

**The Effect of Iron on Pyocyanin production by  
*Pseudomonas aeruginosa***

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## Summary

*Pseudomonas aeruginosa* an opportunistic human pathogen causes both acute and chronic lung disease. *P. aeruginosa* exerts many of its pathophysiological effects by secreting virulence factors, including pyocyanin, a redox-active compound that increases intracellular oxidant stress. Because so little is known in regards to how this active metabolite is regulated by environmental factors the basis of this study involves how the presence of iron affects the ability of *P. aeruginosa* to produce pyocyanin. In this study we examined the regulation of pyocyanin using a defined iron containing media. The amount of pyocyanin produced by *P. aeruginosa* was determined using an acid chloroform extraction protocol. In an iron sufficient GA media, *P. Aeruginosa* strain PAO1 produced substantial amounts of pyocyanin. In contrast, iron deficient GA resulted in significantly lower amounts of pyocyanin. Studies were also performed to determine the exact time that iron effected the production of pyocyanin. This was done by monitoring the pyocyanin levels relative to the rate of growth of the bacterium over a twenty four hour period of time. We found that iron effect the production of pyocyanin in the late stationary phases of growth. In addition to varying iron concentrations, we also examined the effect of iron on transcription of the pyocyanin synthesis genes (*phzA* genes). We first attempted to determine which of the two pyocyanin genes where affected by iron. We did this by using a LacZ chromosomal fusion strain found within one of the two operons and monitoring the Beta-galactosidase activity overtime. Upon performing this study we found that the *phzA1* operon is affected somewhat by iron where as the *phzA2* operon is not. Results of the above transcriptional studies where confirmed by real time PCR analysis. The level of *PhzA1* mRNA in the presence of iron was increased by two fold while that of the *PhzA2* was not changed. These differences are likely to be due to the difference in the sensitivity of both the assays.

## Introduction

The opportunistic pathogen *Pseudomonas aeruginosa* is a Gram-negative bacterium that infects immunodeficient or otherwise compromised patients and causes a wide range of infections. Among those infected with this bacterium are patients with cystic fibrosis, severe burn wounds and especially those with nosocomial infections. Pathophysiological effects of *P. aeruginosa* are often associated with a number of virulence factors secreted by this bacterium. Among these virulence factors secreted by *P. aeruginosa* is pyocyanin, a low molecular weight phenazine redox pigment. From 90 to 95% of *P. aeruginosa* isolates produce pyocyanin and the presence of high concentrations of pyocyanin in the sputum of cystic fibrosis patients has suggested that this compound plays a role in pulmonary tissue damage observed with chronic lung infections. This idea is supported by several recent studies which demonstrated that pyocyanin contributes in a variety of ways to the pathophysiological effects observed in airways infected by *P. aeruginosa*. Pyocyanin interferes with the regulation of ion transport, ciliary beat frequency, and mucus secretion in airway epithelial cells by altering the cytosolic concentration of calcium. The unusually broad range of biological activity associated with phenazines is thought to be due to their ability to undergo redox cycling in the presence of various reducing agents and molecular oxygen, which leads to the accumulation of toxic super oxide and hydrogen peroxide and eventually cell injury or death (5). *In vivo* studies have suggested that these radicals created by pyocyanin inhibit epidermal cell growth, disrupt the host immune system and possess antibiotic properties against other microorganisms. (5) The production of *P. Aeruginosa* virulence factors are known to be controlled by different environmental conditions. One such condition includes the level of iron in the environment. Many studies have shown that iron negatively regulates many virulence factors including Exotoxin A and Siderophores. In iron sufficient media insignificant levels of these two virulence factors are observed. In contrast, in iron deficient media significant amounts are detected. At this present time, little is known about the possibilities of iron in the environment positively regulating any *P. aeruginosa* virulence factors.

