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Defining Environmental Stresses that Activate the RNA Repair Operon in Salmonella Typhimurium

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DEFINING ENVIRONMENTAL STRESSES THAT ACTIVATE THE RNA REPAIR OPERON IN *SALMONELLA* TYPHIMURIUM

By

Caleb Michael Gulledge

Honors Scholarship Project

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in

Biology

Scholarship Project Advisor (printed)	Signature	Date
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Honors Council Member (printed)	Signature	Date

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ABSTRACT

Background

RNA polymerase holoenzyme (Eσ) mediates transcription in eubacteria, and is composed of five constant subunits ($\alpha_2\beta\beta'\omega$) and a variable sigma (σ) subunit that is responsible for promoter recognition and initiation of transcription. An alternative sigma factor in *Salmonella* Typhimurium, σ^{54} (also called RpoN), is mechanistically different than classical σ^{70}-type sigmas, requiring a different promoter consensus sequence, an activator, and ATP hydrolysis. The Rtc RNA repair operon lies within the regulon of RpoN in *S.* Typhimurium, but has no known physiological function. Previous work characterized similar systems in archaea and humans, which were determined to function in recovery from environmental stresses. Focusing on recovery from environmental stress as a function of the Rtc RNA repair operon, *I hypothesized that nitrogen limitation, iron limitation and cell wall stress would induce expression of the Rtc RNA repair operon in Salmonella* Typhimurium.

Results

A plasmid encoding the quantifiable expression of LacZ under the control of the Rtc RNA repair operon was used to measure the impact of environmental stresses on expression of the operon. Cefotaxime as a cell wall stressor induced a four-fold increase in expression maximally at a dose of 40 μg/ml, nitrogen limitation exhibited a two-fold increase, and addition of 2,2'-bipyridyl as an iron chelator did not induce any significant increase in expression at 0.2, 0.3, or 0.5 mM. However, these three treatments all fell short of the positive control treatment with Mitomycin C (MMC), which had two and four-fold increases in expression compared to cefotaxime and nitrogen limitation treatments.

Conclusions

Induction of expression upon treatment with MMC, cefotaxime and nitrogen limitation displays the diversity of signals that induce the Rtc RNA repair operon. Induction with cefotaxime indicates the Rtc RNA repair operon may function to repair transcripts essential for metabolites involved in transitioning to anaerobic metabolism. Induction with nitrogen limitation suggests that the repair operon plays some role in adapting to low nitrogen conditions. However, not all sources of environmental stress were able to induce operon expression. Expression upon iron limitation was not observed and indicates a distinct difference in the response of *rtcR* between *E. coli* and *Salmonella.*

KEYWORDS

Sigma54, RpoN, Bacterial enhancer-binding protein, Sigma factor, *Salmonella*, β-galactosidase assay, nitrogen limitation, iron limitation, cefotaxime, repair operon, Mitomycin C

INTRODUCTION

Salmonella enterica subspecies enterica serovar Typhimurium is estimated to cause over 1 million cases of Salmonellosis in the United States, which typically involves the onset of diarrhea, fever and abdominal cramps. 19,000 of these cases require hospitalization and 380 result in death [1]. *Salmonella* Typhimurium is a key serovar, or serotype, that contains clusters of virulence genes (genes that code for molecules that contribute to pathogenicity and permit colonization in the host niche) that allow invasion of epithelial cells, replication within macrophages, and colonization of the gastrointestinal tract [2]. These characteristics place it among the most prevalent food-borne gastrointestinal diseases worldwide [1], with roughly 5% of these cases resulting in invasive bacteremia and requiring treatment with antimicrobials [3]. With the rapid emergence of antimicrobial resistance in *Salmonella*, finding new targets for antimicrobial agents is becoming increasingly important [3]. The transcriptional control system that initiates the timely transcription of virulence genes is also responsible for maintaining an appropriate level of cellular fitness that allows the bacteria to compete with native microbiota. This remarkably intricate system results in a complex transcriptome that remains to be completely described [2]. Further characterization and understanding of the mechanisms that *Salmonella* utilizes in response to changes in its environment may elucidate novel targets for these agents. Apart from its medicinal benefit, *Salmonella,* as a model system, has led to identification of virulence factors and mechanisms of bacterial transmission [3].

