Analysis of Suppressors Overcoming *sodAB* Deletion in a Stringent Deficient Escherichia Coli Background

by

Melissa Flake

A Thesis Presented in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Approved April 2024 by the
Graduate Supervisory Committee:

Rajeev Misra, Chair
Xuan Wang
Dhara Shah

ARIZONA STATE UNIVERSITY

May 2024

ABSTRACT

Reactive oxygen species (ROS) including superoxide, hydrogen peroxide, and hydroxyl radicals occur naturally as a byproduct of aerobic respiration. To mitigate damages caused by ROS, *Escherichia coli* employs defenses including two cytosolic superoxide dismutases (SODs), which convert superoxide to hydrogen peroxide. Deletion of both *sodA* and *sodB*, the genes coding for the cytosolic SOD enzymes, results in a strain that is unable to grow on minimal medium without amino acid supplementation. Additionally, deletion of both cytosolic SOD enzymes in a background containing the *relA1* allele, an inactive version of the *relA* gene that contributes to activation of stringent response by amino acid starvation, results in a strain that is unable to grow aerobically, even on rich medium. These observations point to a relationship between the stringent response and oxidative stress.

To gain insight into this relationship, suppressors were isolated by growing the Δ*sodAB relA1* cells aerobically on rich medium, and seven suppressors were further examined to characterize distinct colony sizes and temperature sensitivity phenotypes. In three of these suppressor-containing strains, the *relA1* allele was successfully replaced by the wild type *relA* allele to allow further study in aerobic conditions. None of those three suppressors were found to increase tolerance to exogenous superoxides produced by paraquat, which shows that these mutations only overcome the superoxide buildup that naturally occurs from deletion of SODs. Because each of these suppressors had unique phenotypes, it is likely that they confer tolerance to SOD-dependent superoxide buildup by different mechanisms. Two of these three suppressors have been sent for whole-

genome sequencing to identify the location of the suppressor mutation and determine the mechanism by which they confer superoxide tolerance.

TABLE OF CONTENTS

Page

LIST OF TABLES v

LIST OF FIGURES vi

CHAPTER

1 INTRODUCTION 1

Overview of Oxidative Stress 1

Damages Caused by Oxidative Stress 1

Oxidative Stress Response in *E. coli* 2

Iron Homeostasis and Oxidative Stress 2

Stringent Response 3

Stringent Response and Superoxide Stress 4

RNA Polymerase Mutation *rpoB58* Overcomes Some Superoxide Stress Phenotypes 4

Specific Aims 4

2 RESULTS 6

Aim 1: Obtaining Suppressors 6

Aim 2: Phenotypic Analysis of Suppressors 7

3 DISCUSSION 13

4 MATERIALS AND METHODS 15

REFERENCES 5

LIST OF TABLES

Table Page

1. Growth of *relA1* Δ*sodAB* Suppressors on Rich and Minimal Media 9

2. Growth of *relA1* Δ*sodAB* Suppressors at Various Temperatures 10

3. Growth of *relA*+ Δ*sodAB* Suppressors on Rich and Minimal Media..... 11

4. Growth of *relA*+ Δ*sodAB* Suppressors at Various Temperatures..... 12

5. *fepA:lacZ* Expression in *relA*+ Δ*sodAB* Suppressors 13

LIST OF FIGURES

Figure Page

1. Representation of the Workflow for Obtaining Revertant Strains to Overcome the Aerobic Growth Defect of a *relA1 ΔsodAB* Strain on Rich Media 8
2. PCR Confirmation of Conversion of *relA1* to *relA*+ During P1 Transduction... 11
3. *fepA:lacZ* Expression in *relA*+ Δ*sodAB* Suppressors 13
4. Paraquat Sensitivity of *relA*+ Δ*sodAB* Suppressors..... 14
5. Model of How Suppressors May Allow Aerobic Growth 16

CHAPTER 1

INTRODUCTION

Overview of Oxidative Stress

Roughly 2.5 billion years ago, microorganisms had to adapt to rapidly increasing oxygen levels in the atmosphere due to oxygen produced by photosynthetic organisms (1). Some species specialized to grow in anaerobic environments, while others developed abilities to withstand differing levels of atmospheric oxygen. Gerschman et al. were the first to identify reactive oxygen species (ROS) as the method of oxygen-mediated damage (2). ROS, including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH), naturally builds up as a byproduct of aerobic respiration (3). Buildup of ROS causes damage to proteins, lipids, and DNA (4, 5). Oxygen tolerant species, including the facultative anaerobe *Escherichia coli,* employ defenses including scavenging enzymes such as superoxide dismutase (SOD) and catalase to mitigate damages caused by reactive oxygen species.

Damages Caused By Oxidative Stress

Most damages caused by superoxide are dependent on iron. Superoxide oxidizes [4Fe-4S] clusters of a family of Fe-S cluster-cofactored dehydratases, causing them to release the catalytic iron atom and become inactive (4). Superoxide also oxidizes and releases iron atoms of mononuclear enzymes, which often leads to mis-metalation with zinc, making the enzymes less catalytically efficient (4). The iron released from damaged enzymes can react with hydrogen peroxide to form hydroxyl radicals via the Fenton reaction:

$Fe^{2+} + H_2O_2 \rightarrow [FeO]^{2+} + H_2O$

$[FeO]^{2+} + H^+ \rightarrow Fe^{3+} + HO\bullet$

These hydroxyl radicals (HO•) damage DNA by oxidizing the nucleotide bases or ribose moieties, creating lesions that must be repaired by error prone polymerases as part of the SOS response (3, 4, 6, 7).