For pyocyanin purification, *P. aeruginosa* is grown in the specifically designed medium, GA. Significant levels of pyocyanin are observed when *P. aeruginosa* is grown in this media. Iron is one component present within this form of media. Using GA, we attempted to determine if iron influences the production of pyocyanin by *P. aeruginosa* and if this influence takes place at the transcriptional level.

## Materials and Methods

### Media and Growth Conditions.

*P. aeruginosa* strains were regularly grown in Luria-Bertani (LB) broth at 37 degrees. For the purpose of pyocyanin production, *P. aeruginosa* strains were grown in glycerol alanine minimal (GA) medium (4) at 37 degrees C for 24h.

### Growth Curve.

The cultures were grown overnight in Luria Bertani medium (LB) at 37 degrees C. Cells were pelleted and resuspended in GA medium. Individual 10ml volumes of GA media were then aliquoted with the resuspended cells. Cultures were then grown at 37 degrees C with maximum aeration. Throughout the growth cycle of each culture (24 h ), samples were collected every 6 hours for analysis of growth, pyocyanin production or Beta-glycosidase activity. (2)

### Pyocyanin Assay.

Pyocyanin was removed from the supernatant fraction of the isolates of *P. aeruginosa* isolates grown in GA medium for 24 hours. A 5ml volume of supernatant was mixed with a 5ml chloroform layer. A 1.5 ml of 0.2 M HCl was added to this layer and the pyocyanin-rich organic layer was extracted. The amount of pyocyanin within the collected sample layer was determined through measuring the A520. (11)

### Real-Time PCR.

The total amount of RNA that was extracted by a hot-phenol method was used for the real-time PCR reactions. DNA was eliminated from the solution of RNA with DNase I in the presence of Rnase inhibitor. These enzymes and the buffer that was used were removed with an Rneasy kit. Each reverse transcriptase reaction that was performed involved DNase-treated RNA, random hexamer, Rnasin, and Stratascript reverse transcriptase. The cDNA that was generated was mixed with reverse and forward primers and SYBR green PCR mix subsequent to reverse transcription. Duplicate samples were loaded on a 96-well PCR plate. Amplification of DNA products were carried out with the ABI prism 7000 Sequence Detection System. The data was then recorded and analyzed with Microsoft Excel. 16S rRNA was used as a standard to compensate for variation in the number of cells harvested and the efficiency of extracting RNA. Quantitative data for each gene are expressed as number of copies per 10 the 10 copies of 16S rRNA. (1)

### Beta-Galactosidase Assay.

Cells were first centrifuged in order to generate a pellet; these pelleted cells were then washed with 0.02 M phosphate buffered saline (PBS), and resuspended in water. These resuspended cells were then sonicated and the Beta-galactosidase activity within the lysate fraction was determined as previously described. (6)

## Results

### GA medium is suitable to examine the regulation of *P. aeruginosa* genes by Iron

One possible argument in interpreting the results above is that GA medium is not a traditional medium to examine the regulation of *P. aeruginosa* genes by iron. To address this problem we tried to determine the level of expression of the *toxA* gene in *P. aeruginosa* which codes for the exotoxin a protein. The expression of this gene this gene was tested in GA media with and without iron. This gene (*toxA*) is stringently regulated by iron (no *toxA* expression occurs when *P. aeruginosa* is grown in iron sufficient media). Plasmid pSW228 (which carries the *toxA lacZ* fusion) was introduced into PA01 and the strain was grown in GA with iron and GA without iron. As shown in Figure 1, the growth of PA01 pSW228 in GA with iron reduced the level of *toxA* expression considerably. This confirms that the GA media is suitable to examine the regulation of *P. aeruginosa* genes by iron due to the fact that the same phenomenon's observed in rich medias are also observed in our minimal GA.