Transcription

Transcription, while absolutely necessary to life, has high-energy costs and therefore is extensively regulated in bacteria. Regulation conserves energy sources as well as prevents the deleterious effects of expression at inappropriate times [4]. RNA

polymerase holoenzyme (Eσ), composed of five constant subunits ($\alpha_2\beta\beta'\omega$) and a variable sigma subunit (σ), mediates transcription in eubacteria. The constant subunits compose the RNA polymerase core (RNAP), which catalyzes polymerization, while the σ factor is responsible for promoter recognition and binding as well as initiation of transcription [5]. Transcription initiation requires isomerization, or the conversion of the transcriptionally inert closed complex of the Eσ, to the active open complex. Given this mechanism, the σ factor can modify the specificity of the Eσ, targeting different promoter sequences.

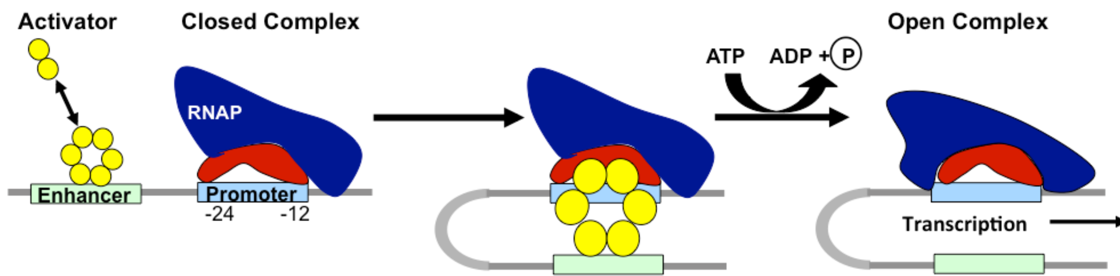
While the primary sigma factor in bacteria, σ^{70}, recognizes promoters for housekeeping genes (those that are constitutively on), alternative sigma factors recognize a different subset of genes [5]. *Salmonella* has 5 alternative sigma factors, though the amount varies greatly among different genera. The first four alternative σ factors control a group of genes that coordinate a response to a particular type of stress [6]; $\sigma^{S/38}$ regulates genes critical to the cells' entry into stationary phase, $\sigma^{H/32}$ controls genes in the heat shock response, $\sigma^{E/24}$ directs the response to envelope stress, and $\sigma^{fliA/28}$ guides expression of flagellar biosynthesis [5]. The fifth alternative sigma factor, σ^{54} (also called RpoN), was initially implicated in the transcription of genes dealing with low nitrogen availability [7] (thus the N in RpoN), yet its known repertoire controls a diverse set of genes in response to very different types of stresses or environmental signals [4]. Its regulon, collection of genes or operons under regulation by σ^{54}, is known to consist of 22 promoters in *S.* Typhimurium [8] and has been shown to be involved in a variety of cellular processes including flagellar biogenesis, transport and metabolism of carbon substrates, tolerance to heavy metals, composition of the cell exterior, and the transport of the precursors of extracellular saccharides [9-13]. Yet this list leaves out a known element of the RpoN regulon, specifically, the Rtc RNA repair operon [4].