Oxidative Stress Response in *E. coli*

To minimize the damage done by ROS, *E. coli* cells employ defenses including superoxide dismutase (SOD) to convert superoxide to hydrogen peroxide, and enzymes such as catalase to break down hydrogen peroxide into water. No enzymes have been found that can neutralize the hydroxyl radical, thus reflecting its potency. *E. coli* has two cytosolic SODs, manganese-dependent MnSOD encoded by *sodA* and iron-dependent FeSOD encoded by *sodB* (3, 4). High iron levels cause the ferric uptake regulator (Fur) to become active and block *sodA* (MnSOD) transcription. However, when iron levels are low, *sodA* (MnSOD) is transcribed but *sodB* (FeSOD) is not synthesized due to translational interference by small RNA RyhB (3, 4). Transcription of *sodA* (MnSOD) is also controlled by the transcriptional regulators ArcA, FNR, IHF, and the products of *soxRS* and *soxQ* (7).

Iron Homeostasis and Oxidative Stress

Fur is activated when it is bound to its corepressor Fe^{2+}. Besides blocking transcription of *sodA*, as has been mentioned previously, activated Fur-Fe^{2+} binds to the promoter region and blocks transcription of iron acquisition genes including the *entCDEBAH* operon, which encodes proteins involved in synthesis of enterobactin (8, 9).

Enterobactin is a siderophore (iron chelator) that binds extracellular Fe^{3+} to bring it back into the cell (9). Transcription of enterobactin transport genes, such as *fepA*, is also regulated by Fur (8–11). It has recently been found that enterobactin synthesis is also induced by oxidative stress and that the compound provides protection against the damages caused by hydrogen peroxide and superoxide (9). The mechanism of enterobactin-mediated protection from oxidative stress is unknown but it has been shown to be independent of enterobactin's iron scavenging activity (9). Superoxide itself can have a direct impact on iron homeostasis in the cell. Superoxide can oxidize and remove iron from proteins, including removing Fe^{2+} from Fur so that it does not strongly associate with its DNA binding site, causing an increase in expression of iron uptake genes (12).

Stringent Response

The stringent response was initially characterized in *E. coli* as a response to amino acid starvation (13–15). Unavailability or starvation for amino acids increase the level of uncharged tRNAs, which causes ribosomes to pause on translating mRNAs. This triggers RelA to bind to the stalled ribosome and activates RelA's synthetase activity. When activated, RelA synthesizes the signaling molecules guanosine 5′-diphosphate 3′-diphosphate (ppGpp) and guanosine 5′-triphosphate 3-diphosphate (pppGpp), collectively known as (p)ppGpp (16). (p)ppGpp and an accessory protein, DksA, bind to RNA polymerase and induce conformational changes that lead to transcriptional changes including upregulation of genes involved in amino acid synthesis and downregulation of genes involved in protein synthesis (16, 17).

RelA is encoded by the gene *relA*. The *relA1* mutation contains an insertion sequence (IS) element between codons 85 and 86 that renders the protein largely inactive. The resulting phenotype of the *relA1* allele is described as "relaxed" as opposed to the wildtype *relA* "stringent" phenotype.

Stringent Response and Superoxide Stress

In a stringent *relA+* background, if both *sodA* and *sodB* are deleted (*relA+* Δ*sodAB*), the resulting strain is able to grow aerobically on rich medium but requires supplementation with amino acids for growth on minimal medium. The branched chain amino acids (isoleucine, leucine, and valine) synthesizing enzymes are especially sensitive to superoxide (4, 18, 19). This is because the enzyme dihydroxy-acid dehydratase, which is a common enzyme in the branched chain amino acid synthesis pathways, is inactivated by superoxide due to extraction of Fe from its [4Fe-4S] catalytic cluster (20–23).

However, in a *relA1* background with *sodAB* deleted (*relaA1* Δ*sodAB)*, the resulting strain is unable to grow aerobically even on rich medium. This suggests an intriguing interplay between the stringent response and superoxide-mediated oxidative stress, where the cells are dependent on a functional RelA protein in order to overcome buildup of superoxide in the absence of MnSOD and FeSOD. This project was designed to potentially uncover information about this mechanism and/or other mechanisms that allow *E. coli* cells to overcome superoxide stress.

RNA Polymerase Mutation *rpoB58* Overcomes Some Superoxide Stress Phenotypes

A mutation, *rpoB58*, in the beta-subunit of the RNA polymerase has been found to confer a phenotype similar to a constitutive stringent response (24). *rpoB58* was first

isolated as a mutation to confer novobiocin and erythromycin resistance to a strain with the antibiotic efflux pumps AcrAB and AcrEF disabled (24). When *rpoB58* is present in a *relA*+ Δ*sodAB* background, it overcomes the amino acid auxotrophy seen in the *relA*+ Δ*sodAB rpoB* wildtype (*rpoB-WT*) strain. However, when *rpoB58* is present in a *relA1* Δ*sodAB* background, it overcomes the aerobic growth defect on rich medium but still requires amino acid supplementation for growth on minimal medium. The pathway(s) by which *rpoB58* overcomes these defects is unknown. Mutations in the RNA polymerase tend to have pleiotropic effects, meaning that multiple pathways are affected by a single mutation. This is because a mutation in RNA polymerase can affect the expression of many different genes at once. Therefore, it is difficult to identify a single underlying mechanism or pathway by which *rpoB58* overcomes the aerobic growth defect of *relA1* Δ*sodAB* or overcomes the amino acid auxotrophy of *relA*+ Δ*sodAB*. For this reason, it was deemed useful to perform a suppressor analysis so that non-pleiotropic mutations can be obtained that overcome growth defects of a *relA1* Δ*sodAB* strain.