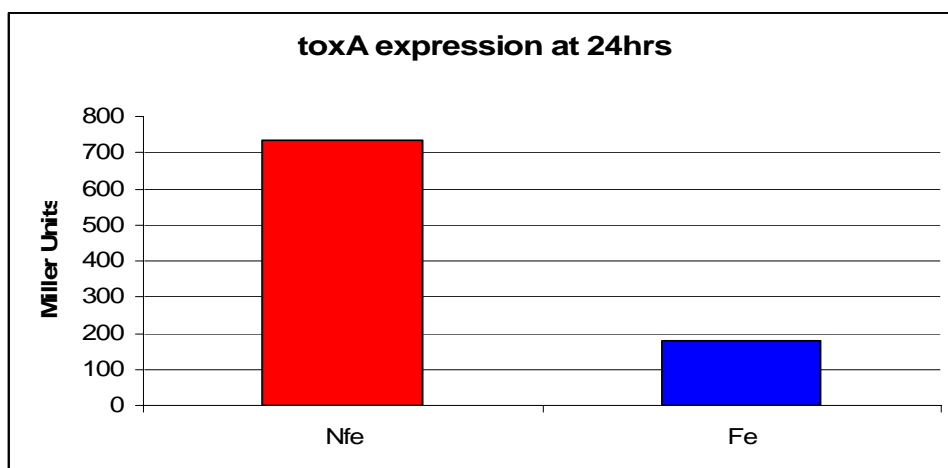


Fig. 1: The effect of iron on the transcription of the *toxA* gene in *P. aeruginosa*. Nfe, No Iron; Fe, With Iron.

### The effect of iron on pyocyanin production by *P. aeruginosa* strain PA01

After determining that our minimal GA media was sufficient to observe gene expression in our bacterial strain the next step was to determine how the minimal media containing iron would affect pyocyanin production. PA01 was grown in GA medium with and without iron for 48 hours as described in materials and method. The level of pyocyanin within the supernatant fraction was determined. As shown figure 2 the amount of pyocyanin produced in the supernatant of PA01 that was grown in iron rich GA medium was significantly higher than that produced by PA01 that was grown in iron deficient GA medium. These results suggest iron enhances pyocyanin production in *P. aeruginosa*.

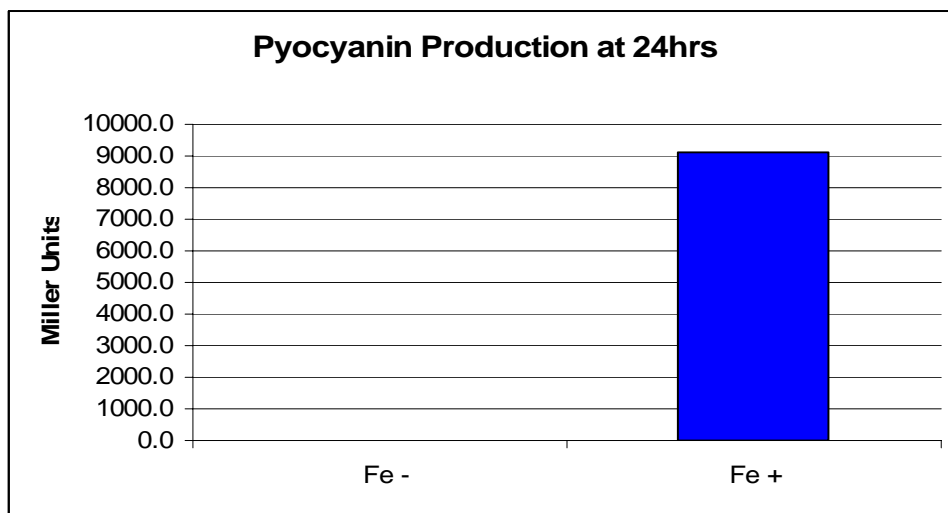


Fig. 2: Level of pyocyanin produced by PA01 that was grown in the presence or the absence of iron. . Fe-, No Iron; Fe+, With Iron.

