **149**: 1633-1648.

Arner,E.S., Holmgren,A. (2000) Physiological functions of thioredoxin and thioredoxin reductase *Eur.J.Biochem.* **267**: 6102-6109.

Aslund,F., Zheng,M., Beckwith,J., and Storz,G. (1999) Regulation of the OxyR transcription factor by hydrogen peroxide and the cellular thiol-disulfide status *Proc.Natl.Acad.Sci.U.S.A* **96**: 6161-6165.

Baev,M.V., Baev,D., Radek,A.J., and Campbell,J.W. (2006) Growth of Escherichia coli MG1655 on LB medium: monitoring utilization of sugars, alcohols, and organic acids with transcriptional microarrays *Appl.Microbiol.Biotechnol.* **71**: 310-316.

Bagg,A., Neilands,J.B. (1987) Ferric uptake regulation protein acts as a repressor, employing iron (II) as a cofactor to bind the operator of an iron transport operon in Escherichia coli *Biochemistry* **26**: 5471-5477.

Balandina,A., Claret,L., Hengge-Aronis,R., and Rouviere-Yaniv,J. (2001) The Escherichia coli histone-like protein HU regulates rpoS translation *Mol.Microbiol.* **39**: 1069-1079.

Ball,P. (2000) Quinolone generations: natural history or natural selection? *J.Antimicrob.Chemother.* **46 Suppl T1**: 17-24.

Bar-Even,A., Paulsson,J., Maheshri,N., Carmi,M., O'Shea,E., Pilpel,Y., and Barkai,N. (2006) Noise in protein expression scales with natural protein abundance *Nat.Genet.* **38**: 636-643.

Barkai,N., Leibler,S. (2000) Circadian clocks limited by noise *Nature* **403**: 267-268.

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

Becker,G., Hengge-Aronis,R. (2001) What makes an Escherichia coli promoter sigma(S) dependent? Role of the -13/-14 nucleotide promoter positions and region 2.5 of sigma(S) *Mol.Microbiol.* **39**: 1153-1165.

Becker,G., Klauck,E., and Hengge-Aronis,R. (1999) Regulation of RpoS proteolysis in Escherichia coli: the response regulator RssB is a recognition factor that interacts with the turnover element in RpoS *Proc.Natl.Acad.Sci.U.S.A* **96**: 6439-6444.

Benov,L.T., Fridovich,I. (1994) Escherichia coli expresses a copper- and zinc-containing superoxide dismutase *J.Biol.Chem.* **269**: 25310-25314.

Blattner,F.R., Plunkett,G., III, Bloch,C.A., Perna,N.T., Burland,V., Riley,M., Collado-Vides,J., Glasner,J.D., Rode,C.K., Mayhew,G.F., Gregor,J., Davis,N.W., Kirkpatrick,H.A., Goeden,M.A., Rose,D.J., Mau,B., and Shao,Y. (1997) The complete genome sequence of Escherichia coli K-12 *Science* **277**: 1453-1474.

Bougdour,A., Wickner,S., and Gottesman,S. (2006) Modulating RssB activity: IraP, a novel regulator of sigma(S) stability in Escherichia coli *Genes Dev.* **20**: 884-897.

Brown,L., Elliott,T. (1996) Efficient translation of the RpoS sigma factor in Salmonella typhimurium requires host factor I, an RNA-binding protein encoded by the hfq gene *J.Bacteriol.* **178**: 3763-3770.

Brown,L., Elliott,T. (1997) Mutations that increase expression of the rpoS gene and decrease its dependence on hfq function in Salmonella typhimurium *J.Bacteriol.* **179**: 656-662.

Brown,M.R., Allison,D.G., and Gilbert,P. (1988) Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? *J.Antimicrob.Chemother.* **22**: 777-780.

Brown,M.R., Gilbert,P. (1993) Sensitivity of biofilms to antimicrobial agents *J.Appl.Bacteriol.* **74 Suppl**: 87S-97S.

Bush,K. (1988) Beta-lactamase inhibitors from laboratory to clinic *Clin.Microbiol.Rev.* **1**: 109-123.

Carlioz,A., Touati,D. (1986) Isolation of superoxide dismutase mutants in Escherichia coli: is superoxide dismutase necessary for aerobic life? *EMBO J.* **5**: 623-630.

Carmel-Harel,O., Storz,G. (2000) Roles of the glutathione- and thioredoxin-dependent reduction systems in the Escherichia coli and saccharomyces cerevisiae responses to oxidative stress *Annu.Rev.Microbiol.* **54**: 439-461.

Carmeli,Y., Eliopoulos,G.M., and Samore,M.H. (2002) Antecedent treatment with different antibiotic agents as a risk factor for vancomycin-resistant Enterococcus *Emerg.Infect.Dis.* **8**: 802-807.

Chelikani,P., Fita,I., and Loewen,P.C. (2004) Diversity of structures and properties among catalases *Cell Mol.Life Sci.* **61**: 192-208.

Chen,G., Patten,C.L., and Schellhorn,H.E. (2004) Positive selection for loss of RpoS function in Escherichia coli *Mutat.Res.* **554**: 193-203.

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

Chung,C.T., Niemela,S.L., and Miller,R.H. (1989) One-step preparation of competent Escherichia coli: transformation and storage of bacterial cells in the same solution *Proc.Natl.Acad.Sci.U.S.A* **86**: 2172-2175.

Cody,C.W., Prasher,D.C., Westler,W.M., Prendergast,F.G., and Ward,W.W. (1993) Chemical structure of the hexapeptide chromophore of the Aequorea green-fluorescent protein *Biochemistry* **32**: 1212-1218.

Courcelle,J., Hanawalt,P.C. (2003) RecA-dependent recovery of arrested DNA replication forks *Annu.Rev.Genet.* **37**: 611-646.

Datsenko,K.A., Wanner,B.L. (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products *Proc.Natl.Acad.Sci.U.S.A* **97**: 6640-6645.

Davies,J., Nomura,M. (1972) The genetics of bacterial ribosomes *Annu.Rev.Genet.* **6**: 203-234.

Davis,B.D. (1987) Mechanism of bactericidal action of aminoglycosides *Microbiol.Rev.* **51**: 341-350.

de Lorenzo,V., Wee,S., Herrero,M., and Neilands,J.B. (1987) Operator sequences of the aerobactin operon of plasmid ColV-K30 binding the ferric uptake regulation (fur) repressor *J.Bacteriol.* **169**: 2624-2630.

Demple,B. (1996) Redox signaling and gene control in the Escherichia coli soxRS oxidative stress regulon--a review *Gene* **179**: 53-57.

Dharmadi,Y., Murarka,A., and Gonzalez,R. (2006) Anaerobic fermentation of glycerol by Escherichia coli: a new platform for metabolic engineering *Biotechnol.Bioeng.* **94**: 821-829.

Ding,H., Hidalgo,E., and Demple,B. (1996) The redox state of the [2Fe-2S] clusters in SoxR protein regulates its activity as a transcription factor *J.Biol.Chem.* **271**: 33173-33175.

Ding,Q., Kusano,S., Villarejo,M., and Ishihama,A. (1995) Promoter selectivity control of Escherichia coli RNA polymerase by ionic strength: differential recognition of osmoregulated promoters by E sigma D and E sigma S holoenzymes *Mol.Microbiol.* **16**: 649-656.

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

Dong,T., Joyce,C., and Schellhorn,H.E. (2008) The role of RpoS in bacterial adaptation. In *Bacterial physiology: a molecular approach.* El-Sharoud,W. (ed). Berlin: Springer, pp. 313-337.

Drake,J.W. (1999) The distribution of rates of spontaneous mutation over viruses, prokaryotes, and eukaryotes *Ann.N.Y.Acad.Sci.* **870**: 100-107.

Drake,J.W. (2007) Too many mutants with multiple mutations *Crit Rev.Biochem.Mol.Biol.* **42**: 247-258.

Drake,J.W., Charlesworth,B., Charlesworth,D., and Crow,J.F. (1998) Rates of spontaneous mutation *Genetics* **148**: 1667-1686.

Dubrac,S., Touati,D. (2000) Fur positive regulation of iron superoxide dismutase in Escherichia coli: functional analysis of the sodB promoter *J.Bacteriol.* **182**: 3802-3808.

Dubrac,S., Touati,D. (2002) Fur-mediated transcriptional and post-transcriptional regulation of FeSOD expression in Escherichia coli *Microbiology* **148**: 147-156.

Dwyer,D.J., Kohanski,M.A., Hayete,B., and Collins,J.J. (2007) Gyrase inhibitors induce an oxidative damage cellular death pathway in Escherichia coli *Mol.Syst.Biol.* **3**: 91.

Edgar,R., Bibi,E. (1997) MdfA, an Escherichia coli multidrug resistance protein with an extraordinarily broad spectrum of drug recognition *J.Bacteriol.* **179**: 2274-2280.

Elowitz,M.B., Levine,A.J., Siggia,E.D., and Swain,P.S. (2002) Stochastic gene expression in a single cell *Science* **297**: 1183-1186.

