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Abstract

Pseudomonas aeruginosa PAO1 is an opportunistic pathogen known to have an arsenal of virulence factors that can affect immunocompromised patients. One of these virulence factors is the redox-active phenazine compound, pyocyanin. The production of pyocyanin is regulated by multiple mechanisms, which include stress response and quorum sensing (QS). In a previous study, we found that the PA2449 gene was required for the biosynthesis of QS homoserine lactones and pyocyanin. This study focused on a particular strain of *P. aeruginosa* that lacked the ability to produce pyocyanin. We restored the production of pyocyanin in this strain by inserting a vector encoding a gene responsible for pyocyanin biosynthesis, *rhlI*. We then did various studies to determine the levels at which the toxin was produced compared to the wild type strain. We found that in smaller volumes of medium, the mutant strain production levels were similar to the wild type, 219 µM. In larger volumes of media the two strains produced a similar amount of pyocyanin although the timing of the production between the two strains was different, 4.5 hours for the wild type and 9 hours for the mutant strain containing the vector. We also analyzed other strains with knockouts relevant to the PA2449 gene. This led us to discover the potential role for PA2449 in pyocyanin production, in repressing the expression of MexEF- OprN allowing the intracellular accumulation of PQS. This study will allow us to further understand the mechanisms that *Pseudomonas*, as well as other bacteria, use when producing toxins.

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Acknowledgements

I would to start out by thanking my parents for always encouraging me to do my best and pursue a career in the sciences. I would also like to thank my high school biology teacher, Mrs. Veronica Bruno for sparking my interest in the field of science. Many thanks to Dr. Nomura and Dr. Ben Lundgren for assisting me in this study.

Introduction

Phenazines are aromatic nitrogen-containing secondary metabolites produced by the soil bacteria *Streptomyces* and *Pseudomonas*. The roles of these compounds are related to their high redox activity, that is they perform such tasks as reduction of molecular oxygen to reactive oxygen species, primary energy metabolism, iron assimilation, and cell-to-cell communication (Heurlier et al., 2005). The type of phenazines this research is interested in is those related to antimicrobial factors. These allow the bacteria to kill competing bacteria in the environment as well as cause disease in some eukaryotic hosts. This study focuses on one of the better known human pathogens, *Pseudomonas aeruginosa*, and its production of the phenazine compound pyocyanin. Pyocyanin is often seen in patients suffering from cystic fibrosis and causes a blueish tint to their sputum (Lundgren et al., 2013).

Phenazine biosynthesis is controlled by a *Pseudomonas* quniolone signal (PQS; 2-heptyl-3-hydroxy-4-quinolone) system. The PQS system along with two other overlapping systems, Las and Rhl, all play key roles in the production of phenazines. Both Las and Rhl responses contain an autoinducer synthase and transcriptional regulator pairs LasI/LasR and RhlI/RhlR which are responsible for the production of 3-oxo-C12-HSL and C4-HSL, respectively (Jimenez et al., 2012). Together, these three systems control the expression of a diversity of virulence factors, e.g. exotoxins, proteases, rhamnolipids, and phenazines.

We have previously discovered that in *P. aeruginosa* strains lacking PA2449, the gene of interest of this study, there is a lack in the production of pyocyanin as well as the inability to use glycine as a carbon source. It was also observed that the efflux pump MexEF OprN was overexpressed in this strain, about a 342 fold increase. This discovery led us to believe that in wild type strains, PAO1, PA2449 continually represses the expression of the MexEF-OprN efflux pump which allows for an accumulation of PQS which in turn can turn on the *rhl* response to begin pyocyanin biosynthesis. In

the mutant lacking PA2449, named PW5126, we thought that by moving further down the biochemical pathway to the *rhl* response we could rescue pyocyanin biosynthesis despite the continual expression of the MexEF-OprN efflux pump (Fig. 1).

Methods

Media and Growth Conditions

Bacterial strains were grown at 37°C, 200 rpm for agitation of broth, in Luria Bertani (LB) 20g/L or peptone broth (PB) 1.5 g $MgSO_4$, 10 g K_2SO_4, and 20 g BD BBL Gelysate peptone. The cultures were grown in 500 mL baffled shake flasks in 50 mL of peptone to create an environment low in phosphate to simulate a condition known to cause pyocyanin production. Media were supplemented with kanamycin (50µg/L) and gentamicin (15µg/L and 30µg/L for *E. coli* and *P. aeruginosa*, respectively) when plasmid selection was necessary. When determining the ability to use glycine as a sole carbon or nitrogen source M9 minimal media was made using 20mM glycine as either the carbon or nitrogen source. When it was the sole nitrogen source, 20mM succinate was used as a carbon source. When it was the carbon source, NH_4Cl was utilized as the nitrogen source. Both of these have been shown to be utilized by *P. aeruginosa* as their respective sources.