### **The effect of iron on the transcription of one of the pyocyanin synthesis gene *phzA1***

It is possible that iron affects pyocyanin production by enhancing the transcription of the pyocyanin synthesis genes (*phzA1*). One way to evaluate this possibility is to determine the effect of iron on the transcription of one of the phenazine synthesis operons *phzA1G1*. To do this we have utilized strain QSC131 which carries a *phzA1-lacZ* chromosomal fusion with one of the pyocyanin synthesis genes. Therefore, an increase in the level of Beta-galactosidase activity in this strain would indicate an enhanced expression of the *phzA1G1* operon. Cells were harvested and the level of Beta-galactosidase activity was determined as described in materials and methods. As shown in figure 3, the level of expression of the *phzA1G1* operon in QSC131 was enhanced in GA media containing iron. However this enhancement was not as considerable as the increase in pyocyanin production (Figs 2 and 3). Thus, based on these result it appears that iron effect pyocyanin production at different levels (i.e. transcription and post-transcriptional levels).

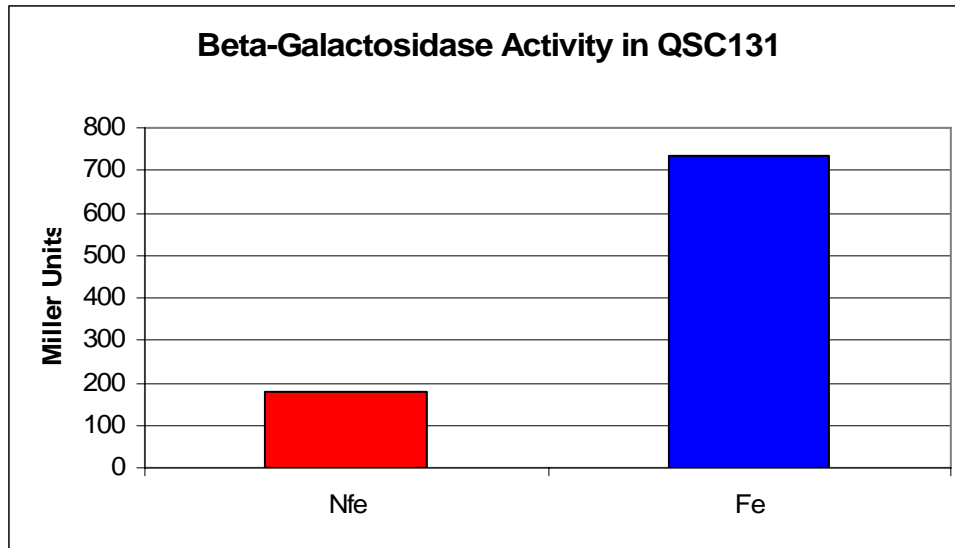


Fig. 3: The level of Beta-galactosidase activity produced by the *phzA lacZ* fusion in *P. aeruginosa* strain QSC131 that was grown in either the presence or the absence of iron. . Nfe, No Iron; Fe, With Iron.

### **The Effect of Iron on Pyocyanin Production throughout the growth cycle of *P.aeruginosa***

Pyocyanin is usually not produced at early stages of growth in *P. aeruginosa*. It is usually produced at mid to late stationary phases of growth. Therefore, we wanted to know if this growth-phase dependent production of pyocyanin is effected by the presence of iron in the growth medium. The *P. aeruginosa* strain PAO1 was grown in iron deficient and iron sufficient GA medium. Samples were harvested every 6 hours and the level of pyocyanin in the supernatant fraction was determined as described in materials and methods. Samples were taken at 6 hours, 12 hours, 18 hours and 24 hours post inoculation. As previously shown by different investigators pyocyanin was not produced at early or mid log phase of growth (Fig. 4) No pyocyanin was detected in GA media with or without iron until late stationary phase of growth (Fig. 4). In GA medium without iron pyocyanin production was detected at 12 hours and the level remained unchanged until the end of the growth cycle. (Fig. 4) By contrast in GA medium with iron the level of pyocyanin was detected at 12 hours, continued to increase, and continued to increase until the end of the growth cycle. (Fig. 4) These results suggest that the enhancement of pyocyanin production in response to iron occurs throughout the growth cycle of *P. aeruginosa*. However, after that, the level of pyocyanin produced in GA iron sufficient media was considerably higher than that produced in iron deficient GA media.