Epe,B., Woolley,P. (1984) The binding of 6-demethylchlortetracycline to 70S, 50S and 30S ribosomal particles: a quantitative study by fluorescence anisotropy *EMBO J.* **3**: 121-126.

Escolar,L., Perez-Martin,J., and de Lorenzo,V. (1999) Opening the iron box: transcriptional metalloregulation by the Fur protein *J.Bacteriol.* **181**: 6223-6229.

European Antimicrobial Resistance Surveillance System. EARSS Annual Report 2007. 1-156. 2008. Bilthoven, Netherlands, EARSS.

Fang,F.C., Libby,S.J., Buchmeier,N.A., Loewen,P.C., Switala,J., Harwood,J., and Guiney,D.G. (1992) The alternative sigma factor katF (rpoS) regulates Salmonella virulence *Proc.Natl.Acad.Sci.U.S.A* **89**: 11978-11982.

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

Fernandes,A.P., Holmgren,A. (2004) Glutaredoxins: glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system *Antioxid.Redox Signal.* **6**: 63-74.

Flint,D.H., Tuminello,J.F., and Emptage,M.H. (1993) The inactivation of Fe-S cluster containing hydro-lyases by superoxide *J.Biol.Chem.* **268**: 22369-22376.

Fridovich,I. (1995) Superoxide radical and superoxide dismutases *Annu.Rev.Biochem.* **64**: 97-112.

Gaal,T., Ross,W., Estrem,S.T., Nguyen,L.H., Burgess,R.R., and Gourse,R.L. (2001) Promoter recognition and discrimination by EsigmaS RNA polymerase *Mol.Microbiol.* **42**: 939-954.

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

Gerding,D.N. (2004) Clindamycin, cephalosporins, fluoroquinolones, and Clostridium difficile-associated diarrhea: this is an antimicrobial resistance problem *Clin.Infect.Dis.* **38**: 646-648.

Goldberg,I.H., Friedman,P.A. (1971) Antibiotics and nucleic acids *Annu.Rev.Biochem.* **40**: 775-810.

Goldman,R.A., Hasan,T., Hall,C.C., Strycharz,W.A., and Cooperman,B.S. (1983) Photoincorporation of tetracycline into Escherichia coli ribosomes. Identification of the

major proteins photolabeled by native tetracycline and tetracycline photoproducts and implications for the inhibitory action of tetracycline on protein synthesis *Biochemistry* **22**: 359-368.

Goldstein,F.W., Kitzis,M.D., and Acar,J.F. (1979) Effect of clavulanic acid and amoxycillin formulation against beta-lactamase producing Gram-negative bacteria in urinary tract infections *J.Antimicrob.Chemother.* **5**: 705-709.

Gonzalez-Flecha,B., Demple,B. (1995) Metabolic sources of hydrogen peroxide in aerobically growing Escherichia coli *J.Biol.Chem.* **270**: 13681-13687.

Gort,A.S., Ferber,D.M., and Imlay,J.A. (1999) The regulation and role of the periplasmic copper, zinc superoxide dismutase of Escherichia coli *Mol.Microbiol.* **32**: 179-191.

Goswami,M., Jawali,N. (2007) Glutathione-mediated augmentation of beta-lactam antibacterial activity against Escherichia coli *J.Antimicrob.Chemother.* **60**: 184-185.

Goswami,M., Mangoli,S.H., and Jawali,N. (2007) Effects of glutathione and ascorbic acid on streptomycin sensitivity of Escherichia coli *Antimicrob.Agents Chemother.* **51**: 1119-1122.

Goulian,M., van der Woude,M. (2006) A simple system for converting lacZ to gfp reporter fusions in diverse bacteria *Gene* **372**: 219-226.

Grace,S.C. (1990) Phylogenetic distribution of superoxide dismutase supports an endosymbiotic origin for chloroplasts and mitochondria *Life Sci.* **47**: 1875-1886.

Hassett,D.J., Imlay,J.A. (2007) Bactericidal antibiotics and oxidative stress: a radical proposal *ACS Chem.Biol.* **2**: 708-710.

Hawkey,P.M. (2003) Mechanisms of quinolone action and microbial response *J.Antimicrob.Chemother.* **51 Suppl 1**: 29-35.

Hazan,R., Sat,B., and Engelberg-Kulka,H. (2004) Escherichia coli mazEF-mediated cell death is triggered by various stressful conditions *J.Bacteriol.* **186**: 3663-3669.

Heim,R., Prasher,D.C., and Tsien,R.Y. (1994) Wavelength mutations and posttranslational autoxidation of green fluorescent protein *Proc.Natl.Acad.Sci.U.S.A* **91**: 12501-12504.

Hengge-Aronis,R. (2002a) Recent insights into the general stress response regulatory network in Escherichia coli *J.Mol.Microbiol.Biotechnol.* **4**: 341-346.

Hengge-Aronis,R. (2002b) Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase *Microbiol.Mol.Biol.Rev.* **66**: 373-95, table.

Hengge-Aronis,R., Lange,R., Henneberg,N., and Fischer,D. (1993) Osmotic regulation of rpoS-dependent genes in Escherichia coli *J.Bacteriol.* **175**: 259-265.

Hirsch,M., Elliott,T. (2002) Role of ppGpp in rpoS stationary-phase regulation in Escherichia coli *J.Bacteriol.* **184**: 5077-5087.

Hirsch,M., Elliott,T. (2005) Fis regulates transcriptional induction of RpoS in Salmonella enterica *J.Bacteriol.* **187**: 1568-1580.

Huovinen,P. (1987) Trimethoprim resistance *Antimicrob.Agents Chemother.* **31**: 1451-1456.

Hutchinson,F. (1996) Mutagenesis. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*. Washington, D.C.: ASM Press, pp. 2218-2235.

Imlay,J.A. (2003) Pathways of oxidative damage *Annu.Rev.Microbiol.* **57**: 395-418.

Imlay,J.A., Fridovich,I. (1991) Assay of metabolic superoxide production in Escherichia coli *J.Biol.Chem.* **266**: 6957-6965.

Inoue,H., Nojima,H., and Okayama,H. (1990) High efficiency transformation of Escherichia coli with plasmids *Gene* **96**: 23-28.

Ishihama,A. (2000) Functional modulation of Escherichia coli RNA polymerase *Annu.Rev.Microbiol.* **54**: 499-518.

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

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

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

Jishage,M., Kvint,K., Shingler,V., and Nystrom,T. (2002) Regulation of sigma factor competition by the alarmone ppGpp *Genes Dev.* **16**: 1260-1270.

Joyce, C. Correlation of RpoS-dependent gene expression with gene copy number and characterization of the distribution of the expression level in *Escherichia coli*. 12-21-2006.

Kargalioglu,Y., Imlay,J.A. (1994) Importance of anaerobic superoxide dismutase synthesis in facilitating outgrowth of Escherichia coli upon entry into an aerobic habitat *J.Bacteriol.* **176**: 7653-7658.

Kitagawa,M., Ara,T., Arifuzzaman,M., Ioka-Nakamichi,T., Inamoto,E., Toyonaga,H., and Mori,H. (2005) Complete set of ORF clones of Escherichia coli ASKA library (a complete set of E. coli K-12 ORF archive): unique resources for biological research *DNA Res.* **12**: 291-299.

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

Klauck,E., Typas,A., and Hengge,R. (2007) The sigmaS subunit of RNA polymerase as a signal integrator and network master regulator in the general stress response in Escherichia coli *Sci.Prog.* **90**: 103-127.

Kobayashi,A., Hirakawa,H., Hirata,T., Nishino,K., and Yamaguchi,A. (2006) Growth phase-dependent expression of drug exporters in Escherichia coli and its contribution to drug tolerance *J.Bacteriol.* **188**: 5693-5703.

Kohanski,M.A., Dwyer,D.J., Hayete,B., Lawrence,C.A., and Collins,J.J. (2007) A common mechanism of cellular death induced by bactericidal antibiotics *Cell* **130**: 797-810.

Kohanski,M.A., Dwyer,D.J., Wierzbowski,J., Cottarel,G., and Collins,J.J. (2008) Mistranslation of membrane proteins and two-component system activation trigger antibiotic-mediated cell death *Cell* **135**: 679-690.

Kolodkin-Gal,I., Engelberg-Kulka,H. (2009) The stationary-phase sigma factor sigma(S) is responsible for the resistance of Escherichia coli stationary-phase cells to mazEF-mediated cell death *J.Bacteriol.* **191**: 3177-3182.

Kolodkin-Gal,I., Sat,B., Keshet,A., and Engelberg-Kulka,H. (2008) The communication factor EDF and the toxin-antitoxin module mazEF determine the mode of action of antibiotics *PLoS.Biol.* **6**: e319.

Kornberg,A., Rao,N.N., and Ault-Riche,D. (1999) Inorganic polyphosphate: a molecule of many functions *Annu.Rev.Biochem.* **68**: 89-125.

Korshunov,S., Imlay,J.A. (2006) Detection and quantification of superoxide formed within the periplasm of Escherichia coli *J.Bacteriol.* **188**: 6326-6334.