σ^{54} versus σ^{70}- family

σ^{54} is mechanistically different from the primary and alternative sigma factors (σ^{70}- family), as it requires a different promoter consensus sequence. The promoter sequences recognized by σ^{70}- type sigma factors are located at -35 and -10 bp relative to the transcription start site (TSS). These consensus sequences, TTGACA and TATAAT, are not highly conserved and can maintain function with insertion of 2-3 bp between the two sites [14]. However, the promoter sequences essential for σ^{54} recognition and binding, center on highly conserved GG and GC consensus sequences located at -24 and -10 bp upstream of the TSS [15]. Insertion of even 1 bp will completely abolish recognition and binding by σ^{54} [15].

Another difference between the σ^{70}- family and σ^{54} is the mechanism of activation (transcription initiation). Activation of $E\sigma^{54}$ is energy-dependent and requires a bacterial enhancer binding protein (bEBP) [5]. Upon recognizing an environmental stimulus, the bEBP becomes activated to form a hexamer, which interacts with an enhancer sequence that is ~100 bp upstream of the TSS [8, 16]. Then, a DNA looping event allows the bEBP to interact with the holoenzyme ($E\sigma^{54}$) bound to the promoter in a closed complex. ATP hydrolysis by the bEBP provides the energy needed for $E\sigma^{54}$ to transition into the open complex and thus initiate transcription [8] (figure 1). These characteristics (i.e., reliance on an enhancer sequence, ATP hydrolysis, and DNA looping) are often found in eukaryotic polymerase II, and indicate that $E\sigma^{54}$ may be more similar to eukaryotic polymerases than other bacterial sigma factors [17].

Enhancer-dependent activation:



Activates at any σ^{54}-dependent promoter:

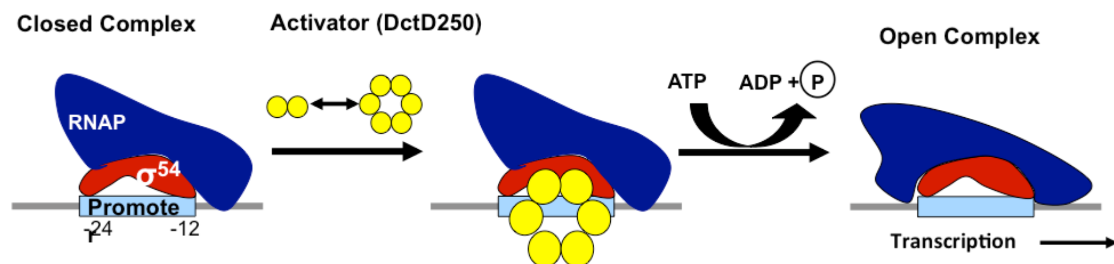


Figure 1: Activation of σ^{54}-dependent transcription.
σ^{54} (red), interacting with core RNA Polymerase (blue), directs binding of the holoenzyme to the -12, -24 promoter sequence (light blue). The closed complex is stable on the promoter sequence and cannot enter the open complex without an activator. The activator (yellow) oligomerizes in response to a cellular signal and binds to the enhancer sequence (green), which is typically 80-150 bp upstream of the promoter. DNA looping brings the activator in contact with $E\sigma^{54}$ and hydrolysis of ATP causes $E\sigma^{54}$ conformation change into the open complex, allowing transcription. In these studies, σ^{54} was activated by DctD250, a constitutive, promiscuous activator of sigma-54-dependent expression, that should activate expression from all sigma-54-dependent promoters simultaneously [Figure modified from Samuels et al., 2013].

Rtc RNA repair operon

The Rtc RNA repair operon in *Salmonella* is a σ^{54}-dependent operon that supposedly encodes an RNA repair system. This operon contains 3 structural genes and 2 small RNAs. The first gene, *rsr*, is a Ro-sixty related ribonucleoprotein; the second, *rtcB*, is an RNA ligase; the third, *rtcA*, is an RNA phosphate cyclase. The 2 small RNAs, partners of Rsr, *yrlA* and *yrlB*, are encoded between *rsr* and *rtcB* [4]. The activator for this σ^{54} dependent pathway is encoded by *rtcR*, which is adjacent to the RNA repair operon, but transcribed in the opposite direction (Figure 2). The physiological function of this operon is, to date, unknown, but based on similar RNA repair systems in other organisms, such as humans and archaea, some inferences can be made. The potential

functions include tRNA splicing, alternative mRNA splicing, recovery from ribotoxin damage, and recovery from environmental stresses [4]. Recovery from environmental stress, as a function of the Rtc RNA repair operon, is the focus of this study.

