

Effect of Ferric Uptake Regulatory Protein on the Transcription of
the Superoxide Dismutase Gene of *Escherichia coli*

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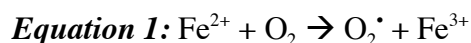
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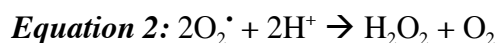
Introduction

Superoxide radicals (O_2^{\bullet}) produced during aerobic respiration can damage DNA. The superoxide dismutase gene (*sodA*) protects DNA by producing the superoxide dismutase protein (1). To control the expression of superoxide radicals, gene expression can be modified using regulatory proteins. In general, the expression of a gene into a protein takes place through two steps: transcription and translation. Transcription is the process through which mRNA is made from DNA and translation is the process by which the mRNA is converted into the protein (2,3). One way in which gene expression can be moderated is at the level of transcription (2). Ferric uptake regulatory protein (Fur) inhibits or enhances transcription due to its ability to bind to ferrous ions (Fe^{2+}) present in the cell (3). The focus of my research was to study gene expression of *sodA* in the bacteria *E. coli* in the presence and absence of Fur and use an internal standard to measure the quantity of mRNA produced during transcription. If Fur protein is proven to regulate gene expression, over and under expression of genes could be corrected, thus reducing the formation of cancer-causing superoxide radicals.

Superoxide radicals are formed through cellular metabolism when ferrous ions in cells react with oxygen during aerobic respiration, as seen in Equation 1. These superoxide radicals are harmful to DNA (4).



SodA protects the cell by producing superoxide dismutase to break superoxide radicals into less harmful substances. As seen in Equation 2, when superoxide radicals react with superoxide dismutase, hydrogen peroxide (H_2O_2) and oxygen (O_2) are produced. The hydrogen peroxide is then broken down by an enzyme, catalase (4).



The SOD protein contains an Mn^{+2} ion that facilitates the reaction to detoxify superoxide radicals. Fur protein binds to ferrous ions and attaches to the promoter (DNA polymerase binding sites) found upstream of the *sodA* gene. A study by Friedman in 2004 showed that Fur protein inhibits transcription of iron-responsive genes in bacteria (3). Fur protein regulates expression of the *sodA* gene when it is stressed by superoxide radicals (5,6). Because the Friedman study showed that superoxide is formed when there is excess Fe^{+2} , and Fur binds Fe^{+2} , I hypothesized that Fur would activate *sodA* transcription in order to protect against increased superoxide production.

A reverse transcription polymerase chain reaction (RT-PCR) was used to demonstrate the transcription activation with the help of the internal standard using gel electrophoresis.

Complimentary DNA (cDNA) was transcribed and amplified through RT-PCR. An internal standard for RT-PCR was designed starting from *sodA* template and a series of deletions by using restriction digests. This internal standard will be used for the standardization of intensities during RT-PCR. This process was used to determine how the amount of transcribed cDNA fluctuated due to the addition of Fur protein by comparing the strength of the signal produced.

Methods

Making the PCR Template: A PCR template was made using 25 μL of 2X Master Mix (dNTPs, Taq polymerase, and PCR buffer), 17 μL of nano-pure water, 4 μL of PCR template, and 2 μL of *sodA* primers 1 and 2. The reagents were mixed by pipetting repeatedly to avoid formation of bubbles. The resulting solution was amplified through PCR using a thermal-cycler with Program 01 BIOCHEM. In this process, the solution was heated to 95 °C for one minute, cooled to 55 °C for one minute, and reheated to 72 °C for one minute.

Making of the 1/10 Fur Dilution: The Fur protein was diluted with Tris buffer by using 10 μL of concentrated Fur and 90 μL of Tris buffer. This solution was then mixed and stored in the $-80\text{ }^{\circ}\text{C}$ freezer for later use in the transcription process.