Specific Aims

The goals of this thesis project were (a) to determine why stringent incompetent (*relA1*) cells lacking *sodAB* are unable to grow on rich medium, and (b) how cells can overcome this growth defect. In order to address these goals, suppressor analysis was conducted to isolate mutations that overcome the aerobic growth defect of a *relA1* Δ*sodAB* on rich medium. Next, phenotypes of suppressor mutants were analyzed to determine similarity and/or distinction among suppressor mutations they carry.

CHAPTER 2

RESULTS

Aim 1: Obtaining suppressors

A method for isolating suppressors

In order to understand why *relA1* Δ*sodAB* cells are unable to grow aerobically, a suppressor analysis experiment was designed to obtain revertant strains that overcome the growth defect. From a frozen stock, a *relA1* Δ*sodAB* strain was streaked on LBA and incubated anaerobically for 48 hours at 37°C. Multiple anaerobically-grown colonies were then streaked on LBA and plates were incubated aerobically at 37°C. Each independently-streaked colony gave rise to different numbers of revertants after 24 to 48 hours of incubation. Twenty-four independent revertant colonies were re-streaked on LBA and grown aerobically for 48 hours at 37°C. Eleven of 24 revertants formed homogenous (uniform) colonies upon re-streaking, reflecting a stable nature of the suppressor mutations, and were stored at -80°C. Of those eleven, four were obtained by another student in the research group, so this project focuses on the other seven revertants. Figure 1 shows a graphic depicting the procedure used to obtain these revertant strains.

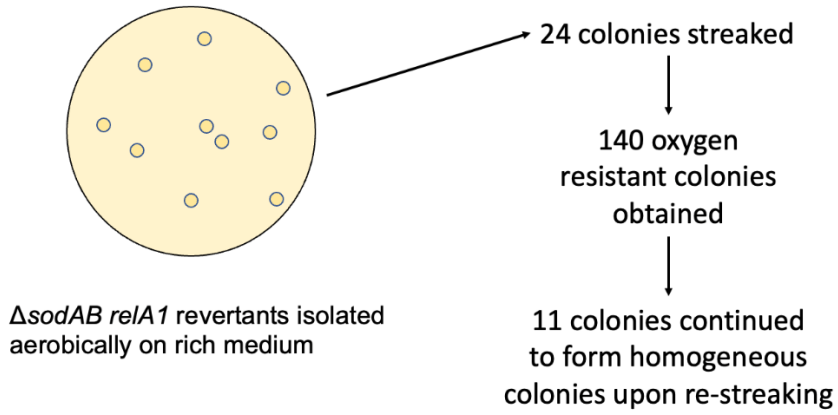

Figure 1. Representation of the workflow for obtaining revertant strains to overcome the aerobic growth defect of a *relA1* Δ*sodAB* strain on rich media.

Aim 2: Phenotypic analysis of suppressors

Analysis of suppressor mutations in a *relA1* background:

Growth of the seven stable revertants was compared against each other and a control *relA1* Δ*sodAB rpoB58* strain. Recall, the *rpoB58* mutation overcomes the *relA1* Δ*sodAB* growth defect but has pleiotropic effects, making it difficult to identify the specific pathway that allows it to overcome superoxide stress. By comparing the *rpoB58* phenotype to the phenotypes of the unknown suppressor mutations, it is possible to rule out mutations that may be similar to *rpoB58* and therefore may not provide as much new information.

Based on the colony size, revertants were categorized into three groups, with some resembling the *relA1* Δ*sodAB rpoB58* strain. When their growth was tested on minimal medium, none of the revertants were able to grow, reflecting acute amino acid auxotrophy. This phenotype was similar to the *relA1* Δ*sodAB rpoB58.* When minimal medium was supplemented with casamino acids, some revertants displayed partial

growth but the growth was much poorer than that displayed by the *relA1* Δ*sodAB rpoB58* strain. This indicated that suppressor mutations differ from *rpoB58*.

Table 1. Growth of *relA1* Δ*sodAB* suppressors on rich and minimal media. Glu refers to glucose. Su+ refers to any unknown suppressor mutation.

Strain	**Genotype**	**Growth***		
		LBA	**M63+glucose +amino acids**	**M63+glucose**
3329	*relA1* Δ*sodAB*	0	0	0
3332	*relA1* Δ*sodAB rpoB58*	3	3	0
2-1A	*relA1* Δ*sodAB* Su+	2	0	0
2-4B	*relA1* Δ*sodAB* Su+	3	1	0
1-1	*relA1* Δ*sodAB* Su+	3	2	0
6-2	*relA1* Δ*sodAB* Su+	3	2	0
7-1	*relA1* Δ*sodAB* Su+	3	1	0
7-3	*relA1* Δ*sodAB* Su+	3	0	0
12-1	*relA1* Δ*sodAB* Su+	1	0	0

* 0 = no growth; 1 to 3 = small to large colony size growth

In addition to testing growth on different media, the growth of the revertant strains was also tested at various temperatures. There was significant variation in temperature sensitivity. Two revertant strains, 6-2 and 7-1, showed cold sensitivity at 30°C and robust growth at 37°C, 42°C, and 45°C, similar to the *relA1* Δ*sodAB rpoB58* strain. The other revertants displayed a variety of temperature sensitivity phenotypes.