Mitomycin C (MMC) belongs to the mitomycin family of antibiotics, derived from species of *Streptomyces.* Furthermore, MMC is commonly used as a chemotherapeutic agent, and its mechanism of cell death is typically attributed to nuclear DNA damage. Recently, MMC has been shown to lead to the degradation of RNA as well [18]. In light of these two mechanisms, a study was performed investigating the treatment of *Salmonella* with MMC as an antibiotic, and demonstrated increased expression of the Rtc RNA repair operon [4].

Figure 2. The Rtc RNA repair operon.
The σ^{54} dependent promoter is delegated as P^{rsr} and the promoter for the enhancer binding protein, *rtcR*, is delegated as P^{rtcR}. These two genes are transcribed in opposite directions.

Project focus

Here, we investigated whether environmental stressors such as carbon starvation, nitrogen limitation, and cell wall stress could induce $E\sigma^{54}$ expression of the Rtc RNA repair operon. These conditions have been observed to activate the regulators of other RpoN-dependent promoters [9-13] as well as toxin-antitoxin systems, which are known to cleave RNA and to be activated by the SOS response [19, 20]. *Therefore, I hypothesized that these environmental stresses would induce expression of the Rtc RNA repair operon in Salmonella* Typhimurium. If identified, this would provide avenues

to determining more specifically the mechanism of activation of the Rtc RNA repair operon. Characterization of the induction of this operon in *Salmonella* may lead to identification of virulence factors and mechanisms of bacterial transmission that can be targeted.

MATERIALS AND METHODS

Preparing *S.* Typhimurium

Construction of a reporter plasmid for expression from the promoter for the Rtc RNA Repair Operon was initiated by Caleb Gulledge and then completed by Ashley Bono and Dr. Anna Karls. Preparation of *S.* Typhimurium strains carrying the plasmid was carried out by Ashley Bono and Dr. Anna Karls of the University of Georgia and then shipped to ONU for my study. Briefly, the reporter plasmid pCMG23 was created by ligation of a ~200 bp DNA fragment containing the promoter for the Rtc RNA repair operon into pNN387 [21], which is a single copy reporter plasmid that contains a multi-cloning site upstream of a promoterless *lac* gene. Cloning was done in *E.coli* DH5α and then transformed into pathogenic *S.* Typhimurium 14028s via electroporation after passage through a *hsdR*$^{-}$ *hsdM*$^{+}$ *Salmonella* strain.

Growth media and conditions

Bacteria was grown at 37°C with aeration in MOPS minimal media (MOPS) [22]. The antibiotic, chloramphenicol (15 μg/ml), was added to cultures to select for the reporter plasmid. Overnight cultures were diluted 1:9 in fresh media with antibiotics, grown to mid-exponential phase (OD_{600}= 0.4-0.6), and then treated. Treatments were with MMC as the positive control (3 μg/ml), iron limitation (0.2, 0.3, & 0.5 mM 2,2′-bipyridyl), nitrogen limitation (2.5 mM arginine as sole nitrogen source), and cell wall stress (2, 10, 30, 40, 50, 65, & 130 μg/ml of cefotaxime).

MMC treated cultures were grown for an additional 90 minutes for use in the β-galactosidase assay. The iron chelator was added during the initial subculture, and then grown for 10 hours. In the case of nitrogen limitation, once the subculture reached mid-exponential phase, the culture was pelleted at 12,000 rpm for 10 min, washed in nitrogen limiting MOPS, pelleted again, and then resuspended in nitrogen limiting MOPS prior to culturing for 3 hours. Cefotaxime treated cultures were grown for an additional 3 hours once the cells reached mid-exponential phase.