Lando,D., Cousin,M.A., and Privat,d.G. (1973) Misreading, a fundamental aspect of the mechanism of action of several aminoglycosides *Biochemistry* **12**: 4528-4533.

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

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

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

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

Lesprit,P., Brun-Buisson,C. (2008) Hospital antibiotic stewardship *Curr.Opin.Infect.Dis.* **21**: 344-349.

Li,Y., Schellhorn,H.E. (2007) Rapid kinetic microassay for catalase activity *J.Biomol.Tech.* **18**: 185-187.

Lin,E.C. (1976) Glycerol dissimilation and its regulation in bacteria *Annu.Rev.Microbiol.* **30**: 535-578.

Livermore,D.M. (2002) Multiple mechanisms of antimicrobial resistance in Pseudomonas aeruginosa: our worst nightmare? *Clin.Infect.Dis.* **34**: 634-640.

Lodmell,J.S., Dahlberg,A.E. (1997) A conformational switch in Escherichia coli 16S ribosomal RNA during decoding of messenger RNA *Science* **277**: 1262-1267.

Loewen,P.C., Stauffer,G.V. (1990) Nucleotide sequence of katG of Salmonella typhimurium LT2 and characterization of its product, hydroperoxidase I *Mol.Gen.Genet.* **224**: 147-151.

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

Maamar,H., Raj,A., and Dubnau,D. (2007) Noise in gene expression determines cell fate in Bacillus subtilis *Science* **317**: 526-529.

Maeda,H., Fujita,N., and Ishihama,A. (2000) Competition among seven Escherichia coli sigma subunits: relative binding affinities to the core RNA polymerase *Nucleic Acids Res.* **28**: 3497-3503.

Majdalani,N., Chen,S., Murrow,J., St John,K., and Gottesman,S. (2001) Regulation of RpoS by a novel small RNA: the characterization of RprA *Mol.Microbiol.* **39**: 1382-1394.

Marshall,O.J. (2004) PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR *Bioinformatics.* **20**: 2471-2472.

Massey,V. (1994) Activation of molecular oxygen by flavins and flavoproteins *J.Biol.Chem.* **269**: 22459-22462.

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

Messner,K.R., Imlay,J.A. (2002) Mechanism of superoxide and hydrogen peroxide formation by fumarate reductase, succinate dehydrogenase, and aspartate oxidase *J.Biol.Chem.* **277**: 42563-42571.

Mika,F., Hengge,R. (2005) A two-component phosphotransfer network involving ArcB, ArcA, and RssB coordinates synthesis and proteolysis of sigmaS (RpoS) in E. coli *Genes Dev.* **19**: 2770-2781.

Miller,J.H. (1992) *A short course in bacterial genetics: A laboratory manual and handbookfor Escherichia coli and related bacteria.* Cold Spring Harbor Press (ed). Cold Spring Harbor, N.Y..

Moellering,R.C., Jr. (1983) Rationale for use of antimicrobial combinations *Am.J.Med.* **75**: 4-8.

Morimyo,M., Hongo,E., Hama-Inaba,H., and Machida,I. (1992) Cloning and characterization of the mvrC gene of Escherichia coli K-12 which confers resistance against methyl viologen toxicity *Nucleic Acids Res.* **20**: 3159-3165.

Morita,Y., Kodama,K., Shiota,S., Mine,T., Kataoka,A., Mizushima,T., and Tsuchiya,T. (1998) NorM, a putative multidrug efflux protein, of Vibrio parahaemolyticus and its homolog in Escherichia coli *Antimicrob.Agents Chemother.* **42**: 1778-1782.

Muffler,A., Barth,M., Marschall,C., and Hengge-Aronis,R. (1997) Heat shock regulation of sigmaS turnover: a role for DnaK and relationship between stress responses mediated by sigmaS and sigma32 in Escherichia coli *J.Bacteriol.* **179**: 445-452.

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

Mukhopadhyay,S., Audia,J.P., Roy,R.N., and Schellhorn,H.E. (2000) Transcriptional induction of the conserved alternative sigma factor RpoS in Escherichia coli is dependent on BarA, a probable two-component regulator *Mol.Microbiol.* **37**: 371-381.

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

Nunoshiba,T., Hidalgo,E., Li,Z., and Demple,B. (1993) Negative autoregulation by the Escherichia coli SoxS protein: a dampening mechanism for the soxRS redox stress response *J.Bacteriol.* **175**: 7492-7494.

Ojangu,E.L., Tover,A., Teras,R., and Kivisaar,M. (2000) Effects of combination of different -10 hexamers and downstream sequences on stationary-phase-specific sigma factor sigma(S)-dependent transcription in Pseudomonas putida *J.Bacteriol.* **182**: 6707-6713.

Olofsson,S.K., Marcusson,L.L., Stromback,A., Hughes,D., and Cars,O. (2007) Dose-related selection of fluoroquinolone-resistant Escherichia coli *J.Antimicrob.Chemother.* **60**: 795-801.

Ormo,M., Cubitt,A.B., Kallio,K., Gross,L.A., Tsien,R.Y., and Remington,S.J. (1996) Crystal structure of the Aequorea victoria green fluorescent protein *Science* **273**: 1392-1395.

Ozbudak,E.M., Thattai,M., Kurtser,I., Grossman,A.D., and van Oudenaarden,A. (2002) Regulation of noise in the expression of a single gene *Nat.Genet.* **31**: 69-73.

Pankey,G.A., Sabath,L.D. (2004) Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections *Clin.Infect.Dis.* **38**: 864-870.

Patten,C.L., Kirchhof,M.G., Schertzberg,M.R., Morton,R.A., and Schellhorn,H.E. (2004) Microarray analysis of RpoS-mediated gene expression in Escherichia coli K-12 *Mol.Genet.Genomics* **272**: 580-591.

Peterson,C.N., Carabetta,V.J., Chowdhury,T., and Silhavy,T.J. (2006) LrhA regulates rpoS translation in response to the Rcs phosphorelay system in Escherichia coli *J.Bacteriol.* **188**: 3175-3181.

Peterson,L.R. (2005) Squeezing the antibiotic balloon: the impact of antimicrobial classes on emerging resistance *Clin.Microbiol.Infect.* **11 Suppl 5**: 4-16.

Phadtare,S., Inouye,M. (2001) Role of CspC and CspE in regulation of expression of RpoS and UspA, the stress response proteins in Escherichia coli *J.Bacteriol.* **183**: 1205-1214.

Prasher,D.C., Eckenrode,V.K., Ward,W.W., Prendergast,F.G., and Cormier,M.J. (1992) Primary structure of the Aequorea victoria green-fluorescent protein *Gene* **111**: 229-233.

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

Projan,S.J., Shlaes,D.M. (2004) Antibacterial drug discovery: is it all downhill from here? *Clin.Microbiol.Infect.* **10 Suppl 4**: 18-22.

Pruteanu,M., Hengge-Aronis,R. (2002) The cellular level of the recognition factor RssB is rate-limiting for sigmaS proteolysis: implications for RssB regulation and signal transduction in sigmaS turnover in Escherichia coli *Mol.Microbiol.* **45**: 1701-1713.

Pryor,W.A. (1986) Oxy-radicals and related species: their formation, lifetimes, and reactions *Annu.Rev.Physiol* **48**: 657-667.

Pucci,M.J. (2006) Use of genomics to select antibacterial targets *Biochem.Pharmacol.* **71**: 1066-1072.

Raser,J.M., O'Shea,E.K. (2005) Noise in gene expression: origins, consequences, and control *Science* **309**: 2010-2013.

Roberts,M.C. (2008) Update on macrolide-lincosamide-streptogramin, ketolide, and oxazolidinone resistance genes *FEMS Microbiol.Lett.* **282**: 147-159.

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

Rosenberg,S.M. (2001) Evolving responsively: adaptive mutation *Nat.Rev.Genet.* **2**: 504-515.

Rybak,M.J. (2007) Antimicrobial stewardship *Pharmacotherapy* **27**: 131S-135S.

Schellhorn,H.E., Audia,J.P., Wei,L.I., and Chang,L. (1998) Identification of conserved, RpoS-dependent stationary-phase genes of Escherichia coli *J.Bacteriol.* **180**: 6283-6291.

Schnappinger,D., Hillen,W. (1996) Tetracyclines: antibiotic action, uptake, and resistance mechanisms *Arch.Microbiol.* **165**: 359-369.

Schrum,L.W., Hassan,H.M. (1994) The effects of fur on the transcriptional and post-transcriptional regulation of MnSOD gene (sodA) in Escherichia coli *Arch.Biochem.Biophys.* **309**: 288-292.

Schumacher,A., Trittler,R., Bohnert,J.A., Kummerer,K., Pages,J.M., and Kern,W.V. (2007) Intracellular accumulation of linezolid in Escherichia coli, Citrobacter freundii and Enterobacter aerogenes: role of enhanced efflux pump activity and inactivation *J.Antimicrob.Chemother.* **59**: 1261-1264.