Bacteria and Plasmids

The bacterial strains and plasmids used in this study are described in table 1. The plasmids were originally transformed into and maintained in *E. coli* Top10 and DH5α for cloning purposes, descriptions available in tables 1 and 2. The wild-type *P. aeruginosa* PAO1 strain as well as transposon mutants were acquired from the *P. aeruginosa* PAO1 transposon mutant library. These strains were verified for the proper inserts by PCR as recommended by the library curators. The plasmids were transformed into *P. aeruginosa* through electroporation methods that were previously established (Choi et al., 2006). Plasmids were selected for using gentamicin 30µg/L or kanamycin 50µg/L, depending on the plasmid, at 37°C for 18-24 hours.

Molecular biology methods

DNA was purified using Promega (Madison, WI) nucleic acid purification kits. Restriction enzymes and ligases were products of New England BioLabs (Ipswich, MA). PrimeStar polymerase (TaKaRa Biosciences, Japan) was used for the PCR experiment. The PCR experiments were conducted according to the manufacturer's recommendations. *rhlI* from PAO1 was amplified through PCR and isolated using a gel electrophoresis clean up kit from Promega. It was then ligated into pCR-Blunt as per the manufacturer's instructions (Invitrogen, Carlsbad, CA). DNA was sequenced for verification (Geneqiz, South Plainfield, NJ).

Construction of plasmids pMTG01 and pMTG02

The DNA region containing *rhlI* was amplified by PCR, using primers BL439, forward and reverse (table 3). This was then gel purified and blunt end ligated into pCR-Blunt to give pMTG01. The ORF of *rhlI* was then isolated using a restriction digest with *SacI* and *XbaI*. It was then ligated into pBBR1 MCS-5, which was cut with the same restriction enzymes. This gave us pMTG02 (Fig 2).

Determining the concentration of extracellular pyocyanin

Cells were grown in LB and transferred to PB (0.5% v/v). While cells were being grown in peptone broth, in triplicate, a small amount of culture was removed and spun down by centrifugation (16,000 x g for 3 minutes). The supernatant was passed through an Acrodisc syringe filter with 0.2µm cellulose hemi-acetate membrane. The absorbance of this was then measured at 690 nm and converted into pyocyanin concentration using the molar extinction coefficient for pyocyanin, 4130/M cm.

Growth of cultures with Glycine as a sole carbon or nitrogen source

In triplicate, 3% of a cell culture grown in LB for 20 hours were grown in M9 minimal media, both PAO1 and PA2449 :: PAO1. For glycine as the sole carbon source cultures were grown for 96 hours with measurements being taken every 12 hours. After every measurement, 2.5 mL of 1x M9 salts

(no carbon or nitrogen sources) were added to replenish whatever was lost due to sampling and evaporation. When glycine served as the sole nitrogen source the cultures were only grown for 24 hours, with measurements being taken every 1.5 hours for the first 12 hours and then a final measurement at the 24 hour mark.

Results

Pyocyanin biosynthesis can be restored in PW5126 by over expressing *rhlI*

After the electroporation of pMTG02 and pBBR1 MCS-5 as a control into PAO1 and PW5126 the biosynthesis of pyocyanin was determined. It was found that PW5126/pMTG02 produced about the same level of pyocyanin as the wild-type PAO1/pBBR1 MCS-5, 225 µM and 210 µM respectively. This was extremely different when compared to PW5126/pBBR1 MCS-5 which only produced pyocyanin to levels of 15.25 µM, which can be attributed to background absorption as no blue color was present in this culture (Fig 3).

We also conducted growth and production curves of the same strains. It showed similar results in that PW5126/pBBR1 MCS-5 was the only strain to not produce pyocyanin. All strains grew at the same rate as shown by their OD 600nm curve (Fig 4). The pyocyanin production results were surprising. The transposon strain with the insert showed a delay in production compared to the two wild-type strains, 9 hours compared to 4.5, respectively.

Over expression of MexEF-OprN efflux pump in PW5126 ceases the production of pyocyanin

To further investigate the role of PA2449 and its relation to the *rhl* response we did similar tests on a variety of transposon strains. One strain that strongly supported our hypothesis was the *mexF*::PW5126. This strain showed pyocyanin biosynthesis to a level of 100µM, (Fig 5). This result allows us to believe that the reason PW5126 is unable to produce pyocyanin is the over expression of the MexEF-OprN efflux pump. Another strain that showed equally supportive results is the PA2449:: Δ*mexF* PAO1. This strain showed a pyocyanin concentration comparable to that of the

wild-type at 182µM (Fig 5). It shows that when both of these genes are unable to be expressed, pyocyanin is synthesized.