β-galactosidase assay

The expression of the *lacZ* gene, which is controlled by the Rtc RNA repair promoter on the reporter plasmid, was assessed as follows. Treated cultures were chilled on ice to stop cell growth, and then 0.5 ml aliquots were combined with a cell lysing mixture (Z buffer with β-mercaptoethanol, chloroform, 0.1% SDS) to expose the intracellular matrix. Cell debris was then spun for 10 seconds and warmed for 5 minutes at 37°. The reaction to begin color change was then initiated with the addition of ortho-Nitrophenyl-β-galactosidase and incubated at 37° until a color reaction occurred, at which time the reaction was stopped with 1M Na_2CO_3. The solution was centrifuged at 15000xg for 5 minutes and the supernatant was used to measure absorbance at 420nm and 550nm. Activity was calculated as Miller units: $\{1000 \times [OD_{420} – (1.75 \times OD_{550})]/[Time (min) \times Volume (ml) \times OD_{600}]\}$ [23]. At least 3 biological replicates for each condition were used. Activity in treated cultures versus untreated cultures were compared and analyzed using a 2-tailed Student's T-test. Error bars indicate standard deviation amongst replicates.

RESULTS

To determine the activity of the Rtc RNA repair operon under environmental stressors we performed a quantifiable colorimetric assay, β-galactosidase assay, using

WT+pCMG23 comparing treated and untreated cultures. Cell wall stress by cefotaxime treatment, which inhibits cell wall synthesis by binding to penicillin binding proteins [24], revealed significant increases in expression at 10, 30, 40, and 50 µg/ml doses (figure 3). Maximal expression of 8.28 Miller units occurred at 40 µg/ml, which is greater than a four-fold increase in expression compared to the 1.90 miller unit baseline activity. However, this level of activity is half that of the positive control MMC treatment, which exhibited activity of 16.26 miller units. Doses of 2 µg/ml and 130 µg/ml caused a significant decrease in expression when compared to the untreated samples measuring -0.20 and -3.12 miller units, respectively.