Table 2. Growth of *relA1* Δ*sodAB* suppressors at various temperatures. Su+ refers to any unknown suppressor mutation.

Strain	**Genotype**	**Growth (LBA)***			
		30°C	**37°C**	**42°C**	**45°C**
3329	*relA1* Δ*sodAB*	0	0	0	0
3332	*relA1* Δ*sodAB rpoB58*	1	3	3	3
2-1A	*relA1* Δ*sodAB* Su+	0	2	0	0
2-4B	*relA1* Δ*sodAB* Su+	0	3	1	0
1-1	*relA1* Δ*sodAB* Su+	1	3	0	0
6-2	*relA1* Δ*sodAB* Su+	1	3	3	3
7-1	*relA1* Δ*sodAB* Su+	1	3	2	2
7-3	*relA1* Δ*sodAB* Su+	0	3	2	0
12-1	*relA1* Δ*sodAB* Su+	0	1	1	1

* 0 = no growth; 1 to 3 = small to large colony size growth

Analysis of suppressor mutations in *relA*+ background:

In order to further characterize the unknown mutations, it was attempted to convert the *relA1* allele in the unknown suppressor strains to *relA*+ by P1 phage transduction using a *relA*+-linked kanamycin resistant marker. In three of the suppressor strains, the *relA1* allele was successfully replaced by the *relA*+ allele, as determined by amplifying *relA*-specific DNA by polymerase chain reaction (PCR). Owing to the presence of an IS element in the *relA1* allele, the PCR-amplified DNA segment is roughly one kilobase larger than the corresponding *relA*+ region (Figure 2).

The growth of these three *relA*+ suppressor strains was characterized on LBA and minimal media and compared to the *relA*+ Δ*sodAB rpoB58* strain. As previously observed, *rpoB58* overcame amino acid auxotrophy of Δ*sodAB* in the *relA*+ background. Notably, the suppressor mutations did not overcome amino acid auxotrophy, indicating that they must not be the same mutation as *rpoB58* and therefore they can likely offer some useful new information upon further study.

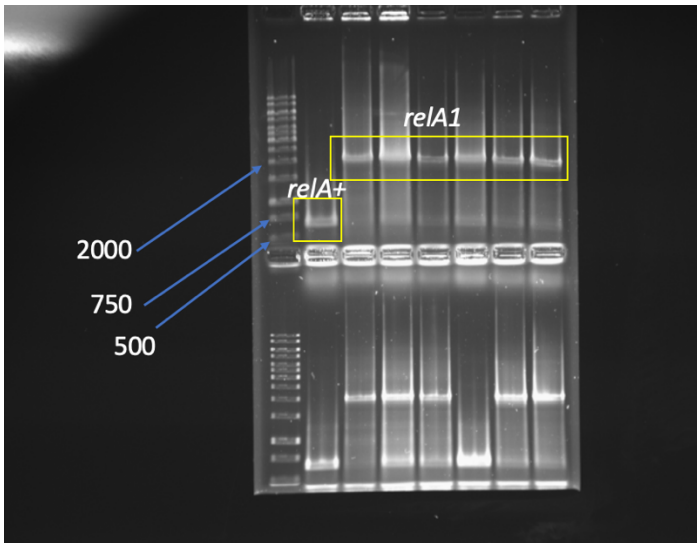

Figure 2. PCR confirmation of conversion of *relA1* to *relA*+ during P1 transduction. This is an example of a gel showing PCR results to confirm conversion of *relA1* to *relA*+. The DNA fragments between 750 and 500 base pairs are *relA*+ while the longer DNA fragments near 2000 base pairs contain the IS element in the *relA1* allele. Three revertant strains were successfully converted from *relA1* to *relA*+.

Table 3. Growth of *relA*+ Δ*sodAB* suppressors on rich and minimal media. Glu refers to glucose. Su+ refers to any unknown suppressor mutation.

Strain	**Genotype**	**Growth***		
		LBA	**M63+glucose +amino acids**	**M63+glucose**
3383	*relA*+ Δ*sodAB*	3	2	0
3385	*relA*+ Δ*sodAB rpoB58*	3	3	2
6-2	*relA*+ Δ*sodAB* Su+	3	2	0
7-1	*relA*+ Δ*sodAB* Su+	3	2	0
7-3	*relA*+ Δ*sodAB* Su+	3	2	0

* 0 = no growth; 1 to 3 = small to large colony size growth

The growth of the *relA*+ suppressors was also tested at various temperatures. In the *relA*+ background, 6-2 and 7-1 grew robustly at 37°C, 42°C, and 45°C and showed only slightly weaker growth at 30°C, similar to the *relA*+ Δ*sodAB* control. 7-3 was able

to grow at all four temperatures, but was still sensitive to the cold at 30°C and the heat at 45°C.

Table 4. Growth of *relA*+ Δ*sodAB* suppressors at various temperatures. Su+ refers to any unknown suppressor mutation.

Strain	**Genotype**	**Growth (LBA)***			
		30°C	**37°C**	**42°C**	**45°C**
3383	*relA*+ *ΔsodAB*	2	3	3	3
3385	*relA*+ *ΔsodAB rpoB58*	1	3	3	3
6-2	*relA*+ *ΔsodAB* Su+	2	3	3	3
7-1	*relA*+ *ΔsodAB* Su+	2	3	3	3
7-3	*relA*+ *ΔsodAB* Su+	1	3	2	2

* 0 = no growth; 1 to 3 = small to large colony size growth

The state of iron homeostasis was assessed by measuring the activity of a *fepA:lacZ* fusion construct that responds to changes in Fur-Fe^{2+} activity. All three suppressor strains expressed higher *fepA* than the isogenic *rpoB58* strain, further confirming that the unknown mutations are different from *rpoB58*. In addition, mutant strain 7-3 expressed significantly higher *fepA* compared to the other two strains. Strains 6-2 and 7-1 had very similar phenotypes in all tests conducted, suggesting that they may contain similar mutations affecting the same pathway or potentially even in the same gene.