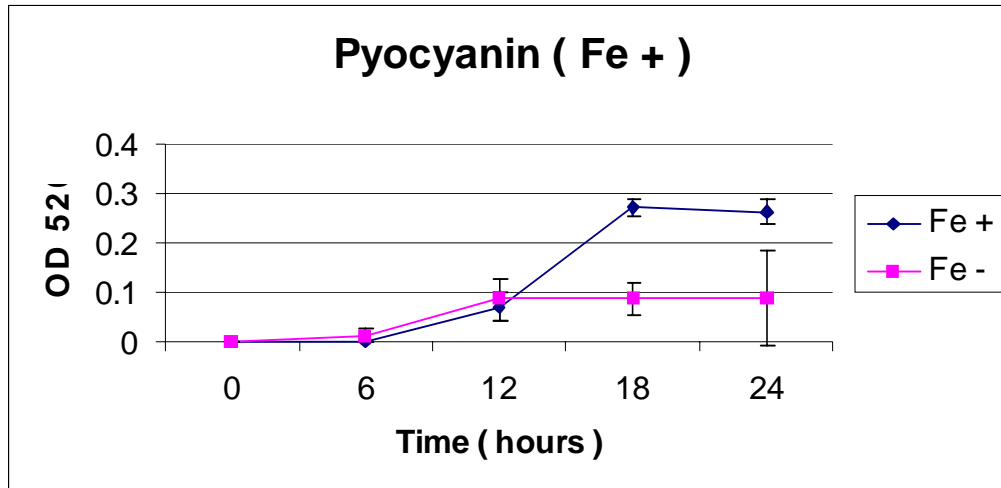


Fig. 4: Level of pyocyanin produced by PAO1 that was grown in iron deficient and iron sufficient GA medium. Each value reflects the average of three independent experiments plus or minus standard deviation. . Fe-, No Iron; Fe+, With Iron.

### The Effect of Iron on the Expression of Pyocyanin Genes

In *P. aeruginosa* the pyocyanin structural genes are organized in the operons; *phzA<sub>1</sub>* and *phzA<sub>2</sub>*. The two operons are 98% identical. However, the upstream promoter regions of the two genes differ from one another. As a result of this difference we tried to determine the specific operon that is targeted by iron. This was done using a specific *P. aeruginosa* strain, PAO1:: *phzB2-lacZ* that was obtained from the University of Washington Genome Center. This strain contains a *phzA2-lacZ* chromosomal fusion. Analysis of this strain would provide us with important clues regarding the effect of iron on pyocyanin production. A change in the level of Beta-galactosidase activity would indicate an effect on the *phz2* operon. The strain contains intact *phz1*, therefore, analysis of pyocyanin level would indicate if iron affects *phzA1*. More importantly, comparison of the pyocyanin level produced by PAO1:: *phzB2-lacZ* and its parent strain would indicate the amount of contribution of *phzA1* and *phzA2*.

PAO1:: *phzB2-lacZ* and its parent strain were grown in iron deficient and iron sufficient GA media. The level of B-galactosidase activity within the lysate was determined as previously described. The amount of pyocyanin within the supernatant fraction was determined. As shown in Fig. 5A, The PAO1:: *phzB2-lacZ* strain produced comparable levels of B. galactosidase activity in the iron deficient and iron sufficient medium. However, the mutant was still producing significantly higher level of pyocyanin in the iron sufficient than in the iron deficient media (Fig. 5B) Therefore based on these results it is possible that iron regulates the expression of PhzA1 but not PhzA2.