PA2449 is crucial to glycine metabolism

The OD 600nm was measured to determine the growth of the cultures. It was found that when PA2449 was deleted from PAO1 glycine was unable to be used as either a carbon or nitrogen source (Fig 6). It stayed at a similar density the entire time, indicating zero growth. The wild-types however, did not have difficulties utilizing glycine as either a carbon or nitrogen source (Fig 7).

Discussion/Conclusion

PA2449 functions as a repressor to *mexEF-oprN* efflux pump genes

By activating the biosyntheses of pyocyanin further downstream than at the accumulation of the intracellular PQS signal we were able to show that the lack of the buildup in the PQS signal is responsible for the lack of pyocyanin biosynthesis. When we over expressed *rhlI* in a mutant deficient in PA2449 we showed that the primary role of this protein was to allow for the intracellular accumulation of PQS, which in turn, would turn on the *rhl* response. In strains in which both PA2449 or MexF were not expressed pyocyanin biosynthesis still occurred at levels comparable to that of the wild-type. This along with our restoration of biosynthesis in the PA2449 transposon, by insertion of *rhlI*, refutes the hypothesis proposed by Dr. Lundgren that PA2449 activates the *rhl* response directly. This would have explained the lack of pyocyanin biosynthesis in PW5126, and restoration of the biosynthesis in PW5126. The fact that when *mexF* was removed from PW5126 pyocyanin biosynthesis was restored also supports the idea that the role of PA2449 is to repress the expression of this efflux pump.

Further studies will be done to determine the levels of various signaling compounds at various stages that activate the biosynthesis of pyocyanin. Extraction times, using the growth and production curve experiments, will be determined to discover the cause of the time delay between

the mutant strain and the wild-type. Furthermore, we will observe any other differences amongst the two strains and plan studies accordingly.

Glycine metabolism can be related to pyocyanin biosynthesis

As previously stated, it was previously proposed that the accumulation of glycine had a direct effect on the production of pyocyanin. When glycine served as the sole carbon or nitrogen source no growth occurred. Due to the lack of growth here we cannot determine if an extremely high level of glycine can be attributed to the lack of pyocyanin production. In the wild-type, PAO1, a slight blue color was observed when glycine served as either the sole nitrogen or carbon source. However, analysis of the PB cultures would need to be done to determine if the levels of glycine reached levels that could deter this production as proposed by Lundgren et al.

Works Cited

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Jimenez, Pol Nadal, Gudrun Koch, Jessica A. Thompson, Karina B. Xavier, Robbert H. Cool, and Wim J. Quax. "The Multiple Signaling Systems Regulating Virulence in Pseudomonas Aeruginosa." *Microbiology and Molecular Biology Reviews* (2012): 46-65. Web.

Lundgren, Benjamin R., W. Thornton, M. H. Doran, L. R. Villegas-Penaranda, C. N. Bobby, and Christopher T. Nomura. "Gene PA2449 Is Essential for Glycine Metabolism and Pyocyanin Biosynthesis in Pseudomonas Aeruginosa PAO1." *Journal of Bacteriology* 100th ser. 195(9).2087 (2013): n. pag. Print.