SodA Transcription of DNA to mRNA: To measure the amount of mRNA produced during transcription in the presence and absence of the ferric uptake regulatory protein, mRNA was made from DNA. A mix of NTP and initiators was made using 10 μL each of ATP, GTP, and UTP, 5 μL each of APA and CPC (the dinucleotide initiators), 5 μL of CTP, and 59.5 μL of nano-pure water to bring the solution to 100 μL . This solution was aliquotted into three Eppendorf tubes (33.3 μL of the solution in each) and frozen. For transcription, 14.9 μL of transcription buffer (amounts of 2 μL and 4 μL were substituted with the 1/10 Fur dilution in the samples with Fur), 1 μL of RNase inhibitor, 0.5 μL of template DNA, and 0.5 μL of RNA polymerase were combined in an Eppendorf tube. This solution was incubated at $37\text{ }^{\circ}\text{C}$ for 20 minutes. Next, 8.1 μL of the initiator and NTP mix were added and the tube was incubated at $37\text{ }^{\circ}\text{C}$ for 45 minutes. Next, 20 μL of phenol-chloroform were combined and the solution was inverted, vortexed for five seconds, and centrifuged for five minutes. The supernatant was pipetted off and stored, and the pellet was discarded. To the supernatant, 2.9 μL sodium acetate and 58 μL of 2X ethanol were added and the solution was cooled at $-80\text{ }^{\circ}\text{C}$ for 20 minutes. The solution was centrifuged and aspirated. After air-drying for ten minutes, the tube was filled with 17 μL of nano-pure water to resuspend the pellet. The solution was then heated to $95\text{ }^{\circ}\text{C}$ for two minutes to denature the RNA/DNA hybrids and, by adding 2 μL 5 units/ μL DNase and 2 μL of 10X transcription buffer, the DNA was degraded. Lastly, the solution was incubated at $37\text{ }^{\circ}\text{C}$ for one hour and the mRNA was stored in the $-80\text{ }^{\circ}\text{C}$ freezer for future use.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) of mRNA to cDNA: To quantify mRNA produced in transcription, RT-PCR was used. For the reverse transcription reaction, 1.5 μL of mRNA, 2 μL of RT primers #2 and #3, and 5 μL of nano-pure water were combined in an Eppendorf tube. Next, the solution was incubated for ten minutes at 70 °C. The tube was centrifuged and stored on ice for 30 seconds. Then, 4 μL of 2.5mM dNTP mix and 1 μL of RNase inhibitor were added along with 2 μL of NE Buffer for M-Mul V RT 10x and 2 μL of the enzyme, M-Mul V reverse transcriptase. The solution was incubated at 42 °C for one hour to convert the mRNA to cDNA. To amplify the cDNA through PCR, 2 μL of RT#2 primer, RT#3 primer, 25 μL of 2X Master Mix, and 19 μL of nano-pure water were added to 2 μL of cDNA. When adding the Fur protein, the water was substituted with a 1/10 dilution of Fur protein in Tris buffer. The solutions were mixed by pipetting and then amplified using PCR. Once the cDNA was amplified, it was stored in the -40 °C freezer for future use. To ensure that only the cDNA would signal during the gel electrophoresis, negative controls, samples omitting key reagents necessary for signaling during gel electrophoresis, were also made by substituting 2X Master Mix and reverse transcriptase with nano-pure water.

Gel Electrophoresis: Gel electrophoresis was used to quantitate the mRNA produced during the expression of the *sodA* gene in the presence and absence of FUR. To make the 5% acrylamide gel, 2 μL of 5X TBE buffer, 1.25 μL of 40% acrylamide (29:1), 6.75 μL of nano-pure water, 20 μL of tetramethyl-1,2-ethanediamine, and 100 μL of APS were combined in a round-bottom flask and mixed by swirling. (These were added directly before the gel was poured because they caused the solution to polymerize quickly.) The solution was transferred to a gel mold via burette. A comb was inserted into the mold to form wells, and the gel set until polymerized. The gel was then attached to a blank mold and transferred to a tub. A 1X TBE

buffer was used to fill the space in between the two molds and the tub. The cDNA samples, original PCR template, standard (1kb DNA Ladder N32325), and negative controls were added using 3 μL of 6X Gel loading buffer DNA mixed with 5 μL of sample. The samples were pipetted three times to ensure mixing and then loaded into the wells. Electrophoresis was run at 176 volts for 25 minutes. The gel was removed and incubated in 10 mL of 1X TBE buffer and 1 μL of SYBR Green (the fluorescing reagent) on a rocker for five minutes. The gels were then analyzed using a VersaDoc that took a digital image of the gel. An internal standard was constructed to measure the effects of the protein on the expression of the *sodA* gene. The intensity of the signaling bands was evaluated using the internal standard to determine the effects of Fur.

Results

Figure 1 shows negative and positive controls without Fur. The negative controls with no Taq polymerase (2X Master Mix) did not signal, and the positive controls with everything signaled strongly, as expected. However, the negative controls with no enzyme (reverse transcriptase) signaled slightly, indicated by the red box. To solve this problem, new batches of DNase were made in concentrations of 5 units/ μL and 50 units/ μL to find the optimal amount necessary for the bands of the negative controls not to signal as seen in Figure 2.