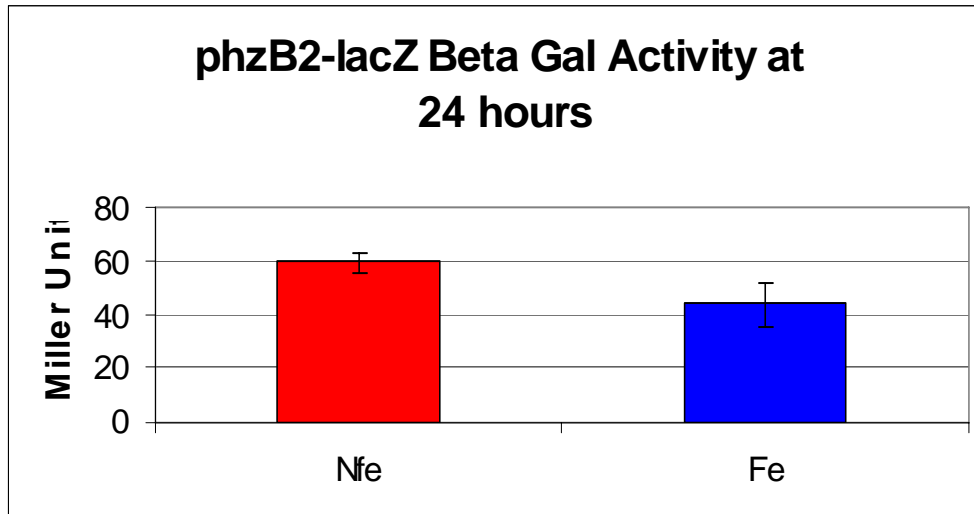


Fig. 5 A: Level of Beta-galactosidase activity produced by PAO1:: *phzB2-lacZ* in iron deficient and iron sufficient media. Nfe, No Iron; Fe, With Iron.

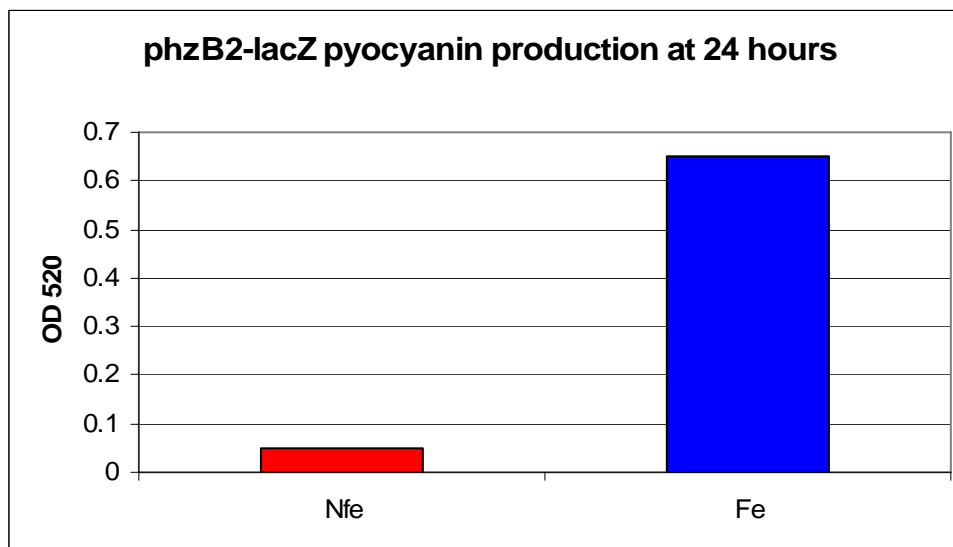


Fig. 5 B: The level of pyocyanin produced by the mutant in iron sufficient and iron deficient media. . Nfe, No Iron; Fe, With Iron.