Appendices

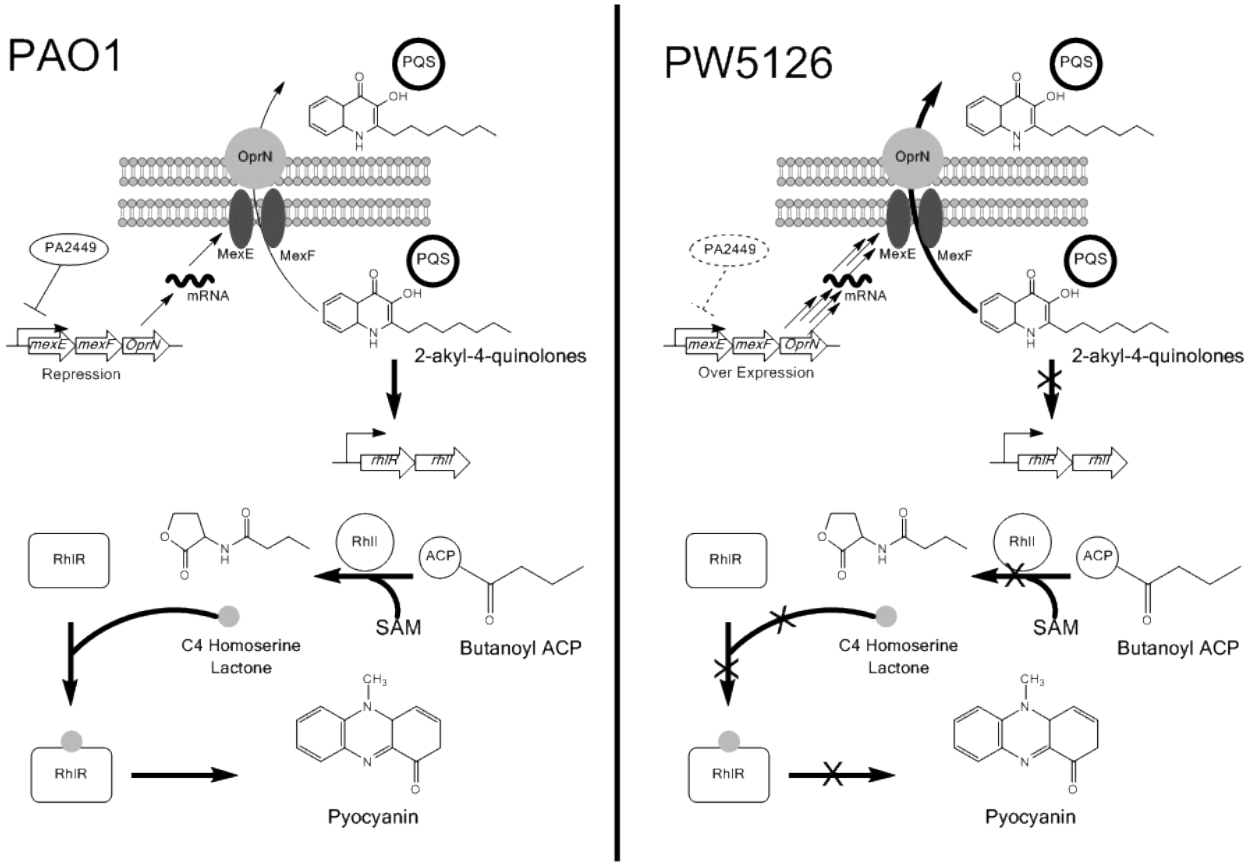

Figure 1. The right half of this image shows the hypothesized role of PA2449 in PAO1. It is suggested that it blocks the expression of the ORF of *mexE, mexF,* and *OprN*. It is still expressed at minimal levels. This limited expression allows for the intracellular accumulation of 2-akyl-4-quinolones (PQS). When this reaches a threshold level it activates the *rhl* response which in turn activates pyocyanin biosynthesis. On the right half of the figure, it indicates that PA2449 is absent in the PW5126 strain and as a result, the ORF of *mexE, mexF,* and *OprN* is continually expressed. This does not allow for the intracellular accumulation of PQS. In turn, the *rhl* response is never activated, therefore, pyocyanin biosynthesis does not occur.

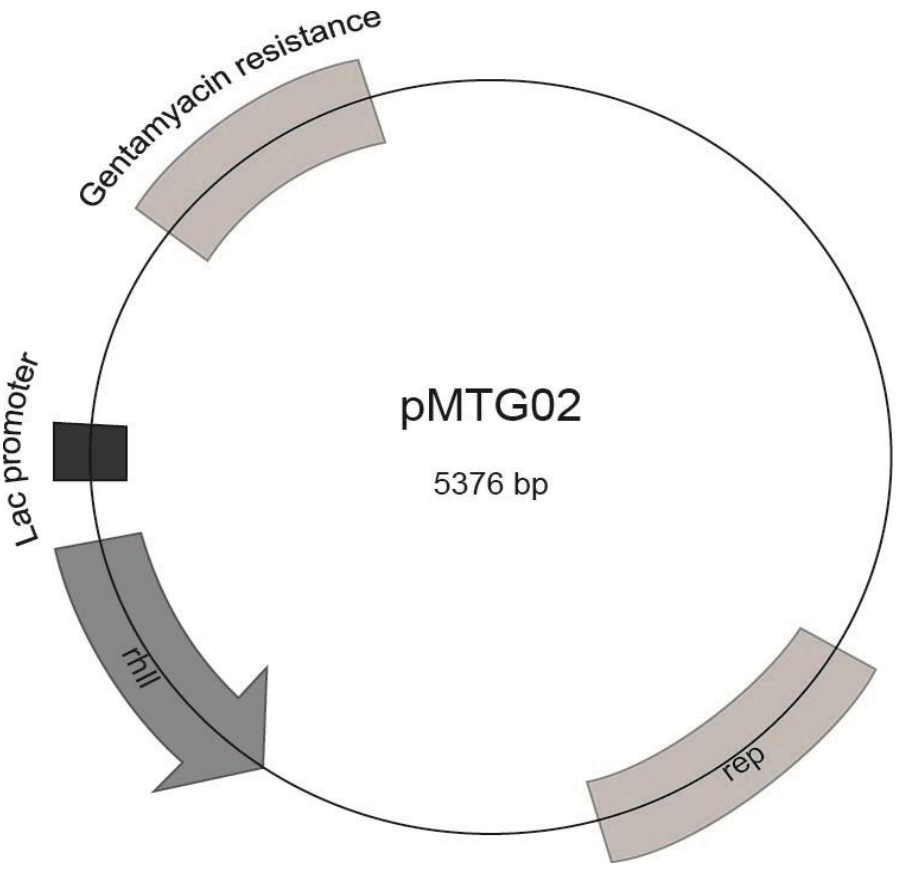

Figure 2. This shows the plasmid map of pMTG02. It is the cloning plamid, pBBR1 MCS-5 with the gene of interest of this study, *rhlI*, inserted.