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

Schweitzer,B.I., Dicker,A.P., and Bertino,J.R. (1990) Dihydrofolate reductase as a therapeutic target *FASEB J.* **4**: 2441-2452.

Seaver,L.C., Imlay,J.A. (2001) Hydrogen peroxide fluxes and compartmentalization inside growing Escherichia coli *J.Bacteriol.* **183**: 7182-7189.

Setsukinai,K., Urano,Y., Kakinuma,K., Majima,H.J., and Nagano,T. (2003) Development of novel fluorescence probes that can reliably detect reactive oxygen species and distinguish specific species *J.Biol.Chem.* **278**: 3170-3175.

Sevinc,M.S., Mate,M.J., Switala,J., Fita,I., and Loewen,P.C. (1999) Role of the lateral channel in catalase HPII of Escherichia coli *Protein Sci.* **8**: 490-498.

Shaner,N.C., Steinbach,P.A., and Tsien,R.Y. (2005) A guide to choosing fluorescent proteins *Nat.Methods* **2**: 905-909.

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

Shimomura,O., Johnson,F.H., and Morise,H. (1974) Mechanism of the luminescent intramolecular reaction of aequorin *Biochemistry* **13**: 3278-3286.

Shimomura,O., Johnson,F.H., and SAIGA,Y. (1962) Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea *J.Cell Comp Physiol* **59**: 223-239.

Skold,O. (2000) Sulfonamide resistance: mechanisms and trends *Drug Resist.Updat.* **3**: 155-160.

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

Slee,A.M., Wuonola,M.A., McRipley,R.J., Zajac,I., Zawada,M.J., Bartholomew,P.T., Gregory,W.A., and Forbes,M. (1987) Oxazolidinones, a new class of synthetic antibacterial agents: in vitro and in vivo activities of DuP 105 and DuP 721 *Antimicrob.Agents Chemother.* **31**: 1791-1797.

Stearns,T. (1995) Green fluorescent protein. The green revolution *Curr.Biol.* **5**: 262-264.

Steenbergen,J.N., Alder,J., Thorne,G.M., and Tally,F.P. (2005) Daptomycin: a lipopeptide antibiotic for the treatment of serious Gram-positive infections *J.Antimicrob.Chemother.* **55**: 283-288.

Stieber, N. 2007. Undergraduate thesis.
Switala,J., Loewen,P.C. (2002) Diversity of properties among catalases *Arch.Biochem.Biophys.* **401**: 145-154.

Tanaka,K., Kusano,S., Fujita,N., Ishihama,A., and Takahashi,H. (1995) Promoter determinants for Escherichia coli RNA polymerase holoenzyme containing sigma 38 (the rpoS gene product) *Nucleic Acids Res.* **23**: 827-834.

Tardat,B., Touati,D. (1991) Two global regulators repress the anaerobic expression of MnSOD in Escherichia coli::Fur (ferric uptake regulation) and Arc (aerobic respiration control) *Mol.Microbiol.* **5**: 455-465.

Thomas,C.L., Maule,A.J. (2000) Limitations on the use of fused green fluorescent protein to investigate structure-function relationships for the cauliflower mosaic virus movement protein *J.Gen.Virol.* **81**: 1851-1855.

Tipper,D.J. (1985) Mode of action of beta-lactam antibiotics *Pharmacol.Ther.* **27**: 1-35.

Touati,D. (2000) Sensing and protecting against superoxide stress in Escherichia coli--how many ways are there to trigger soxRS response? *Redox Rep.* **5**: 287-293.

Touati,E., Dassa,E., and Boquet,P.L. (1986) Pleiotropic mutations in appR reduce pH 2.5 acid phosphatase expression and restore succinate utilisation in CRP-deficient strains of Escherichia coli *Mol.Gen.Genet.* **202**: 257-264.

Tsien,R.Y. (1998) The green fluorescent protein *Annu.Rev.Biochem.* **67**: 509-544.

Tuveson,R.W. (1980) Genetic control of near-UV sensitivity independent of excision deficiency (uvrA6) in Escherichia coli K12 *Photochem.Photobiol.* **32**: 703-705.

Typas,A., Barembruch,C., Possling,A., and Hengge,R. (2007) Stationary phase reorganisation of the Escherichia coli transcription machinery by Crl protein, a fine-tuner of sigmas activity and levels *EMBO J.* **26**: 1569-1578.

Typas,A., Hengge,R. (2006) Role of the spacer between the -35 and -10 regions in sigmas promoter selectivity in Escherichia coli *Mol.Microbiol.* **59**: 1037-1051.

Ueguchi,C., Misonou,N., and Mizuno,T. (2001) Negative control of rpoS expression by phosphoenolpyruvate: carbohydrate phosphotransferase system in Escherichia coli *J.Bacteriol.* **183**: 520-527.

Vakulenko,S.B., Mobashery,S. (2003) Versatility of aminoglycosides and prospects for their future *Clin.Microbiol.Rev.* **16**: 430-450.

Vara Prasad,J.V. (2007) New oxazolidinones *Curr.Opin.Microbiol.* **10**: 454-460.

Vijayakumar,S.R., Kirchhof,M.G., Patten,C.L., and Schellhorn,H.E. (2004) RpoS-regulated genes of Escherichia coli identified by random lacZ fusion mutagenesis *J.Bacteriol.* **186**: 8499-8507.

Villain-Guillot,P., Bastide,L., Gualtieri,M., and Leonetti,J.P. (2007) Progress in targeting bacterial transcription *Drug Discov.Today* **12**: 200-208.

Visick,J.E., Clarke,S. (1997) RpoS- and OxyR-independent induction of HPI catalase at stationary phase in Escherichia coli and identification of rpoS mutations in common laboratory strains *J.Bacteriol.* **179**: 4158-4163.

Wallerstein,R.O., Condit,P.K., Kasper,C.K., Brown,J.W., and Morrison,F.R. (1969) Statewide study of chloramphenicol therapy and fatal aplastic anemia *JAMA* **208**: 2045-2050.

Wasil,M., Halliwell,B., Grootveld,M., Moorhouse,C.P., Hutchison,D.C., and Baum,H. (1987) The specificity of thiourea, dimethylthiourea and dimethyl sulphoxide as scavengers of hydroxyl radicals. Their protection of alpha 1-antiproteinase against inactivation by hypochlorous acid *Biochem.J.* **243**: 867-870.

Wassarman,K.M., Storz,G. (2000) 6S RNA regulates E. coli RNA polymerase activity *Cell* **101**: 613-623.

Webb,C., Moreno,M., Wilmes-Riesenberg,M., Curtiss,R., III, and Foster,J.W. (1999) Effects of DksA and ClpP protease on sigma S production and virulence in Salmonella typhimurium *Mol.Microbiol.* **34**: 112-123.

White,A.R., Kaye,C., Poupard,J., Pypstra,R., Woodnutt,G., and Wynne,B. (2004) Augmentin (amoxicillin/clavulanate) in the treatment of community-acquired respiratory tract infection: a review of the continuing development of an innovative antimicrobial agent *J.Antimicrob.Chemother.* **53 Suppl 1**: i3-20.

Winterbourn,C.C., Metodiewa,D. (1999) Reactivity of biologically important thiol compounds with superoxide and hydrogen peroxide *Free Radic.Biol.Med.* **27**: 322-328.

Wu,A.J., Penner-Hahn,J.E., and Pecoraro,V.L. (2004) Structural, spectroscopic, and reactivity models for the manganese catalases *Chem.Rev.* **104**: 903-938.

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

Yerushalmi,H., Lebendiker,M., and Schuldiner,S. (1995) EmrE, an Escherichia coli 12-kDa multidrug transporter, exchanges toxic cations and H+ and is soluble in organic solvents *J.Biol.Chem.* **270**: 6856-6863.

Yunis,A.A. (1988) Chloramphenicol: relation of structure to activity and toxicity *Annu.Rev.Pharmacol.Toxicol.* **28**: 83-100.

Zambrano,M.M., Siegele,D.A., Almiron,M., Tormo,A., and Kolter,R. (1993) Microbial competition: Escherichia coli mutants that take over stationary phase cultures *Science* **259**: 1757-1760.

Zhang,A., Altuvia,S., Tiwari,A., Argaman,L., Hengge-Aronis,R., and Storz,G. (1998) The OxyS regulatory RNA represses rpoS translation and binds the Hfq (HF-I) protein *EMBO J.* **17**: 6061-6068.

Zhang,Y., Heym,B., Allen,B., Young,D., and Cole,S. (1992) The catalase-peroxidase gene and isoniazid resistance of Mycobacterium tuberculosis *Nature* **358**: 591-593.

Zheng,M., Aslund,F., and Storz,G. (1998) Activation of the OxyR transcription factor by reversible disulfide bond formation *Science* **279**: 1718-1721.

Zheng,M., Doan,B., Schneider,T.D., and Storz,G. (1999) OxyR and SoxRS regulation of fur *J.Bacteriol.* **181**: 4639-4643.