### Confirming the effect of iron on *phzA1* expression using real time PCR analysis

The real time PCR analysis is a very sensitive method that measures the copies of the MRNA rather than expression of the reporter gene. It quantifies the amount of mRNA for a specific gene. Cells were grown in iron sufficient and iron deficient medium and the total RNA was extracted though the hot phenol method. The mRNA for *phzA1* and *phzA2*

was analyzed using specific primers. Results showed that PAO1 produced comparable levels of *phzA2* mRNA in both iron deficient and iron sufficient medium (Fig. 6). However, in iron sufficient media PAO1 produced higher levels of *phzA1* mRNA than in iron deficient media (Fig. 6) Therefore these analyses confirm our above described analysis using the lacZ fusion system and proved that iron regulates the expression of *phzA1* and not *phzA2*.

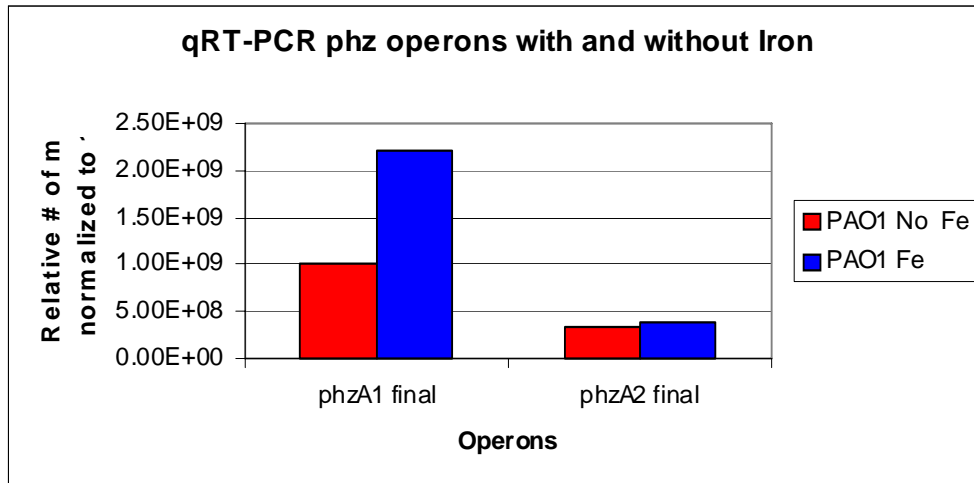


Fig. 5: Analysis of *phzA1* and *phzA2* mRNA in PAO1 that was grown in iron deficient and iron sufficient GA medium . Nfe, No Iron; Fe, With Iron.

## Discussion

Glycerol alanine media (GA media) is a chemically defined minimal media that was essential to the experiments that were performed above. This minimal media contains a number of chemicals, the most notable one being that of iron. Although the growth of *P. aeruginosa* within the media is somewhat reduced relative to rich media it allowed us to analyze the effects iron has upon our bacterium. The nature of defined media versus a rich media is that they allow for the removal and alteration of chemicals found within the media. With the ability to alter the amounts of chemicals one is allowed to observe how they affect a given bacteria.

To ensure that GA was suitable for analyzing the effects of iron on *P. aeruginosa* we made use of a gene known as *toxA*. *ToxA* is a gene within *P. aeruginosa* that is known to be negatively regulated within a rich media. If the *toxA* gene exhibited similar negative regulation within GA media this would validate the fact that it is appropriate for the use analyzing the transcriptional effects on pyocyanin genes. Upon performing this experiment we found that the same negative regulation observed in rich media was also found to take place in GA. As a result of these observations we concluded that GA was sufficient.

Subsequent to determining that GA was sufficient for use within our experiments we turned to the effects that iron has upon *P. aeruginosa*. By altering the level of iron within our media and monitoring the level of pyocyanin produced over various time increments we found that the level of pyocyanin produced by our bacteria was very much affected. In the presence of iron pyocyanin was positively regulated. However, when iron was not present a dramatic decrease in the amount of pyocyanin was observed. This observation is very unique in the fact that most virulence factors generated by *P. aeruginosa* are negatively regulated by the presence of iron.