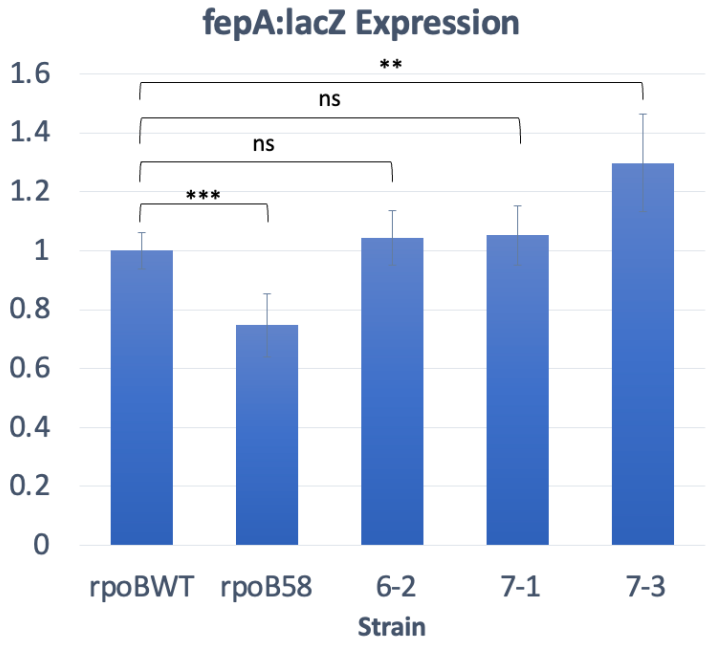

Figure 3. *fepA:lacZ* expression in *relA*+ Δ*sodAB* suppressors. Beta-galactosidase assay results comparing expression of *fepA* in each strain.

Table 5. *fepA:lacZ* expression in *relA*+ Δ*sodAB* suppressors. Data from beta-galactosidase assay shown in table form. Su+ refers to any unknown suppressor. SD refers to standard deviation.

Strain	**Genotype**	***fepA* expression in Miller Units (SD)**
3525	*relA*+ Δ*sodAB*	355 (±22.16)
3528	*relA*+ Δ*sodAB rpoB58*	265 (±37.98)
6-2	*relA*+ Δ*sodAB* Su+	370 (±32.97)
7-1	*relA*+ Δ*sodAB* Su+	373 (±35.54)
7-3	*relA*+ Δ*sodAB* Su+	460 (±58.95)

To assess resistance to additional oxidative stress beyond what occurs naturally within the cell under aerobic growth conditions, a minimum inhibitory concentration (MIC) assay was conducted using the superoxide-generating agent paraquat. Results showed that, while the *relA*+ Δ*sodAB rpoB58* strain showed significantly increased resistance to paraquat, all three *relA*+ suppressor strains showed a similar paraquat

sensitivity to the parental *relA*+ Δ*sodAB* strain. This shows that all three suppressor mutations are able to overcome the oxidative stress induced by deleting *sodAB* but they do not overcome extra oxidative stress caused by addition of paraquat.