Appendix A. Standard Operating Procedures

SEM Transformation protocol (Inoue et al., 1990)

This protocol was used to transform the *pir*$^{+}$ *E. coli* strain BW19851, which does not transform well using the TSS-dependent method.

1. Inoculate O/N culture in LB and grow normally.
2. Dilute into SOB and grow with shaking at 18 °C or RT to OD_{600} 0.6.
3. Place cells on ice for 10 min.
4. Harvest (spin at 2500 x g for 10 min at 4 °C).
5. Resuspend in 0.33 volumes of TB.
6. Incubate in ice for 10 min and spin down again.
7. Resuspend in 0.25 volumes of TB and add DMSO (slowly and with gentle swirling) to 7%.
8. Aliquot and freeze in liquid nitrogen, or use immediately (proceed to step 9).
9. Thaw competent cells (200 µL) on ice in 15 mL tubes.
10. Add plasmid DNA and incubate on ice for 30 min.
11. Heat shock at 42 °C for 30 s and put back on ice.
12. Add 0.8 mL of SOC medium (SOB + 20 mM glucose) and incubate with shaking at 37 °C for 1 h.
13. Plate on selective media.

Reagents:

SOB Medium:

- Tryptone 20 g/L
- Yeast Extract 5 g/L
- NaCl 0.5g/L
- 1M KCl 2.5 mL
- Adjust to 1 L with ddH_2O
- Add $MgCl_2$ to 10 mM after autoclaving

TB buffer:

- 10 mM Pipes
- 55 mM $MnCl_2$ (should be added last)
- 15 mM $CaC1_2$
- 250 mM KCl
- Adjust to pH 6.7

Assaying GFP fluorescence using the Tecan SAFIRE plate reader

This protocol is used to quantify the GFP expression of *E. coli* grown in liquid culture. While not capable of examining single cells, this method provides an overall picture of whether the culture is fluorescent.

1. Inoculate overnight cultures of desired strains (in triplicate) in 5 mL LB broth + any appropriate antibiotics and incubate overnight at 37°C and 200 rpm. Always include a *gfp*$^-$ strain is tested along with the desired strains.

2. The following morning subculture 1:1000 into 5 mL fresh LB broth (no antibiotics) and continue incubation at 37°C and 200 rpm.
3. At desired time points (typically OD_{600} = 0.3 and 1.5), take a 1 ml (exponential phase) or 200 µl (stationary phase) sample.
4. Spin down sample for 2 min.
5. Resuspend in 500 µl 10 mM $MgCl_2$.
6. Repeat steps 4 and 5.
7. Spin down culture again for 2 min.
8. Resuspend in desired amount of 10 mM $MgCl_2$. The final concentration of cells should be OD_{600} ~0.3 to 0.4.
9. Add 200 µl of each sample to a clear-bottom, black 96-well plate. Ensure a series of blanks are loaded as well (10 mM $MgCl_2$ alone).
10. Read both fluorescence and OD_{600} on the Tecan SAFIRE plate reader in Dr. Finan's laboratory.
11. Normalize raw fluorescence readings to OD_{600} (divide fluorescence by OD_{600}), producing fluorescence in arbitrary units (AU).

Disc diffusion assay

This assay is to screen a variety of strains against a variety of antibiotics (in discs) quickly. Accuracy at all steps of this process is crucial, as final results are measured in mm. All media (e.g. 2 x LB, 2 x Agar) should have the same volume, plates should contain as similar an amount of agar as possible, etc.

1. Inoculate overnight cultures of desired strains (in triplicate) in 5 mL LB broth + any appropriate antibiotics and incubate overnight at 37°C and 200 rpm.
2. Prepare the necessary number of LB/agar plates. Ensure that they harden on a surface as flat as possible (so that the LB/soft agar will cover the plate evenly). 6 antibiotics can be examined on a single plate, 2 technical replicates must be done per biological replicate, and three biological replicates must be done per strain. Therefore, if 7 to 12 antibiotics are being examined in four different strains, the number of plates needed are:

   2 x 2 x 3 x 4 = 48 plates
3. The following morning subculture the strains 1:1000 into fresh LB broth (no antibiotics) and continue incubation at 37°C and 200 rpm.
4. Prepare the necessary number of tubes of LB/soft agar. LB/soft agar is 50% 2xLB, 25% 2xAgar, and 25% ddH_2O (autoclaved). It solidifies at 50°C and therefore must be kept in a water bath at 55°C. Add 4 mL of LB/soft agar per tube, and one tube is needed per plate.
5. Once the cultures go turbid (approx. 2 h) subculture them again 1:10 into fresh LB broth (no antibiotics) and continue incubation at 37°C and 200 rpm.
6. Upon reaching OD_{600} = 0.8 (approx. 2 h) add 400 µL of culture into a tube of LB/soft agar, vortex at medium speed (5-6), add to the LB/Agar plate and quickly swirl the plate to spread the LB/soft agar evenly. Let the plates dry on a flat surface (approx. 1.5 h) and ensure that the LB/soft agar tubes do not solidify

during this step. To continue the example from step 2, 400 µL of each replicate culture should be added to 2 x 2 = 4 plates.

7. Remove antibiotic discs from fridge and allow them to warm up 10-15 min prior to use.
8. Apply the antibiotic discs using the disc dispensers. Ensure that a disc is deposited on each plate for each antibiotic, and ensure that the discs are lying flat on the plate. Allow the plates to incubate face-up overnight at 37°C.
9. The following day measure the radii of the inhibition zones. Each radius should be measured towards the centre of the plate (or at least away from any other nearby antibiotics), should be measured twice to ensure that the measurement is accurate, and does not include the disc itself. The disc is 6 mm in diameter – use this to confirm ruler's accuracy and that the measurement is not on an angle).

Viability assay

This assay allows the quantification of the number of CFU present within a liquid *E. coli* culture during antibiotic stress. The protocol can be adapted to exclude the antibiotic, or substitute in a different compound.

1. Inoculate overnight cultures of desired strains (in triplicate) in 5 mL LB broth + any appropriate antibiotics and incubate overnight at 37°C and 200 rpm.
2. The following morning subculture 1:1000 into 5 mL fresh LB broth (no antibiotics) and continue incubation at 37°C and 200 rpm.

3. Once the cultures go turbid (approximately 2 h) subculture 1:10 into 5 mL fresh LB broth (no antibiotics) and continue incubation at 37°C and 200 rpm. Some cultures should be subcultured into two tubes to generate control tubes.
4. Upon reaching desired OD_{600} (0.1 for exponential phase or 1.5 for stationary phase) take 100 µL sample from each culture and add to 900 µL sterile PBS or 10 mM $MgCl_2$. This is considered the 10^{-1} dilution.
5. For the experimental cultures (not the controls!), add the desired antibiotic to the desired concentration. Standard antibiotic concentrations are: kanamycin – 5 µg/ml; chloramphenicol – 15 µg/ml; ampicillin – 25 µg/ml. Resume incubating (with shaking) all cultures.
6. Take each 10^{-1} dilution and serially dilute in PBS or 10 mM $MgCl_2$ as needed (add factor of 10 to dilutions necessary for stationary phase cultures):

Antibiotic	Time (h)	Serial Dilution Needed (Approximate)
Any culture	0	10^{-6}
None (control)	2 or 4	10^{-7}
Chloramphenicol	Any	10^{-5} to 10^{-6}
Kanamycin	2 or 4	10^{-2} to 10^{-4}
Ampicillin	2 or 4	10^{-2} to 10^{-4}

Ensure adequate mixing of each tube by inverting 3-5 times prior to continuing dilution.

7. Plate 100 µl of the desired serial dilution onto an LB/Agar plate (no antibiotics). Plate each culture onto at least two plates. If the number of remaining viable colony-forming units cannot be reliably predicted then the two plates should have different serial dilutions (e.g. 10^{-4} and 10^{-5}).
8. Incubate plates face down at 37°C overnight.
9. After 2 h and 4 h, take a 100 µL sample from each culture and add to 900 µL sterile PBS or 10 mM $MgCl_2$. This is considered the 10^{-1} dilution.
10. Repeat steps 6 to 8 for each sample.
11. After overnight incubation, count the number of colonies on each plate. Disregard plates that contain fewer than 10 or greater than 300 colonies.
12. Calculate the CFU/ml of the culture:

CFU/ml = (# colonies on plate) / (dilution factor) x (volume plated in ml)

HPF assay (Kohanski et al., 2007)

This assay is to quantify the amount of ·OH present within the cells of a liquid culture of *E. coli* resulting from antibiotic exposure. The protocol can be adapted for a different compound / no added compound, if desired.

1. Inoculate overnight cultures of desired strains, in triplicate from isolated colonies, in 5 ml of LB media and incubate @ 37°C and 200 rpm overnight.
2. Subculture 1:1000 from overnight culture into 5 ml fresh LB media and incubate @ 37°C and 200 rpm.
3. Subculture 1:10 into 5 ml fresh LB media and incubate @ 37°C and 200 rpm.