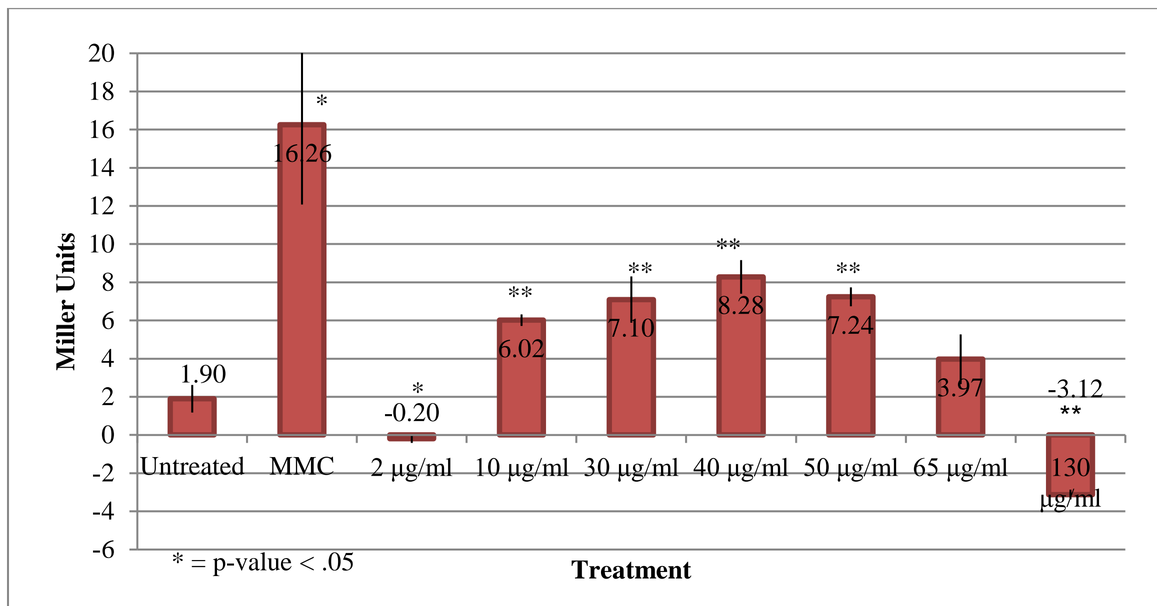


Figure 3. Cell wall stress by cefotaxime induces Rtc RNA repair operon expression.
Dosage titration revealed maximal expression of the Rtc RNA Repair Operon at a dosage of 40 µg/ml cefotaxime.

The iron limitation titration using an iron chelator, 2,2'-bipyridyl, did not induce significant changes in expression at 0.2, 0.3, or 0.5 mM doses (figure 4). Maximal expression of 2.60 miller units was exhibited at the 0.2 mM dose but was not statistically significant (p= .230).

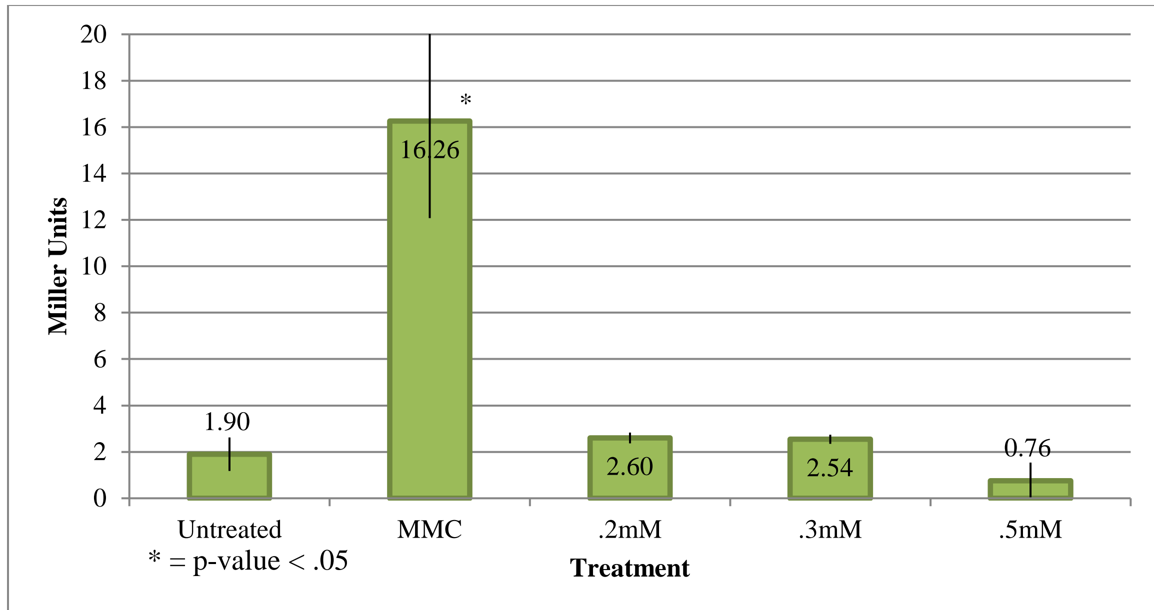


Figure 4. Iron Limitation by 2,2'-bipyridyl does not induce Rtc RNA repair operon expression. Dosage titration revealed maximal expression of the Rtc RNA Repair Operon at a dosage of 0.2 mM 2,2'-bipyridyl. This dose induced 2.60 miller units of activity. However, none of the doses revealed a statistically significant change in expression when compared to untreated culture.

