VIRULENCE FACTOR PRODUCTION IN *pyrE* MUTANTS OF *Pseudomonas aeruginosa*

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It has been shown previously in our lab that mutations in the pyrimidine pathway reduced the ability of *Pseudomonas aeruginosa* to produce virulence factors. Knockout mutations in *pyrB, pyrC* and *pyrD* genes of the pyrimidine pathway showed that virulence factor production was decreased. Pyoverdin, pyocyanin, hemolysin, iron chelation, motility, and adherence are all considered virulence factors. Here I further investigate the effects of mutations in the pyrimidine pathway by studying a *pyrE* mutant. I studied the effect of the *pyrE* mutation on the production of the above virulence factors. Just like the effect of *pyrB, pyrC* and *pyrD* mutations,the *pyrE* mutation also showed that the bacteria were deficient in producing virulence factors when compared to the wild type. The broader impact of this research would be the possibility of finding drugs that could treat patients infected with *P. aeruginosa* and possibly extend the lives of chronically infected patients with cystic fibrosis.

ACKNOWLEDGMENTS

First of all I would like to thank Dr. O'Donovan for all his support, mentally, technically and for simply being a fatherly figure for me the entire time. If it weren't for his encouragements, I would not be where I am right now. I would also like to thank the lab members for their support and special thanks to Todd for his help. I could come to him any time for any question and he would answer me sufficiently.

A special thanks to my little family, my wife Arwa, and two sons, Omar and Elias. My little family is who I am doing all this for. Thank you Arwa for all the support that you have shown me and continue to show me.

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INTRODUCTION

Pseudomonas aeruginosa is a bacterium well known to cause chronic and acute infections in both humans and animals. It is a gram negative, obligate aerobe, rod shaped bacterium that uses oxygen as a final electron acceptor. It also has the ability to use nitrate as a final electron acceptor when there is a lack of oxygen. *P. aeruginosa* is also known to cause infections in burn patients (6).The burn patients suffer from what is called blue pus. It is called that because of a pigment that is produced called pyocyanin that has a blue color to it. It is also notorious for causing nosocomial infections. That means this bacterium has the ability to replicate in hospitals and resist hospital detergents. It can be a risk for patients that have invasive procedures by being able to adhere onto medical equipment such as catheters(1). Samant et al. in 2002 showed that *Escherichia coli* and *Salmonella enterica* had a decreased ability to replicate in the blood if they contained mutations in the pyrimidine and purine pathways (20). In order for *P. aeruginosa* to survive, they need to be able to synthesize most of their requirements *de novo*. One of the rare nutrients that *P. aeruginosa* needs to scavenge is iron. Human blood has 10^{-18} M of iron (5), while *P. aeruginosa* needs 10^{-6}– 10^{-7} M of iron for their growth (24) . One way to take up the iron is by using siderophores. Siderophores are chemicals released by *P. aeruginosa* to obtain iron from the medium to take it up for its own use. Siderophores are produced as florescent green pigment that is called pyoverdin. Another type of siderophore is pyochelin. However, pyoveridin has a higher

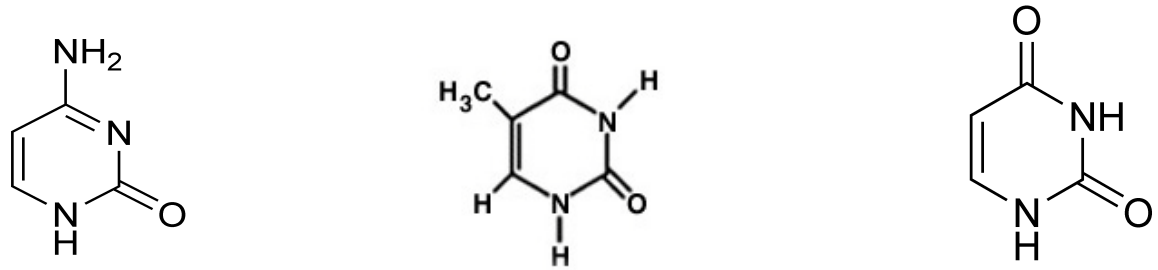
affinity for Fe^{+3} than do other siderophores and is considered to be a potent siderophore for *P. aeruginosa* (25) . When pyocyanin works together with pyochelin and with the support of NADH, they cause tissue injuries by forming reactive hydroxyl and superoxide radicals (4) . Pyocyanin on the other hand has the ability to kill other organisims and even tissue cells. That is because it has the ability to produce reactive oxygen intermediates that can pass through the cell membrane of other microorganisms. These reactive oxygen intermediates also help in killing neutrophils so the bacteria can infect human bodies by lowering the intracellular cAMP(23). *P. aeruginosa* protects itself from the pyocyanin by producing detoxifying agents such as catalase to prevent it from entering the cell (9). *P. aeruginosa* also produces hemolysin which is an enzyme used to breakdown RBCs. The motility abilities of *P. aeruginosa* are also considered to be virulence factors because they need to mobilize themselves to reach their targets and cause disease. These motility abilities are, swimming, swarming, and twitching (19). The iron chelating ability for *Pseudomonas* is also considered to be a virulence factor. The bacterium needs to be able to take up iron in order to grow normally. There are several genes involved in the pyrimidne pathway. *pyrE* is the fifth gene in the pathway and codes for orotate phosphoribosyltransferase. The *pyrE* gene is the target of this study.