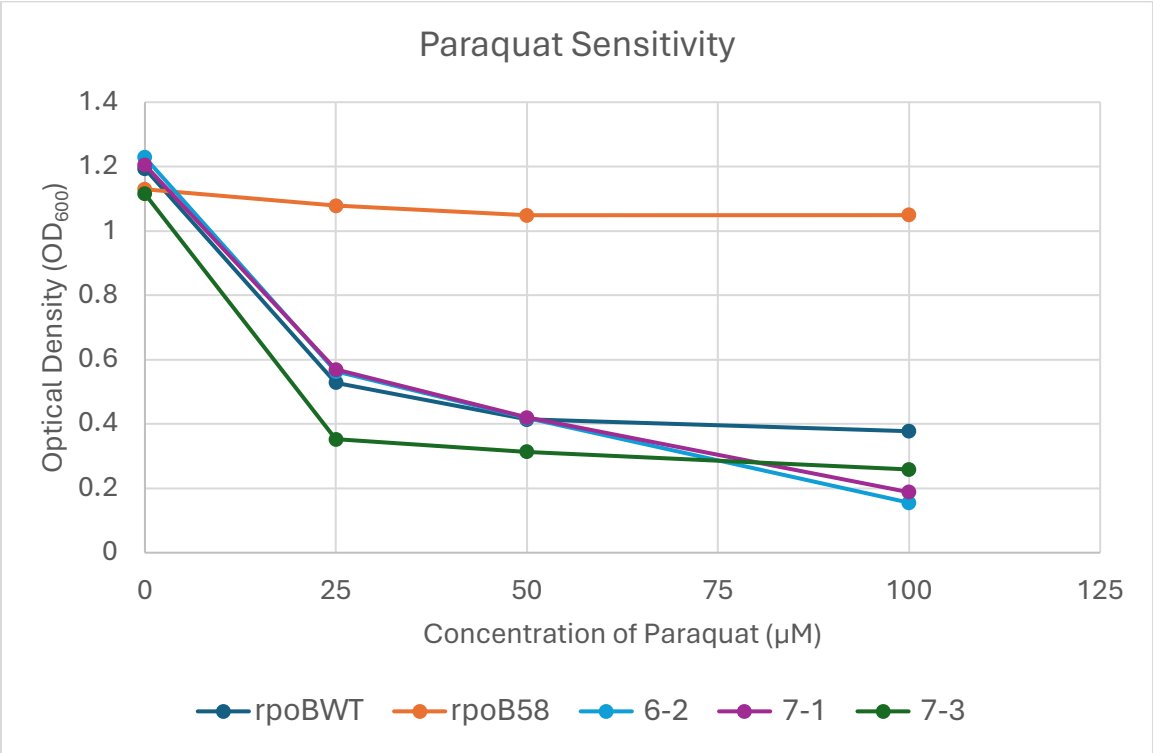

Figure 4. Paraquat sensitivity of *relA*+ Δ*sodAB* suppressors. Overnight growth, as measured by optical density at 600 nm, of cultures exposed to varying concentrations of paraquat.

CHAPTER 3

DISCUSSION

The main purpose of this thesis project was to gain insight into the relationship between the oxidative stress response and stringent response. This was triggered by the observation that the presence of a non-functional *relA1* allele in a Δ*sodAB* background prevented aerobic growth on rich medium. This led to the isolation of seven revertants with suppressor mutations that afforded growth of the *relA1* Δ*sodAB* strain on rich medium. However, the precise nature of suppressor mutations or their effects on specific cellular components or pathways could not be revealed due to delay in obtaining the whole genome sequence data from suppressors. Pinpointing the location of suppressor mutations would have allowed a more directed experimental approach to deduce the mechanism by which these suppressors act to overcome the aerobic growth defect of the parental *relA1* Δ*sodAB* strain. In the absence of the sequencing data, the work described here proceeded largely through conducting phenotypic analysis. The data from suppressor containing strains was compared with a *relA1* Δ*sodAB* strain containing *rpoB58*, which, like the suppressors obtained in this work, allows the *relA1* Δ*sodAB* strain to grow on rich medium.

Growth on rich (LBA) and minimal media, as well as at different temperatures, revealed that with the exception of one revertant, 6-2, others behaved quite differently from *rpoB58*, indicating either that the suppressors affect a gene other than *rpoB* or that they represent different alleles of *rpoB*. Even though the seven revertants could grow on rich medium, their modest growth raised a concern that secondary suppressors may accumulate. Additionally, for growth on minimal medium without amino acid

supplementation, even *rpoB58* required the presence of a *relA*+ allele. The *relA1* allele from the revertant strains was genetically replaced by the *relA*+ allele using a linked antibiotic resistant marker in order to increase genetic stability and compare the growth of the *relA*+ revertants to that of the *relA*+ *rpoB58* strain. In three revertants—6-2, 7-1, and 7-3—a *relA*+ allele was successfully introduced. However, in spite of the presence of *relA*+, the three revertants were unable to grow on minimal medium not supplemented with amino acids, indicating the persistence of oxidative damage to metabolic enzymes, including those involved in amino acid synthesis. It also shows that suppressors in these revertants are specific against a stress that occurs on rich medium and not on minimal medium.

Although it is not known what specific oxidative damages prevent growth of the *relA1* Δ*sodAB* strain on rich medium, a pathway influenced by the RelA-mediated stringent response must play a role in oxidative tolerance. RelA is principally known to lower synthesis of macromolecules, including protein and nucleic acid, in response to nutrient starvation. However, on rich medium nutrient starvation is not expected. Yet, without the oxidative stress defense afforded by SodAB, RelA+ is needed for growth on rich medium (Figure 5). Unlike *rpoB58*, suppressors studied here may not trigger the stringent response in the presence of a non-functional *relA1* allele. It is, however, possible that suppressors overcome the growth defect of Δ*sodAB* on rich medium by influencing a subset of stress-related pathways normally under the stringent response control. Alternatively, they may somehow overcome oxidative stress entirely independent of stringent response (Figure 5). It is worth noting that when tested for paraquat-mediated elevated sensitivity to exogenously-produced superoxide, none of the suppressor showed

increased tolerance, indicating the absence of a novel mechanism that specifically lowers superoxide-mediated oxidative stress. It is also a possibility that the suppressors restore growth on rich medium by some novel mechanism independent of both the oxidative stress response and stringent response (Figure 5).