Nitrogen limitation was also able to significantly increase Rtc RNA repair operon expression, and was compared with cell wall stress and iron limitation treatments (figure 5). The nitrogen limitation treatment induced 4.04 miller units of activity, which accounts for a two-fold increase from baseline, yet four-fold lower when compared to the positive control treatment of MMC. Upon comparing all treatments in the study, MMC was the best inducer of expression, displaying over an eight-fold increase in expression from baseline. Cefotaxime and nitrogen limitation also increased expression by four and two-fold, respectively, while the iron limitation treatment did not affect expression.

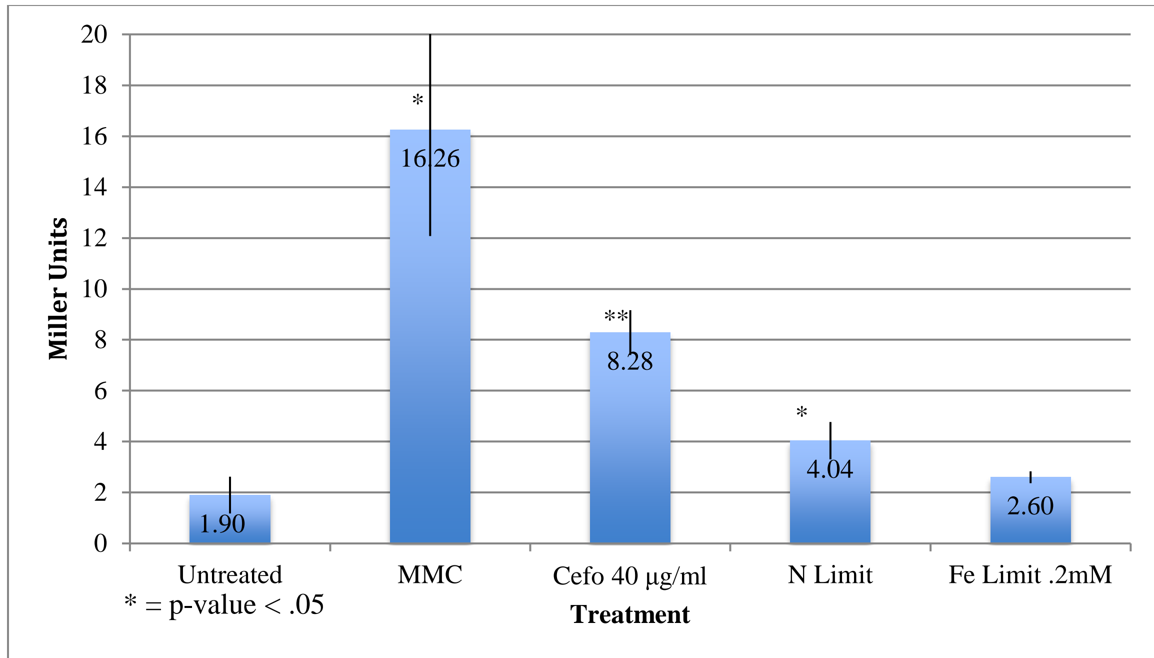


Figure 5. Expression of Rtc RNA repair operon is induced by MMC, cefotaxime, and nitrogen limitation, but not iron limitation.
Treatment with cefotaxime elucidated roughly half the activity of treatment with MMC, but also a four-fold increase from that of untreated culture. Nitrogen limitation revealed a two-fold increase in expression when compared to untreated culture. Iron limitation did not induce expression of the repair operon.

DISCUSSION

Pathogenic virulence is a major area of study. Repair systems in pathogens are often associated with pathogenic virulence, though a repair system in bacteria has yet to be fully characterized [4]. By defining conditions of induction, we move closer to the mechanism of activation and full characterization of the repair system because we gain the ability to trigger the natural response of the pathogen. Understanding these physiological responses of the pathogen may allow for directed and novel antimicrobial targeting. This study sought to characterize the ability of environmental stress to induce Rtc RNA repair operon expression in *S.* Typhimurium, and identified three treatments that induce expression, two of which represent novel discoveries.