As seen in Figure 2, the new batches of DNase were effective in eliminating leftover mRNA in the controls, because no negative controls signaled. Since using only 2 μL of 5 units/ μL DNase worked as well as using 4 μL of 50 units/ μL , 2 μL of 5 units/ μL DNase were used in the revised transcription process.

Figure 3 shows strong signaling of the standard and negative controls without reverse transcriptase, indicated by the green boxes. There was also slight signaling of the positive

controls as seen in the blue box. The negative controls without Master Mix did not signal. This gel shows the greatest signaling of the positive controls with 2 μ L of 1/10 Fur dilution, indicated by the red box.

Conclusion

My hypothesis that Fur would regulate the transcription of the *sodA* gene of *E. coli* was supported. My research showed that the samples with 2 μ L of 1/10 dilution of Fur signaled stronger than those with 4 μ L of Fur or those without Fur. Substituting 2 μ L of transcription buffer with 1/10 dilution of Fur during transcription produced the most mRNA from the samples with no Fur and with 4 μ L Fur added. This means that for optimal signaling of samples, Fur can be added to regulate the amount of mRNA produced.

While my results showed consistent signaling of the positive controls with and without Fur, there was inconsistent signaling of the negative controls from trial to trial. This may have been due to deformities in the wells of the gel. The next step of the project is to run additional gels to confirm that the signals from the negative controls are due to outside factors.

Future work on this project should include running trials with and without Fur and using an internal standard to quantify the mRNA produced. Once the mRNA is quantified, the work that I did to show that Fur does activate transcription can be used to study other regulatory proteins and their effects on gene expression. Research on the regulation of the Fur protein provides information on how cells use defensive proteins to convert harmful radicals into less harmful substances (*1*). This research may then be related to finding a cure for cancer by using regulatory proteins to correct potentially cancerous, over- and under-expressed genes.

Acknowledgements

Dr. Olson guided me through the transcription and RT-PCR protocols and taught me about the background of DNA and gene expression. Additionally, Mrs. Fruen helped me edit my final papers and forms, taught me how to present concisely, and explained material I was not comfortable with presenting. Dr. Miller and Team Research revised my papers and gave me useful tips on what to explain further and what to abridge.

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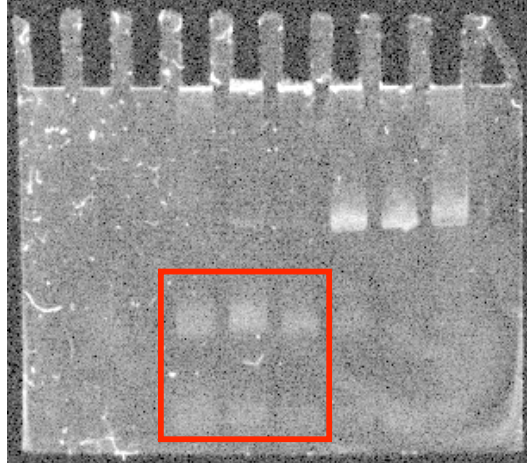
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Appendix

Figure 1: 5% Acrylamide Gel with Negative and Positive Controls in the Absence of Fur



Positive control
Positive control
Positive control
Negative control – no RT
Negative control – no RT
Negative control – no RT
Negative control – no Master Mix
Negative control – no Master Mix
Negative control – no Master Mix

Figure 2: 5% Acrylamide Gel with 6 Negative cDNA Controls with Varying Concentrations of DNase

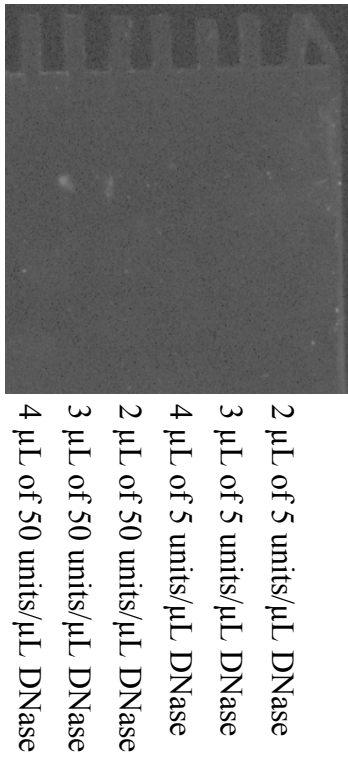


Figure 3: 5% Acrylamide Gel with Positive and Negative cDNA Controls with 0 μL , 2 μL , and 4 μL of the 1/10 Fur