4. At OD_{600} = 0.1 (exponential phase) or 1.5 (stationary phase), take a 1 ml or 0.2 ml sample, respectively, and spin down at 13000 g for 2 min. Add desired antibiotic to remainder of culture and continue incubating.
5. Remove the supernatant and resuspend in PBS buffer.
6. Wash the cells twice more.
7. After final wash, add 5 μM HPF to cell suspension. Incubate in darkness for 30 min at room temperature.
8. Wash the cells 3 times in PBS buffer. On final wash, adjust amount of PBS buffer to concentrate or dilute cells as necessary to achieve proper OD600 range in plate reader (200 μl of OD_{600} 0.2 to 0.4 sample).
9. Read samples on the fluorescence plate reader in LSB 506.
10. At desired time intervals, sample incubating cultures and read samples (as done in steps 4 through 9), with any PBS buffer also containing the antibiotic used.

qPCR Standard Operating Protocol

This protocol is to examine gene expression in *E. coli* liquid cultures exposed to antibiotics. This protocol can be adapted for cultures not exposed to antibiotics or exposed to a different compound.

RNA Isolation

1. Grow overnight cultures of desired strains in triplicate in LB and incubate at 37°C and 200 rpm overnight.

2. Subculture overnight cultures 1:1000 into fresh media (at least 25 ml) and incubate at 37°C and 200 rpm to OD_{600} = 0.3 (exponential phase) or 1.5 (stationary phase).
3. Add 2.5 ml of 5% phenol in 100% EtOH to 50 ml tubes and keep on ice.
4. Add 25 ml (exponential phase) or 5 ml (stationary phase) of culture to tubes containing phenol-EtOH.
5. Spin at 4000 rpm for 7 min at 4°C.
6. Discard the supernatant and freeze pellets in liquid nitrogen. Keep at -80°C prior to use.
7. Defrost pellet on ice. Resuspend pellet in 960 µl of resuspension buffer (at 4°C).
8. Vortex for 15-30 s.
9. Split sample (480 µl each) into two eppendorf tubes with an equal volume of hot phenol solution (400 µl resuspension buffer, 80 µl phenol, 4 µl β-mercaptoethanol).
10. Vortex for 15 s and incubate at 95°C for 1 min.
11. Spin down cell debris at 12000 rpm for 1 min at room temperature.
12. Transfer supernatant to new tubes and add 600 µl phenol-chloroform (1:1), vortex for 10 s, and spin down at 12000 rpm for 1 min at room temperature.
13. Repeat step 12 until no white stuff is visible at the interface between layers, and add 150 µl DEPC-ddH_2O as needed to make up any loss of volume (i.e. if decreases below 500 µl).

14. Transfer supernatant to RNase-free tubes with RNase-free tips (RNase free from here down).
15. Add 2.5 volumes of 100% EtOH and 0.1 volumes 3 M NaAcetate, and invert to mix.
16. Keep at -20°C for at least 5 min (may be left overnight).
17. Spin at 12000 g for 15 min at 4°C.
18. Wash pellet with 1 ml 75% EtOH and vortex for 15 s at half speed.
19. Spin at 12000 g for 15 min at 4°C.
20. Discard the supernatant and spin 5 s to be able to pipet out any remaining EtOH.
21. Dry pellet in the biosafety cabinet until no liquid is visible.
22. Resuspend RNA in 100 µl of DEPC-ddH_2O and mix by tapping the tubes. Keep on ice.
23. Check RNA quality by loading 5 µl samples on a 1% agarose gel and quantify the RNA through OD_{260} measurement.
24. Store RNA at -80°C.

DNase Treatment (taken from Fermentas; all reagents from Fermentas)

1. Add 10 µl of 10x DNase buffer to each RNA sample (all steps should be done with RNase-free tips).
2. Add 10 µl of DNase I to each RNA sample (alter as needed, based on amount of DNA seen in agarose gel above).
3. Incubate mixture at 37°C for 30 min.

4. Add 0.1 volumes of EDTA and incubate for 10 min at 65°C to inactivate the enzyme.
5. Check loss of DNA through a 1% agarose gel and by running a PCR.
6. Quantify the remaining RNA through OD_{260} measurement.
7. Return RNA samples to -80°C.

Reverse Transcription (taken from New England Biolabs with modification; all reagents from New England Biolabs)

1. Make master mix # 1, multiplying each reagent by the number of samples + 1.

Reagent	**Volume**
Random hexamers	2 µl
dNTP	1.6 µl
DEPC ddH_2O	12.4 µl less the Vol of RNA sample added

2. Aliquot master mix and add necessary volume of RNA sample (should be 1 µg to 2 µg of RNA present for reaction).
3. Heat at 70°C for 5 min using the PCR machine.
4. Make master mix # 2, multiplying each reagent by the number of samples + 1.

Reagent	**Volume**
10 x RT buffer	2 µl
RNase inhibitor	0.25 µl

MULV reverse transcriptase	0.125 µl
DEPC ddH_2O	1.625 µl

5. Aliquot master mix # 2 into each tube of RNA + hexamers + dNTP.
6. Heat at 42°C for 1 h using the PCR machine.
7. Heat at 95°C for 5 min to inactivate the enzyme.
8. Add 230 µl of ddH_2O to each tube.
9. Run a PCR to confirm that cDNA has been generated.

qPCR

1. Warm up machine lamp for at least 15 min (qPCR machine is in LSB-205).
2. Make master mix of SYBR green (10 µl), primers (0.2 µl), and ROX dye (0.2 µl).
3. Pipet 10 µl of mixture into each well used of the PCR plate.
4. Make genomic DNA dilutions from $3x10^5$ copies to $3x10^1$ copies.
5. Add 10 µl of cDNA or genomic DNA to each well. Five genomic DNA wells, one negative control well, and the necessary number of sample wells should be present for each gene being examined.
6. Spin down PCR plate for 1 min.
7. Insert plate and set reaction protocol.
8. Start qPCR run and wait to ensure that the machine does begin to warm up.
9. Check on machine periodically.
10. Collect PCR plate and data when run completed and power down machine.

Appendix B. Thesis defense

Impact of Catalases and Superoxide Dismutases on Antibiotic-Induced Oxidative Stress

Charlie Joyce
M.Sc. Defence
Department of Biology, McMaster University
July 30 2009

2

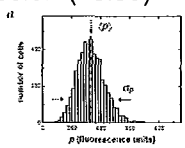
Outline

- Gene expression noise in the RpoS regulon
  - Noise in gene expression
  - RpoS
  - Objectives
  - Results
  - Conclusions
- Antibiotic-induced oxidative stress
  - Antibiotics: action and efficacy
  - Oxidative stress response proteins
  - Objectives
  - Results
  - Conclusions and significance

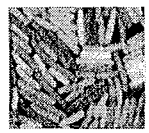
3

Stochastics in gene expression (noise)

- Noise (η) = $\langle p \rangle / \sigma_p$
  - Causes variation in expression between isogenic cells
  - Due to random microconcentration and orientation effects
- Primary factor in several phenomena
  - Lysis/lysogeny decisions by λ phages
  - Induction of competence in stationary phase of *Bacillus subtilis*



Variation in expression from cell to cell. Ozbudak et al., 2002



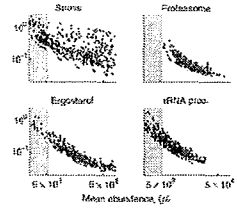
Noise in expression of two equivalent FP reporters. Elowitz et al., 2002

Arkin et al., 1998; Elowitz et al., 2002; Ozbudak et al., 2002; Maamar et al., 2007

4

Stochastics in gene expression (noise)

- Noise is suppressed in essential pathways of *Saccharomyces cerevisiae*
  - Variation in concentration of such enzymes would be detrimental
- Noise is not suppressed in stress response pathways
  - Allows individuals to "specialize" for particular conditions?
  - Noisy gene expression might improve an isogenic population's fitness



Noise vs. protein abundance in *S. cerevisiae* grouped by protein function. Bar-Even et al., 2006

Bar-Even et al., 2006; Raser and O'Shea, 2005

5

RpoS

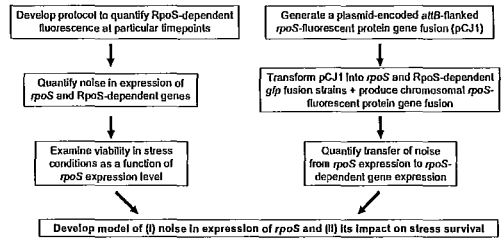
- An alternative sigma factor of RNA polymerase
  - Primarily found in β- and γ-proteobacteria
- Activated during stresses and stationary phase of growth
  - Induces expression of a large regulon to respond to these conditions
  - Reduces expression of non-RpoS controlled functions
  - Regulates approximately 10% of the genome
- RpoS is an integration point for a variety of signals
  - Transcription, translation, activity, and degradation are all regulated
- *rpoS* mutants commonly found in environmental isolates
  - Both advantages and drawbacks to RpoS expression

Patten et al. 2004; Dong et al., 2008

6

Objectives

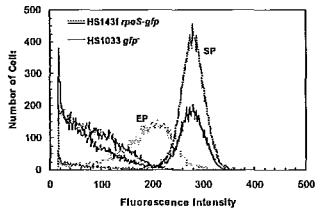
- Global objective:
  - To quantify the noise in gene expression in the RpoS regulon



7

Protocol development: flow cytometry

- Fluorescence microscopy originally used for noise quantification but *osmY-gfp* strain was not detected
  - Based on *lacZ* data, *osmY-gfp* should be fluorescent at OD_{600} = 1.5 but was not
  - Flow cytometry was selected as an alternative approach
- Using flow cytometry, HS143f (*rpoS-gfp*) was fluorescent in stationary phase
  - Shown at right



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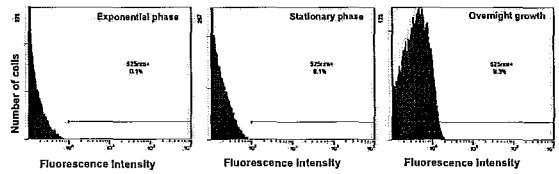
Protocol development: flow cytometry



- Results:
  - HS1091f (*osmY-gfp*): fluorescent after overnight growth only
- Conclusions:
  - At stationary phase, *rpoS-gfp* was fluorescent but *osmY-gfp* was not
  - How long is the lag between GFP expression and fluorescence?