Cefotaxime

A recent study revealed that at sub-inhibitory treatments of cefotaxime, genes related to anaerobic metabolism, biosynthesis of purines, pyrimidines, amino acids and other metabolites were necessary to survive [25]. The paper concluded that with exposure to sub-lethal concentrations of cefotaxime, the systemic colonization of *S.* Typhimurium increased, establishing fitness alterations that deal with the new environment. This infers that the cellular mechanisms in response to stress, or the SOS response, are up regulated for survival. They also found that upon exposure to cefotaxime the cell switches to anaerobic metabolism to sustain growth, even when incubated aerobically. This is because cefotaxime inhibits *S.* Typhimurium's ability to consume oxygen [25]. Though my study initially identified cefotaxime as a cell wall stressor, this provides evidence that the mechanism targets the cell's consumption of oxygen and causes oxidative damage. As the sub-lethal levels of cefotaxime intrude, preventing ideal growth, the cell expresses different genes in response, in this case, genes helpful in anaerobic metabolism.

This study identified the minimum inhibitory concentration (MIC) for cefotaxime as 130 µg/ml, and used a concentration of 65 µg/ml (0.5 x MIC) for the sub-lethal concentration [25]. My study reinforced their findings of the MIC, however my findings revealed that maximum expression of the Rtc repair operon was induced with a lower dose of cefotaxime (40 µg/ml) in *S.* Typhimurium. Cefotaxime's known effects to switch to anaerobic conditions, coupled with the increased expression of the Rtc repair operon upon treatment with cefotaxime that I found, suggest a possible mechanism of action for the Rtc RNA repair operon. The operon may aid in the recovery and repair of transcripts of essential metabolites that are involved in the transition to anaerobic metabolism.

Nitrogen limitation

Despite sigma-54's known function in genes involved with low nitrogen availability [7], the nitrogen limiting treatment was less robust, but statistically significant in inducing rtc RNA repair operon expression. This finding, along with the increased expression upon treatment with MMC and cefotaxime, further demonstrate the diversity of responses that sigma-54 is involved in. Though it was not the largest response, nitrogen availability did exhibit a two-fold increase in expression, suggesting that the RNA repair operon does play some role in low nitrogen conditions. Further investigation into different levels of nitrogen availability may reveal greater responses from the repair operon.

Iron limitation

The lack of expression during the iron limitation treatment indicates a stark difference in expression of the repair operon between *E. coli* and *Salmonella.* One study induced a three-fold increase in expression of *rtcA* in *E. coli* upon addition of the iron chelator 2,2'-bipyridyl at 0.2 mM, utilizing essentially the same conditions and concentrations as in my own study [26]. One difference in methods was their use of real time-PCR, which would give more sensitive results. However, it is unlikely that sensitivity could account for a three-fold difference. It is more likely the difference in expression between the two organisms indicates a difference in how *rtcR* responds to environmental signals. Though *Salmonella* RtcB and RtcA are 88% and 68% identical, respectively, to those proteins in *E. coli* [4], the response to stress is controlled by RtcR. While the homology of RtcA and RtcB suggest a similar function of the RNA repair operon, it is apparent the signals they respond to, which initiate RtcR, are different.

Conclusion

This work adds to the current body of knowledge, and identifies two novel

inducers of the Rtc RNA repair operon by sigma-54 allowing other researchers to build on the conditions that I have defined. Moreover, the long-term benefits of this study have real potential through application in industrial pharmaceuticals. Studies that target the mechanisms of pathogenic virulence have potential to be exploited in pharmaceutical production of antimicrobials, which expands their relevance to every part of the globe.

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