Upon determining the effect that iron has upon *P. aeruginosa* we began to search for when this effect was taking place. We attempted to find exactly when in the growth cycle of the *P. aeruginosa* was the increase in pyocyanin production in the presence of iron observed. This question was answered by a series of growth curve analysis that pointed to the fact this positive regulation of pyocyanin was taking place in late stationary phase or around the twelve hour time period. By observing the production of pyocyanin versus growth we determined that the different types of media (Iron sufficient and Iron deficient media) had no considerable effect on the ability of the bacteria to grow. However, the effect of iron did appear to have a significant effect on the ability of the bacterial strain to generate pyocyanin. This is significant because if the growth has been different our observations could possibly be discounted because the observed increase in pyocyanin product could be dismissed as the presence or absence of iron affecting the growth.

After identifying when the observed increase in pyocyanin levels took place we tried to uncover at what level did this increase take place. Analyzing the transcription of the pyocyanin operons was the most logical place to start in attempting to answer this question. It is important to again note that *Pseudomonas* has two operons that have 99%

homology. To take advantage of this homology we utilized a strain that contained a Beta-galactosidase fusion at a region in the operon where homology does not exist. This enabled us to monitor the activity of that particular operon and determine which of the two if any were affected by the presence of iron. We did this because the presence of the fusion inactivates the operon that contains it. This allows us to analyze the Beta-galactosidase activity in the presence and absence of iron to determine if the transcription of that operon is affected. Simultaneously we can also analyze the pyocyanin production of the second operon in the presence and absence of iron. Although we are analyzing a product versus transcriptional activity it gives us a rough understanding of what is taking place in these two different medias/environments in terms of the operons.

After analyzing this rough assessment of operon activity we came to the conclusion that the one of the operons was affected very little by iron in regards to transcription (*phzA2*) while the other experienced a ten fold increase in the generation of a pyocyanin product (*phzA1*). We then looked at the total transcripts that resulted from the presence and absence of iron through real time PCR analysis. Upon performing this experiment we observed that the data supported our understanding that one of the operons was affected very little if any by the presence of iron (*phzA2*). One operon (*phzA1*) however, did experience a two fold increase in the number of transcripts however, this does not account for the ten fold increase in pyocyanin product.

These differences observed in the activity of the *phzA1* operon can be explained by two likely possibilities. The first possibility lies in the sensitivities of the two assays. The second possibility may be in the parameters that are examined by the two assays. Real time PCR analyzes reports the accumulation of mRNA overtime. While a Beta-galactosidase assay analyzes the activity of a promoter at specific time. These two characteristic differences could account for the two fold increase in Beta-galactosidase activity versus the ten fold increase found in the PCR results.

The results presented above pose one major question. The question being, what is the actual role of the interaction of iron and pyocyanin *in vivo*. The central dogma concerning iron in an *in vivo* environments suggest that virulence factors of *P. aeruginosa* are negatively regulated in its presence. However, the results above show that iron does not negatively regulate pyocyanin but positively regulates it. This suggests that there is a deeper underlying significance to pyocyanin other than just a virulence factor or simply for the purpose of tissue destruction. This role of iron on pyocyanin appears to be essential because the regulation occurs at the transcriptional level. One possibility in explaining this complex interaction of iron and pyocyanin could lie in the type of infection. *P. aeruginosa* is a bacterium known for its ability to generate a number of different types of infections (9). These infections range from lung to urinary tract infections. Levels of iron vary throughout the body. It seems logical to think that these varying levels of iron could affect the amount of pyocyanin generated by *P. aeruginosa*. These new findings and the importance of searching for new approaches to combating this bacteria force us to reexamine the dogma.

## **Acknowledgments**

The author would like to thank the McNair Scholars program at Texas Tech University for the time, money and effort placed in to helping complete this project. This work was supported by a grant from the National Institute of Health.

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