The goal of this thesis is to detect the effect of the *pyrE* mutation on the production of virulence factors in *P. aeruginosa*. I have assayed the production of pyocyanin and pyoverdin in both wild type and *pyrE* mutants. Hemolysin, which is used by *P. aeruginosa* to breakdown RBCs, production has also been tested to see the effect

of the *pyrE* mutation. In addition to pigment production and hemolysin, the iron chelating capacity of both wild type and *pyrE* mutants are compared. The motility ability of *P. aeruginosa* was also tested since it is considered to be a virulence factor. All three type of motility, swimming, swarming, and twitching are tested. Since being able to adhere to the host is an important factor for *P. aeruginosa* to cause disease, adherence is tested as well. The results of both wild type and mutant *P. aeruginosa* are compared to each other. This research will be further extended when I complete my PhD in molecular biology.

PROJECT DESCRIPTION

The *de novo* pathway for pyrimidine synthesis has long been demonstrated to be almost exactly the same in all organisms (Fig. 4). The pyrimidines involved in DNA reproduction are cytosine and thymine for DNA and cytosine and uracil for RNA (Fig. 1).



Cytosine Thymine Uracil

FIG. 1. Showing the chemical structure of the three pyrimidines, cytosine, thymine and uracil.

There are nine genes that are known until now to be responsible for coding the enzymes involved in the pyrimidine pathway. The first one in the pathway is *carAB*. The gene encodes the enzyme carbamoylphosphate synthetase which converts 2ATP, HCO_3 and glutamine or NH_3 to carbamoyl phosphate as the first intermediate in the pyrimidine pathway. The second gene in the pathway is *pyrB* which codes for the enzyme aspartate transcarbamoylase. Aspartate transcarbamoylase catalysis the conversion of aspartate and carbamoyl phosphate to carbamoyl aspartate. The gene *pyrC* codes for the enzyme dihydroorotase. This catalyzes the formation of dihydroorotate from carbomoylaspartate while removing a water molecule in the process(2).

Dihydroorotate is then converted to orotate by the enzyme dihydroorotate dehydrogenase which is coded for by the gene *pyrD*. At this step, there is an introduction of an NAD+ and removing of an NADH and a proton. The next step in the *de novo* pathway is the transformation of orotate to OMP. This reaction is catalyzed by the enzyme orotate phosphoribosyltransferase coded by the gene *pyrE*. This reversible reaction happens in the presence of orotate and phosphoribosyl 1-pyrophosphate (PRPP) . The reaction results in the production of a pyrophosphate (PPi) and the 5th intermediate of the pyrimidine pathway orotate monophosphate (OMP; Fig. 2).

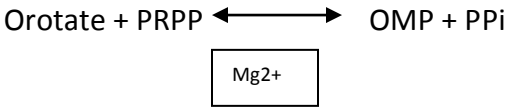


FIG. 2. Showing the conversion of Orotate into OMP.

The enzyme orotate phosphoryltransferase is inhibited by orotate and also by some other analogs, such as 4-uracilsulfonate. The enzyme is highly specific for PRPP and PPi. OMP in this reaction is the first nucleotide to be formed in this pathway. The next step in the pathway is where OMP is converted to UMP. This occurs by a decarboxylation of the OMP catalyzed by the enzyme OMP decarboxylase (Fig. 3). OMP decarboxylase is coded by the gene *pyrF* (14).