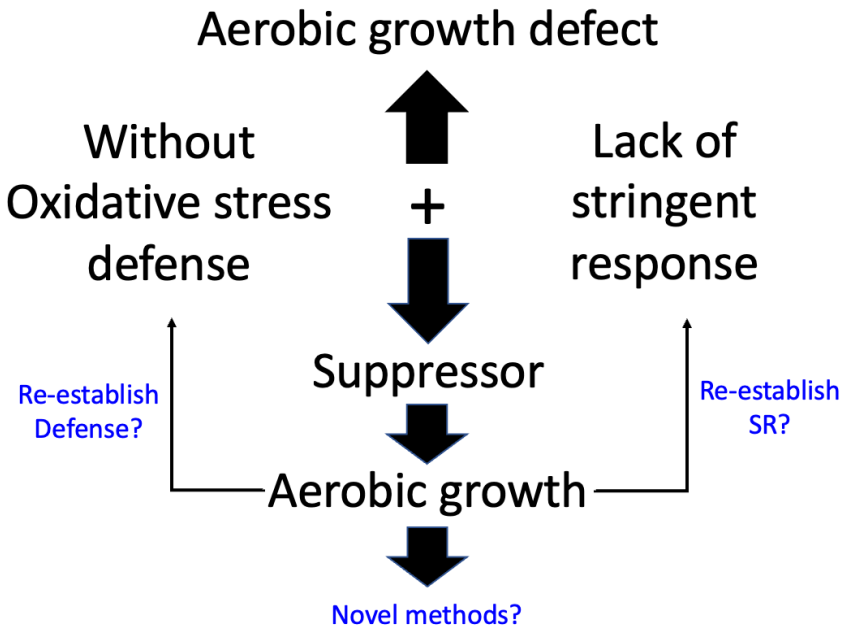

Figure 5. Model of how suppressors may allow aerobic growth. Without the oxidative stress defense and lacking stringent response, Δ*sodAB relA1* cells show an aerobic growth defect. With the unknown suppressors, the cells are able to grow aerobically. This may be because the suppressors are somehow re-establishing the oxidative stress defense, or re-establishing the stringent response (or some pathway controlled by the stringent response), or it may be by some novel method independent of the oxidative stress and stringent responses.

6-2 and 7-1 were able to grow at an elevated growth temperature of 45°C in both a *relA1* and *relA*+ background. Heat tolerance and oxidative stress have been linked in a paper by Kogoma et al. (25) showing that deletion of *rpoH*, the sigma factor regulating the heat shock response, resulted in increased paraquat sensitivity in Δ*sodAB E. coli* cells.

It is possible that superoxide-mediated damage to iron-sulfur-cluster containing proteins leads to their misfolding, which could trigger the heat-shock response and increase the level of molecular chaperones and proteases. 6-2 and 7-1 may contain mutations that directly or indirectly affect the heat shock response pathway or protein homeostasis and also increase tolerance to superoxide stress.

The accumulation of superoxide without SodAB is expected to alter iron homeostasis, since superoxide can extract iron from susceptible [4Fe-S] clusters. Indeed, another graduate student in the lab has shown that the absence of SodAB elevates expression of *fepA*::*lacZ*, which is under the control Fur. In 6-2 and 7-1, *fepA*::*lacZ* activity was similar to the parental *relA*+ Δ*sodAB* strain. However, there was a small but significant increase in *fepA*::*lacZ* activity in 7-3. This may be due to lower levels of active, Fe^{2+}-bound Fur. Increased ferric uptake may not be beneficial for cells experiencing superoxide stress, since increased iron and hydrogen peroxide levels can lead to the generation of hydroxyl radicals through the Fenton reaction. As there are no scavenging enzymes that neutralize hydroxyl radicals, the only way to prevent damages from hydroxyl radicals is to prevent their formation in the first place.

However, the genes regulated by Fur also include the *entCDEBAH* operon, which includes genes that synthesize the siderophore enterobactin. Peralta et al. recently found that enterobactin plays a role in protection from oxidative stress, independent of its iron scavenging ability (9). Therefore, it is possible that suppressor strain 7-3 utilizes the enterobactin mediated protection from oxidative stress to grow aerobically on rich medium.

Gene sequencing data is required to identify the location of the suppressor mutations. Once the mutations have been identified, further testing will be necessary to identify and understand the mechanism(s) by which they overcome the aerobic rich medium growth defect of *relA1* Δ*sodAB*.

CHAPTER 4

MATERIALS AND METHODS

Obtaining suppressors

A *relA1 ΔsodAB* strain of *E. coli* K-12 MG1655 was incubated anaerobically on LBA for 48 hours at 37°C. Multiple colonies were streaked on LBA and incubated aerobically at 37°C. Revertants arising from each of these colonies were selected and restreaked on LBA and incubated for 48 hours at 37°C. Revertants that formed uniform colonies upon re-streaking were selected and stored at -80°C.

Growth assays on plates

The same colony from each strain was streaked on LBA, M63 supplemented with casamino acids and glucose, and M63 supplemented with glucose. LBA plates were incubated for 24 hours at 37°C and M63 plates were incubated for 48 hours at 37°C.

Temperature sensitivity assays

Two colonies from each strain were streaked on four LBA plates. These LBA plates were incubated for 24 hours at 30°C, 37°C, 42°C, and 45°C, respectively.

relA+ P1 phage transduction and PCR confirmation

Revertant strains were converted from *relA1* to *relA*+ via P1 phage transduction using the kanamycin resistance gene as a marker. To confirm that *relA*+ strains had been constructed, PCR was used to amplify a segment of the *relA* gene. Gel electrophoresis showed that the *relA1* gene produced a segment of about 2000 bp while the *relA*+ segment was 600 bp.

Beta-galactosidase assay

In each of the *relA*+ revertant strains, the kanamycin resistance gene linked to *relA*+ was scarred out using the pCP20 plasmid. Then, a *fepA::lacZ* strain was constructed from each *relA*+ revertant strain using P1 phage transduction with a kanamycin resistance gene linked to *fepA::lacZ* as a marker. β-galactosidase activities were measured from three independent overnight cultures in duplicate using the method described by Miller (26).

Minimum inhibitory concentration assay

In a 96-well plate, overnight cultures of two biological duplicates of each strain were diluted 1000 fold (approximately 10^6 cells per well) in LB with 0μM, 25μM, 50μM, or 100μM paraquat. Paraquat concentrations were achieved using the 2-fold serial dilution method. The plate was incubated on a rocker at 37°C for 16 to 18 hours. Optical density of overnight cultures was measured using a spectrophotometer at wavelength 600 nanometers.