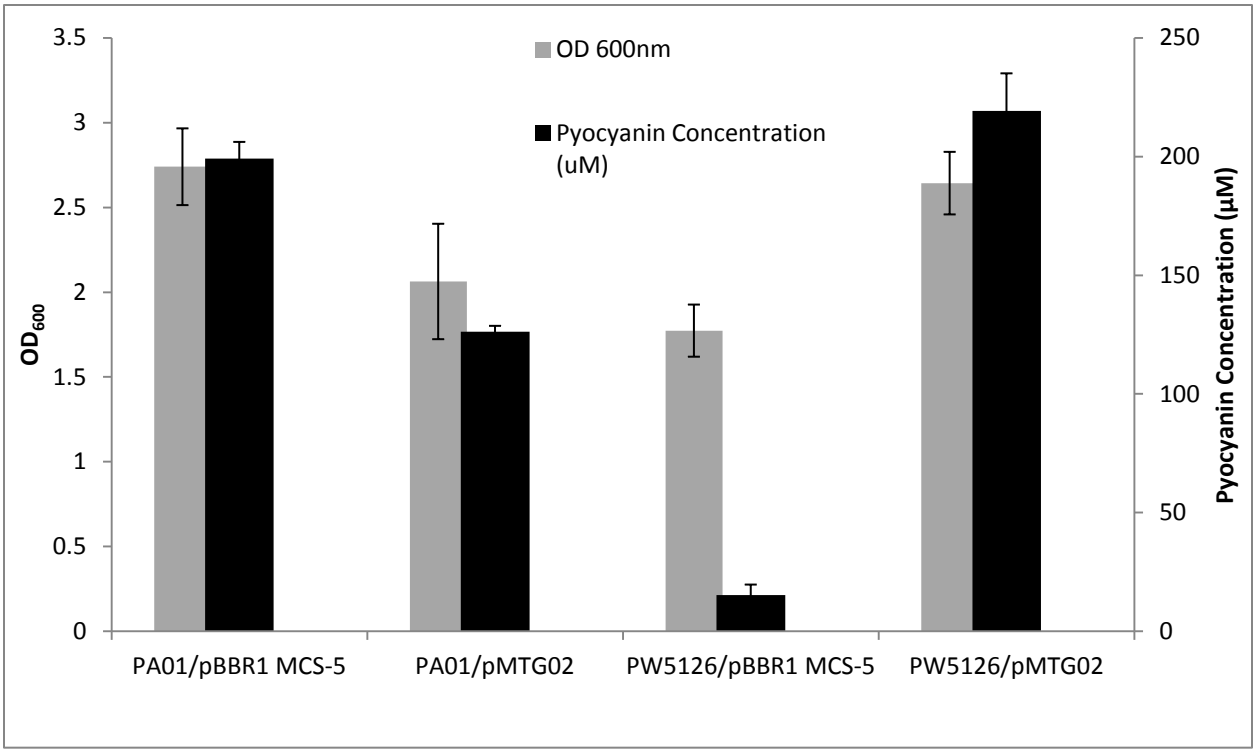

Figure 3. This graph shows the OD 600nm and concentration of pyocyanin found using Beer's Law of the 4 strains . The production seen in PW5126/pMTG02 compared to PW5126/pBBR1 MCS-5 shows that the insertion of *rhlI* rescued the biosynthesis of pyocyanin.