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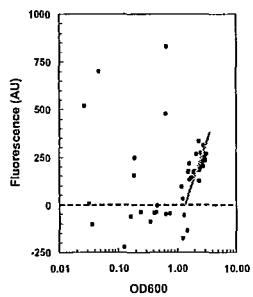
Protocol development: kinetic assay

- Methods:
  - HS1091f (*osmY-gfp*) was grown in LB
  - Fluorescence and OD_{600} were assayed periodically
- Results:
  - Fluorescence begins to increase at OD_{600} = 1.05
- Conclusion:
  - Approximately 40 min lag between GFP expression and fluorescence



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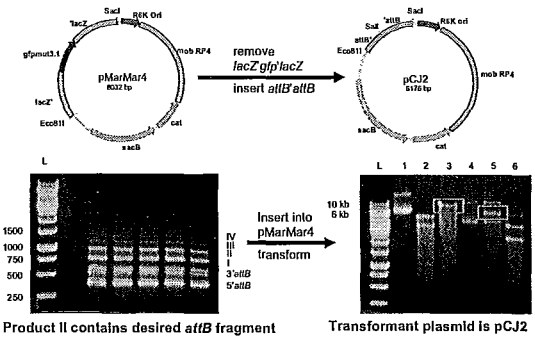
pCJ1 construction: pCJ2 intermediate



Product II contains desired *attB* fragment

Transformant plasmid is pCJ2

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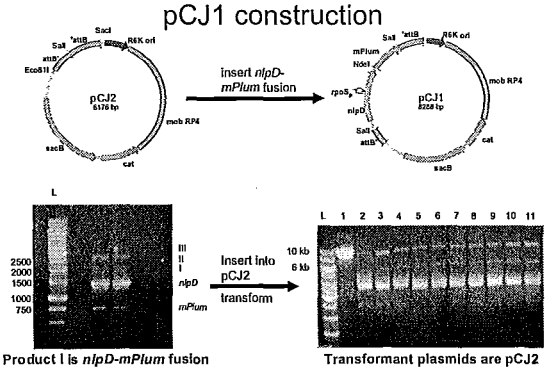
pCJ1 construction



Product I is *nlpD-mPlum* fusion

Transformant plasmids are pCJ2

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Conclusions

- Protocol development and noise quantification:
  - Lag between GFP expression and detectable fluorescence was observed
  - Lag was approximately 40 min long
  - Future student can examine noise in expression of RpoS-dependent genes incorporating the 40 min delay into protocol
- pCJ1 construction:
  - Intermediate vector pCJ2 was successfully constructed
  - Putative pCJ1 transformants actually contained pCJ2
  - Future student can complete construction of pCJ1 and examine noise in expression of *rpoS* and RpoS-dependent genes in the same cell

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Outline

- Gene expression noise in the RpoS regulon
  - Noise in gene expression
  - RpoS and its regulon
  - Objectives
  - Results
  - Summary and future directions
- Antibiotic-induced oxidative stress
  - Antibiotics: action and efficacy
  - Oxidative stress response proteins
  - Objectives
  - Results
  - Conclusions and significance

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Antibiotics

- Inhibit microorganism growth and target essential processes that are not present or are divergent in humans
  - Cell wall synthesis (β-lactams, glycopeptides)
  - Translation (aminoglycosides, tetracyclines, chloramphenicol)
  - DNA gyrase / topoisomerase IV (fluoroquinolones)
- Though central to modern medicine, resistance to antibiotics is increasing in pathogenic bacteria
  - Vast majority of antibiotic classes were developed = 30 years ago
- Possible ways to maintain utility of current antibiotics:
  - Behavioral changes?
  - Adding a second compound to enhance the antibiotic's potency?

Projan and Shales, 2004; Pucci, 2006; Rybak, 2007

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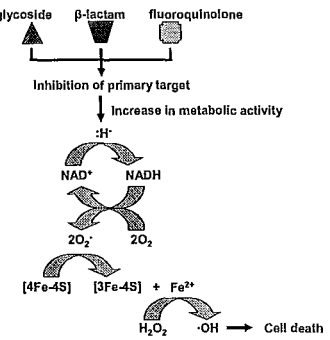
Bactericidal antibiotics: ROS generators?

- Model:
  1. Bactericidal antibiotic inhibits primary target
  2. Metabolic surge produces NADH
  3. NADH non-specifically reduces O_2 to O_2^-
  4. O_2^- increases intracellular [Fe^{2+}]
  5. Fe^{2+} catalyzes production of ·OH
  6. Massive cell damage results in death



Kohanski et al., 2007

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Catalases and superoxide dismutases

- Catalases:
  - Catalyze $2H_2O_2$? $2H_2O + O_2$
  - HPI (*katG*) is a catalase/peroxidase regulated by OxyR
  - HPII (*katE*) is a monofunctional catalase regulated by RpoS
- Superoxide dismutases (SOD):
  - Catalyze $2O_2^- + 2H^+$? $O_2 + H_2O_2$
  - Mn-SOD (*sodA*) is regulated by SoxRS
  - Fe-SOD (*sodB*) is regulated by Fur
  - Cu/Zn-SOD (*sodC*) is regulated by RpoS

Catalase HPII at 1.9 Å resolution. PDB ID: 1GG9

Mn-SOD at 0.9 Å resolution. PDB ID: 1IXB

Benov and Fridovich, 1994; Dubrac and Touati, 2002; Ding et al., 1996; Fridovich, 1997; Imlay, 2003; Loewen, 1984; Zheng et al., 1998

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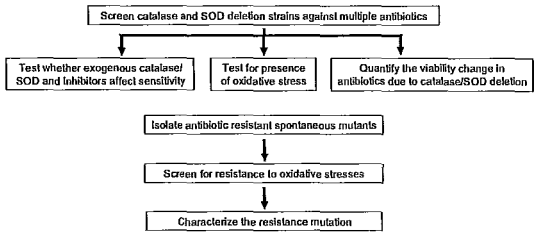
Objective

- Global objective:
  - To determine whether ROS-detoxifying enzymes affect antibiotic sensitivity



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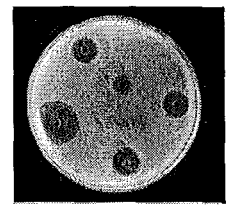
Screen of catalase and SOD mutants for antibiotic sensitivity

- Screen was done via a disc diffusion assay
- Methods:
  - Strains grown to OD_{600} = 0.8
  - Strains were plated, antibiotic discs added, and incubated at 37°C
  - Zones of inhibition measured the following day
- Larger zone = more sensitive
- Smaller zone = less sensitive



www.actionbioscience.org/figures/kardarphoto.jpg

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Loss of catalase activity increases antibiotic sensitivity

Luria-Bertani media (rich media) – in mm									
Strain	Genotype	Amp†	Kan†	Nal†	Nor†	Str†	Tmp	Chl	Tet
MG1655	Wild type	12.8±0.3	10.7±0.2	18.2±0.7	12.8±0.5	9.5±0.2	20.0±0.4	16.2±0.4	18.8±0.4
HS2605	Δ *katE*	15.3±0.3	11.7±0.2	21.3±0.5	16.2±0.4	9.2±0.3	23.7±0.3	17.8±0.2	19.8±0.2
HS2606	Δ *katG*	15.5±0.3	12.0±0.0	20.5±0.5	16.8±0.4	9.3±0.2	22.2±0.2	17.5±1.0	18.7±0.7
HS2607	Δ *katE* Δ *katG*	15.8±0.2	12.5±0.3	21.3±0.3	16.0±0.7	9.7±0.2	22.3±0.4	16.5±0.5	18.5±0.3
MG1655	Wild type	11.5±0.3	11.0±0.3	11.3±0.3	13.5±0.3	9.5±0.2	14.5±0.6	12.7±0.2	12.3±0.2
HS2210	Δ *rpoS*	12.5±0.2	12.2±0.5	13.0±0.4	17.0±0.4	9.8±0.2	17.0±0.4	13.2±0.2	12.3±0.3
HS2824	Δ *oxyR*	12.7±0.2	12.2±0.7	13.8±0.4	16.2±0.5	9.7±0.2	16.2±0.2	14.2±0.4	13.5±0.3
HS2828	Δ *rpoS* Δ *oxyR*	13.2±0.2	13.3±0.2	13.5±0.3	17.8±0.9	10.0±0.3	17.5±0.2	14.5±0.4	13.5±0.3