REFERENCES

1. Krumova K, Cosa G. 2016. Overview of Reactive Oxygen Species, p. 5–18. *In* Nonell, S, Flors Cristina (eds.), Singlet Oxygen: Applications in Biosciences and Nanosciencesillustrated. Royal Society of Chemistry.

2. Gerschman R, Gilbert DL, Nye SW, Dwyer P, Fenn WO. 1954. Oxygen poisoning and X-irradiation: A mechanism in common. Science (1979) 119:623–626.

3. Chiang SM, Schellhorn HE. 2012. Regulators of oxidative stress response genes in Escherichia coli and their functional conservation in bacteria. Arch Biochem Biophys 525:161–169.

4. Imlay JA. 2013. The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium https://doi.org/10.1038/nrmicro3032.

5. Lynch AS, Lin ECC. 1996. Responses to Molecular Oxygen, p. 1526–1538. *In* Neidhardt, FC, Curtiss III, R, Ingraham, JL, Lin, ECC, Low, KB, Magasanik, B, Reznikoff, WS, Riley, M, Schaechter, M, Umbarger, HE (eds.), Escherichia coli and Salmonella: Cellular and Molecular Biology2nd Edition. ASM Press, Washington, D.C.

6. Nunoshiba T, Watanabe T, Nakabeppu Y, Yamamoto K. 2002. Mutagenic target for hydroxyl radicals generated in Escherichia coli mutant deficient in Mn-and Fe-superoxide dismutases and Fur, a repressor for iron-uptake systems. DNA Repair (Amst) 1:411–418.

7. Earhart CF. 1996. Uptake and Metabolism of Iron and Molybdenum, p. 1075–1090. *In* Neidhardt, FC, Curtiss III, R, Ingraham, JL, Lin, ECC, Low, KB, Magasanik, B, Reznikoff, WS, Riley, M, Schaechter, M, Umbarger, HE (eds.), Escherichia coli and Salmonella: Cellular and Molecular Biology2nd Edition. ASM Press, Washington, D.C.

8. Andrews SC, Robinson AK, Rodriguez-Quinones F. Bacterial iron homeostasis https://doi.org/10.1016/S0168-6445(03)00055-X.

9. Peralta DR, Adler C, Corbalán NS, Carlos Paz García E, Fernanda Pomares M, Vincent PA. 2016. Enterobactin as Part of the Oxidative Stress Response Repertoire https://doi.org/10.1371/journal.pone.0157799.

10. Hollifield WC, Neilands JB, Hollifield WC. 1978. Ferric enterobactin transport system in Escherichia coli K-12. Extraction, assay, and specificity of the outer membrane receptor. Biochemistry 17:1922–1928.

11. Fiss EH, Stanley-Samuelson P, Neilands JB. 1982. Properties and proteolysis of ferric enterobactin outer membrane receptor in Escherichia coli K12. Biochemistry 21:4517–4522.

12. Gu M, Imlay JA. 2013. Superoxide poisons mononuclear iron enzymes by causing mismetallation. Mol Microbiol 89:123–134.

13. Cashel M. 1969. The Control of Ribonucleic Acid Synthesis in Escherichia coli. Journal of Biological Chemistry 244:3133–3141.

14. Cashel M, Gallant J. 1969. Two Compounds implicated in the Function of the RC Gene of Escherichia coli.

15. Stent GS, Brenner S. 1961. A Genetic Locus for the Regulation of Ribonucleic Acid SynthesisBrookhaven Symposia in Biology. Cambridge University Press.

16. Bange G, Brodersen DE, Liuzzi A, Steinchen W. 2021. Annual Review of Microbiology Two P or Not Two P: Understanding Regulation by the Bacterial Second Messengers (p)ppGpp https://doi.org/10.1146/annurev-micro-042621.

17. Irving SE, Choudhury NR, Corrigan RM. 2021. The stringent response and physiological roles of (pp)pGpp in bacteria. Nat Rev Microbiol. Nature Research https://doi.org/10.1038/s41579-020-00470-y.

18. Carlioz A, Touati D, Radman M. 1986. Isolation of superoxide dismutase mutants in Escherichia coli: is superoxide dismutase necessary for aerobic life? EMBO J 5:623–630.

19. BOEHME DE, VINCENT K, BROWN OR. 1976. Oxygen and toxicity inhibition of amino acid biosynthesis. Nature 262:418–420.

20. BROWN O, SEITHER R. 1983. Oxygen and redox-active drugs: Shared toxicity sites. Fundamental and Applied Toxicology 3:209–214.

21. Brown OR, Yein F. 1978. Dihydroxyacid dehydratase: The site of hyperbaric oxygen poisoning in branch-chain amino acid biosynthesis. Biochem Biophys Res Commun 85:1219–1224.

22. Fee JA, Lees AC, Bloch PL. 1980. Oxygen stasis of bacterial growth: analogy between the stasis of E. coli by hyperbaric oxygen and by aerobic paraquat. Biochem Int 1:304–311.

23. Kuo CF, Mashino T, Fridovich I. 1987. alpha, beta-Dihydroxyisovalerate dehydratase. A superoxide-sensitive enzyme. Journal of Biological Chemistry 262:4724–4727.

24. Cho H, Misra R. 2021. Mutational Activation of Antibiotic-Resistant Mechanisms in the Absence of Major Drug Efflux Systems of Escherichia coli https://doi.org/10.1128/JB.00109-21.

25. Kogoma T, Yura T. 1992. Sensitization of Escherichia coli cells to oxidative stress by deletion of the rpoH gene, which encodes the heat shock sigma factor. J Bacteriol 174:630–632.

26. Miller J. 1992. A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia Coli and Related Bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.