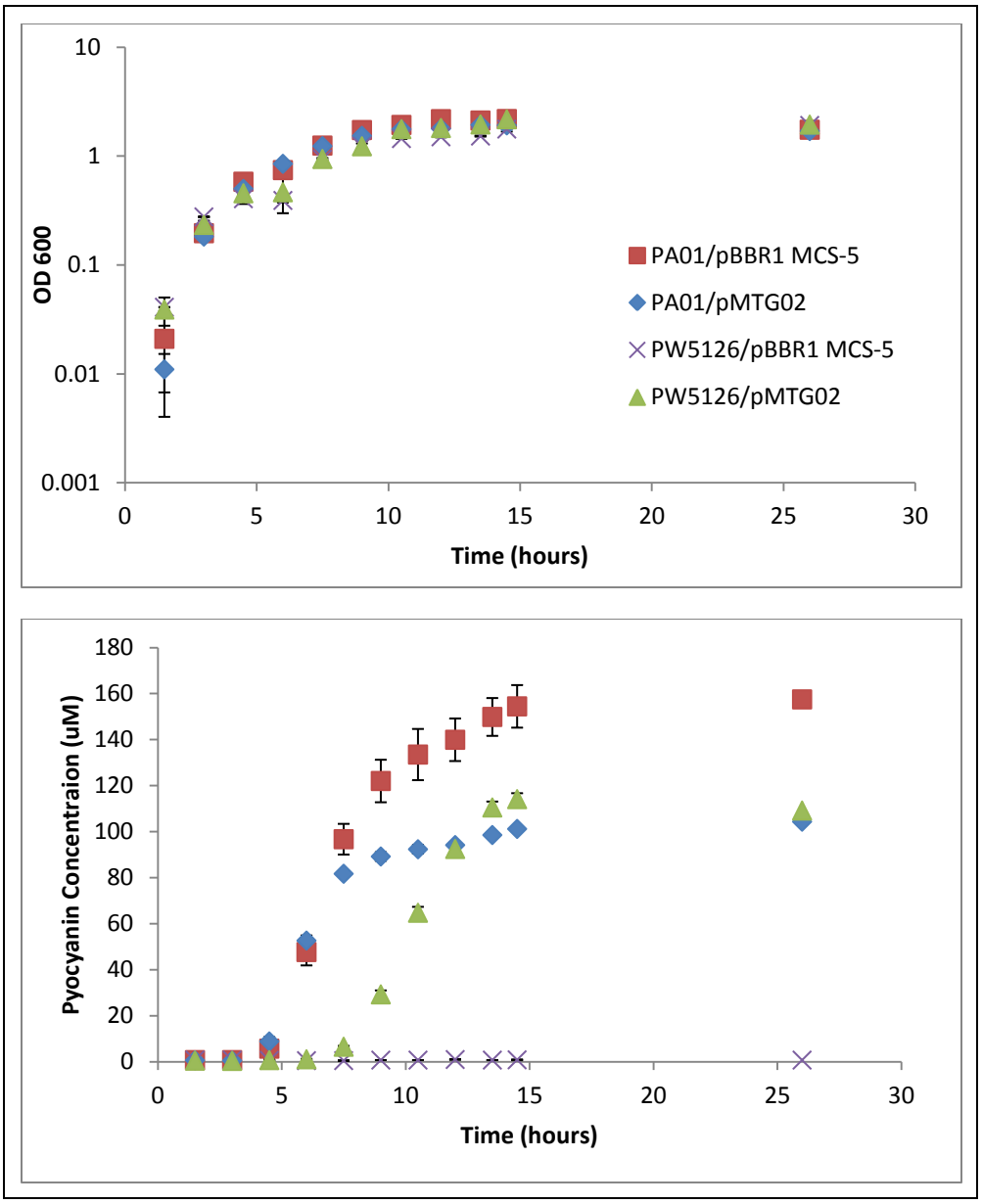

Figure 4. These graphs show the OD 600nm and pyocyanin concentration of the strains PAO1/pBBR1 MCS-5, PAO1/pMTG02, PW5126/pBBR1 MCS-5, and PW5126/pMTG02. It too shows that the insertion of *rhlI* into a PA2449 deficient mutant can restore the pyocyanin biosynthesis.