N = 6 Blue = significantly higher at P=0.05 † = bactericidal

- Catalase and catalase regulator deletion strains were screened
- Results:
  - Loss of catalase activity ? sensitivity to Amp, Kan, Nal, Nor, and Tmp
  - Loss of catalase regulators ? sensitivity to all antibiotics except Str

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SOD have little effect on antibiotic sensitivity

Luria-Bertani media – in mm									
Strain	Genotype	Amp†	Kan†	Nal†	Nor†	Str†	Tmp	Chl	Tet
MG1655	Wild type	15.3±0.2	11.5±0.1	13.8±0.1	16.8±0.2	10.8±0.2	15.8±0.1	15.8±0.2	15.8±0.1
HS2906	Δ *sodA*	14.8±0.2	12.0±0.2	13.8±0.2	16.5±0.3	10.0±0.2	15.5±0.1	15.5±0.1	15.5±0.1
HS2910	Δ *sodB*	14.7±0.1	12.0±0.2	13.0±0.2	16.3±0.2	9.8±0.1	15.7±0.1	15.7±0.1	15.7±0.1
HS2911	Δ *sodA* Δ *sodB*	10.7±0.1	10.3±0.1	15.7±0.2	17.7±0.2	8.7±0.1	17.0±0.2	17.2±0.3	17.0±0.2

N = 6 Blue = significantly higher at P=0.05 † = bactericidal
Red = significantly lower at P=0.05

- SOD deletion strains were screened using the same method
- Results:
  - Few changes in sensitivity were observed with only one SOD lost
  - Loss of both SOD altered sensitivity to a variety of antibiotics
    - Effect of slower growth?

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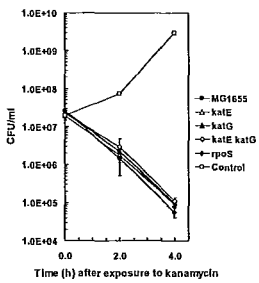
Exogenous catalase, inhibitors, and viability

- Addition of exogenous catalase:
  - Reduces sensitivity to antibiotics in catalase deletion strains
- Addition of NH_2OH:
  - Reduces sensitivity difference between mutant and wild type
- Viability in Kan (right):
  - No difference in viability between wild type and mutants in first 4 h



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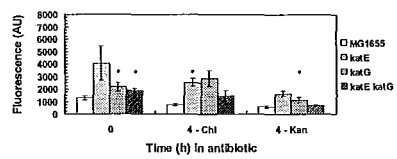
Detection of oxidative stress



- Methods:
  - Strains were grown to exponential or stationary phase, exposed to antibiotic for 4 h, then sampled and stained with HPF (·OH reporter)
- Results:
  - Only loss of *katE* resulted in ? ·OH production vs. the wild type in antibiotics in either growth phase
  - All SOD deletion strains had similar ·OH as the wild type

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Mutant isolation and screening

Total CFU plated	# mutants	# mutants/ CFU	Cross Resistance to	
			H_2O_2	Paraquat
3.965×10^{11}	241	6.08×10^{-10}	0	87

- Hypothesis:
  - If antibiotics act via ROS generation, then selecting on multiple bactericidal antibiotics should generate ROS-resistant mutants
- Methods:
  - 14 isolates grown to saturation and selected on Amp, Kan, and Nor
  - Mutants were screened against oxidative stress-inducing agents
- Results:
  - 241 resistant mutants were obtained from 3.965×10^{11} CFU
  - 36.1% were resistant to paraquat (an O_2^- generator) and 0% to H_2O_2

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Paraquat screening

- Methods:
  - The 87 putative paraquat^R mutants were screened against a broader range of concentrations
- Results:
  - 37 were confirmed paraquat^R beyond wild type level (1 mM)
    - 15.1% of total mutants
- Conclusion:
  - Selection on bactericidal antibiotics can result in oxidative stress resistance

Paraquat concentration (μM)	Number of resistant mutants	% of Total
0	87	100.0
200	83	95.4
400	78	89.7
600	76	87.4
800	71	81.6
1000	55	63.2
1500	37	42.5
2000	23	26.4
3000	3	3.4
4000	0	0.0

Blue = concentrations at which wild type is sensitive

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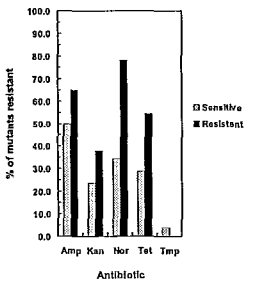
Paraquat screening

- Hypothesis:
  - Paraquat^R mutants are more likely to be resistant to antibiotics than paraquat^S mutants
- Methods:
  - Resistance to a set of antibiotics was compared for the 37 paraquat^R and 204 paraquat^S mutants
- Results:
  - Paraquat^R mutants were more likely to be resistant to each of Amp, Kan, Nor, and Tet



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Conclusions

- Catalase:
  - Loss of catalase activity increased antibiotic sensitivity
  - Increased H_2O_2 scavenging not detected in antibiotic^R mutants
- SOD:
  - Loss of both SodA and SodB affected sensitivity to a variety of antibiotics tested
  - Increase in O_2^- scavenging correlated with increased antibiotic resistance
- ROS generated in bactericidal and bacteriostatic antibiotics
  - Catalase activity affects sensitivity to bactericidal antibiotics and Tmp
  - Paraquat^R mutants are more likely to be Tet^R

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Significance

- Further elucidates the mechanism of antibiotic action
  - Supports and expands upon model of ROS generation by antibiotics
  - Does this mechanism also apply in pathogenic organisms?
    - Effect varies depending on strain background (MG1655 vs. MC4100)
    - Similar effect in pathogenic *E. coli*?
    - Similar effect in (e.g.) *Salmonella*?
- Provides a possible new avenue for therapeutic research
  - Catalase inhibitors may increase the efficiency of antibiotics if administered together
  - Currently no non-heme inhibitors of catalase exist, yet such inhibitors could be developed

Kolodkin-Gal et al., 2008

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References

A. Arkin, J. Ross, H. H. McAdams, *Genetics* 149, 1633-1648 (1998).

A. Bar-Even et al., *Nat.Genet.* 38, 636-643 (2006).

L. T. Benov and I. Fridovich, *J.Biol.Chem.* 269, 25310-25314 (1994).

H. Ding, E. Hidalgo, B. Demple, *J.Biol.Chem.* 271, 33173-33175 (1996).

T. Dong, C. Joyce, H. E. Schellhorn, In *Bacterial physiology: a molecular approach*, W. El-Sharoud, Ed. (Springer, Berlin, 2008).

S. Dubrac and D. Touati, *Microbiology* 148, 147-156 (2002).

M. B. Elowitz, A. J. Levine, E. D. Siggia, P. S. Swain, *Science* 297, 1183-1186 (2002).

I. Fridovich, *J.Biol.Chem.* 272, 18515-18517 (1997).

J. A. Imlay, *Annu.Rev.Microbiol.* 57, 395-418 (2003).

M. A. Kohanski, D. J. Dwyer, B. Hayete, C. A. Lawrence, J. J. Collins, *Cell* 130, 797-810 (2007).

I. Kolodkin-Gal, B. Sat, A. Keshet, H. Engelberg-Kulka, *PLoS.Biol.* 6, e319 (2008).

P. C. Loewen and B. L. Triggs, *J.Bacteriol.* 160, 668-675 (1984).

H. Maamar, A. Raj, D. Dubnau, *Science* 317, 526-529 (2007).

E. M. Ozbudak, M. Thattai, I. Kurtser, A. D. Grossman, A. van Oudenaarden, *Nat.Genet.* 31, 69-73 (2002).

C. L. Patten, M. G. Kirchhof, M. R. Schertzberg, R. A. Morton, H. E. Schellhorn, *Mol.Genet.Genomics* 272, 580-591 (2004).

S. J. Projan and D. M. Shlaes, *Clin.Microbiol.Infect.* 10 Suppl 4, 18-22 (2004).

M. J. Pucci, *Biochem.Pharmacol.* 71, 1066-1072 (2006).

J. M. Raser and E. K. O'Shea, *Science* 309, 2010-2013 (2005).

M. J. Rybak, *Pharmacotherapy* 27, 1311S-1355 (2007).

M. Zheng, F. Aslund, G. Storz, *Science* 279, 1718-1721 (1998).