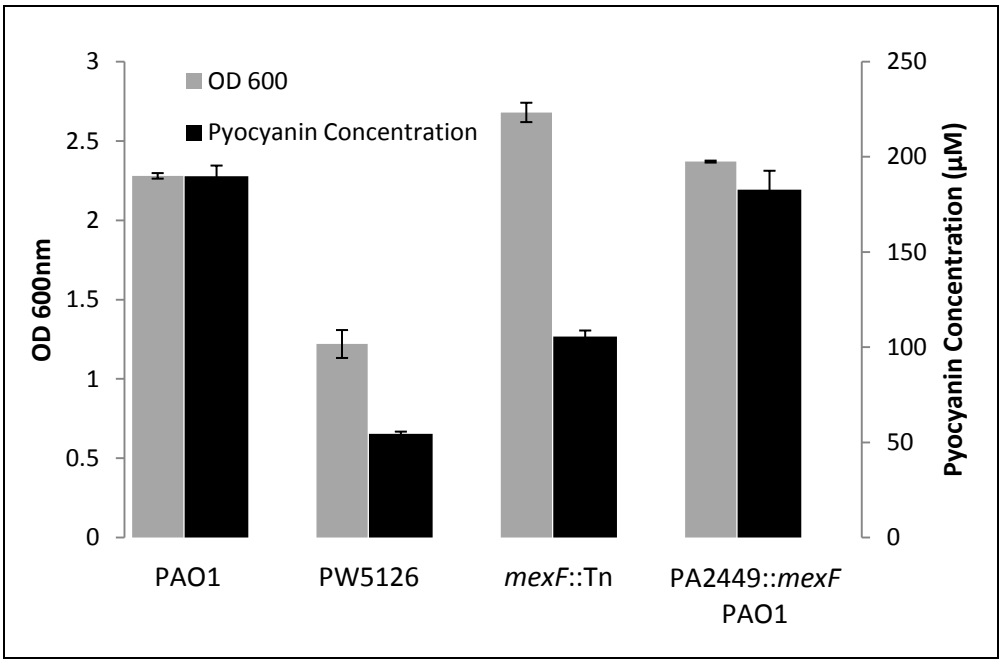

Figure 5. This graph shows the production level of various strains. Most importantly it shows that when both PA2449 and *mexF* are not expressed in the wild type, pyocyanin biosynthesis still occurs. Similarly, it shows that when *mexF* is knocked out of the transposon mutant, pyocyanin biosynthesis is also restored.

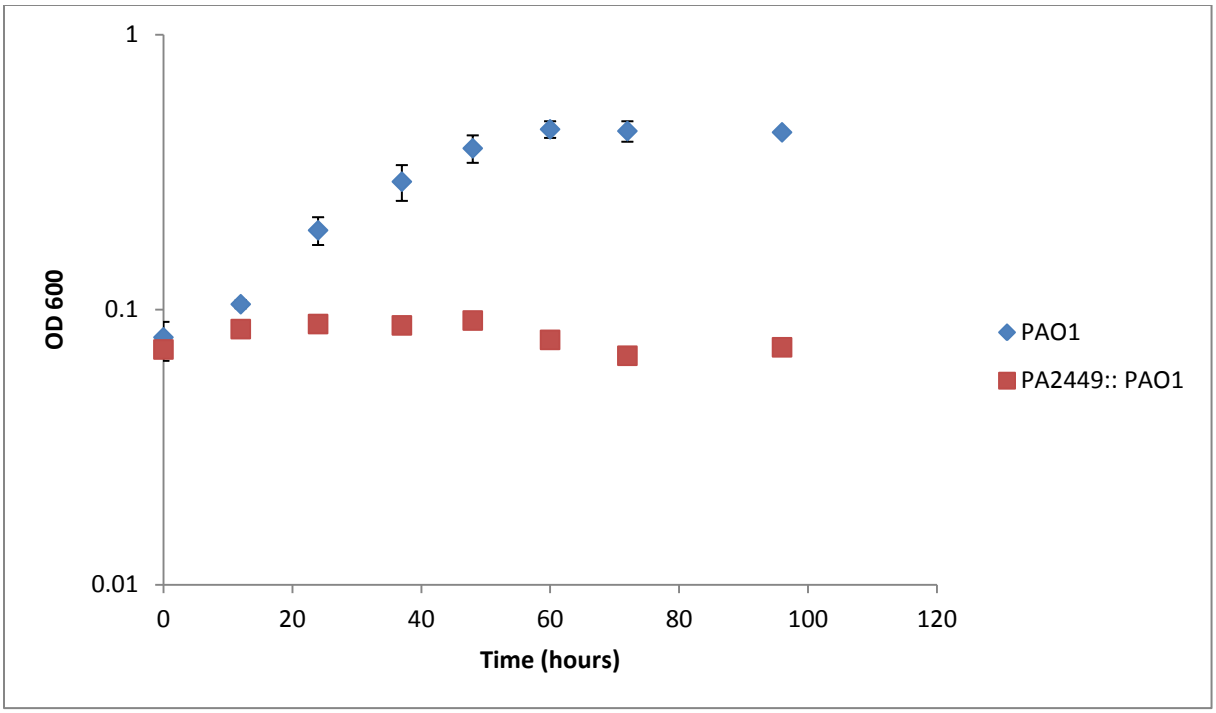

Figure 6. This graph shows the growth of PAO1 and PA2449 deficient strain of PAO1. Without PA2449, PAO1 was not able to utilize glycine as a carbon source. When PA2449 was present, glycine was able to be utilized as a carbon source.

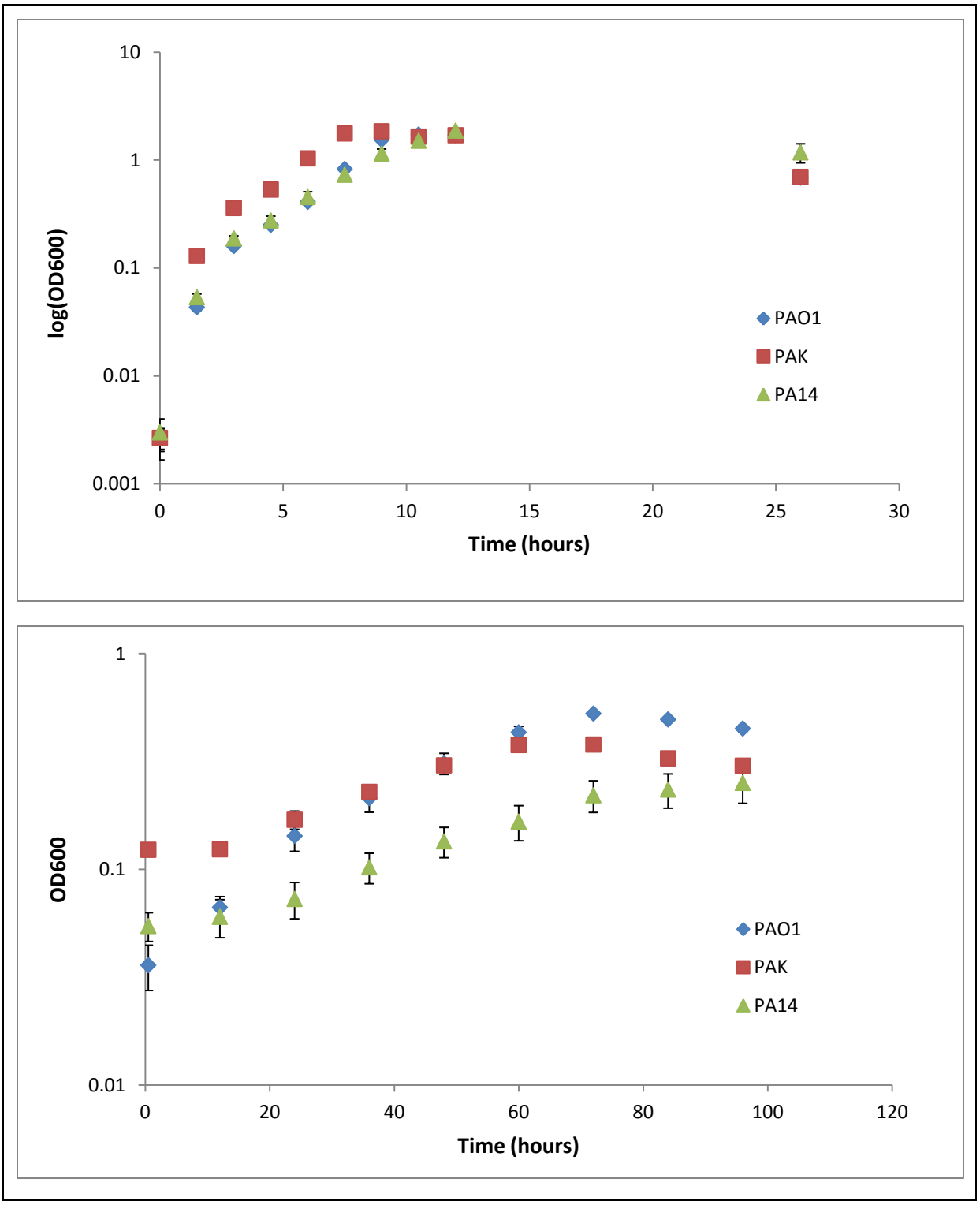

Figure 7. These graphs show the ability of the wild-types PAO1, PAK, and PA14 to utilize glycine as both a carbon and nitrogen source. The graph on top is the strains grown with glycine as the sole nitrogen source, while the graph below is the strains utilizing glycine as a carbon source. Because glycine is a very unfavorable carbon source, the curve took place over 96 hours compared to the 24 hours for glycine as a nitrogen source.

Table 1. All *Pseudomonas* strains used in this study were obtained from the *P. aeruginosa* PAO1 transposon mutant library. The *E. coli* strains were obtained from Invitrogen with the pCR-BLUNT cloning kit.

Strain	Relevant genotype
P. aeruginosa	
PAO1	Wild type
PA14	Wild type
PAK	Wild type
PW5126	PA2449-E03::ISphoA/hah
E. coli	
Top10	Electrocompetent strain
DH5-α	Electrocompetent strain

Table 2. All of the plasmids used in this study. Plasmids code for either kanamycin (Kn^R) or gentamicin (Gn^R) resistance.

Plasmid	Characteristics	Source
pCR BLUNT	*E. coli* cloning plasmid; Kn^R	Invitrogen
pBBR1MCS-5	Broad-host range expression plasmid; Gn^R	
pMTG01	*P. aeruginosa rhlI* inpCR BLUNT	This study
pMTG02	*P. aeruginosa rhlI* in pBBR1MCS-5	This study

Table 3. Oligonucleotides used in this study. Name, sequence, and target gene are given for each oligonucleotide.

Name	Sequence	Target gene
BL439.f		*rhlI*
BL439.r		*rhlI*