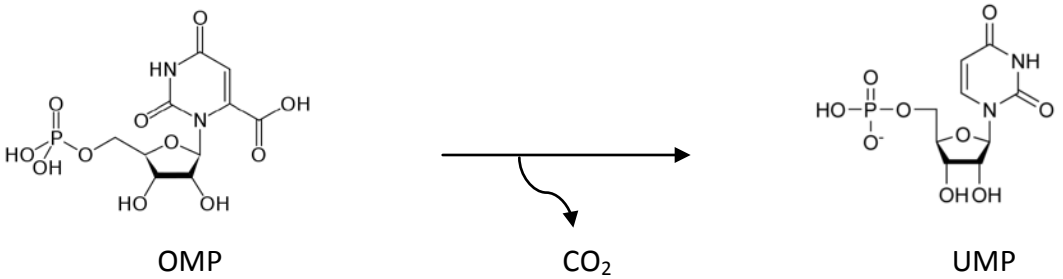


FIG. 3. Showing the conversion of OMP into UMP with the release of CO_2

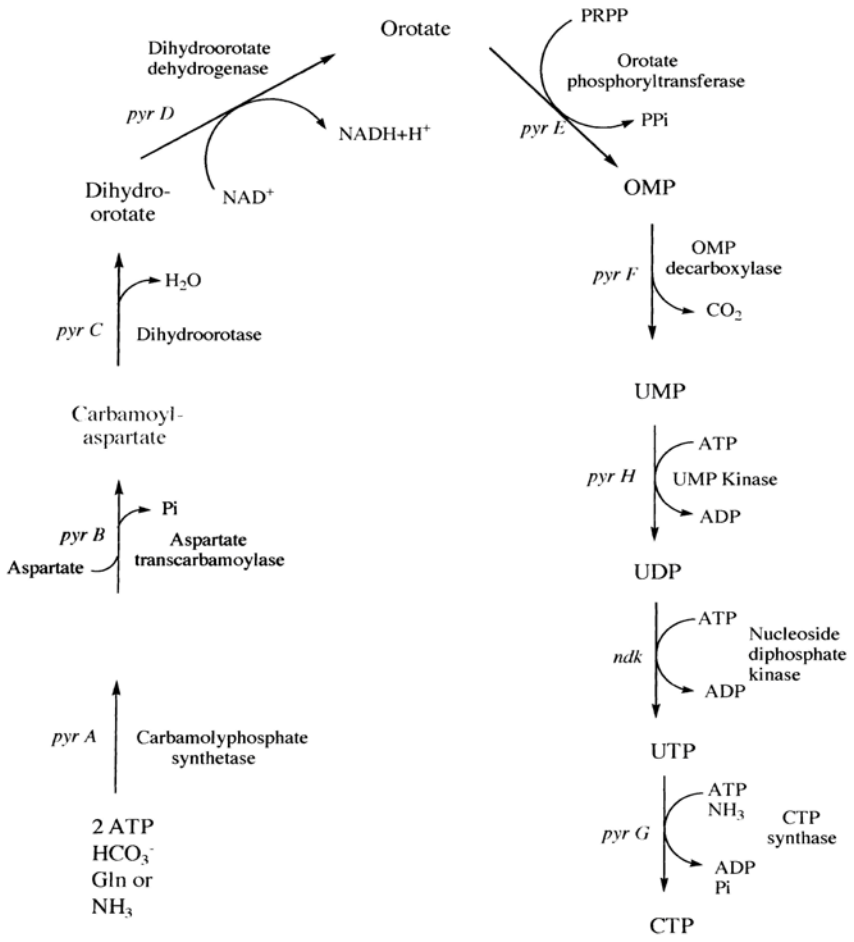


FIG. 4. Showing the pyrimidine *de novo* biosynthetic pathway.

The enzyme UMP kinase converts UMP to UDP by introducing a phosphate from ATP and releasing ADP. This enzyme is coded by the gene *pyrH*. UDP is then converted to a UTP by the enzyme nucleoside diphosphate kinase. This again requires an ATP to donate a phosphate and releasing an ADP. The final step is where the CTP is formed. The enzyme CTP synthase, which is coded by the gene *pyrG*, converts UTP to NH_3 to a CTP and releasing ADP and Pi (15).

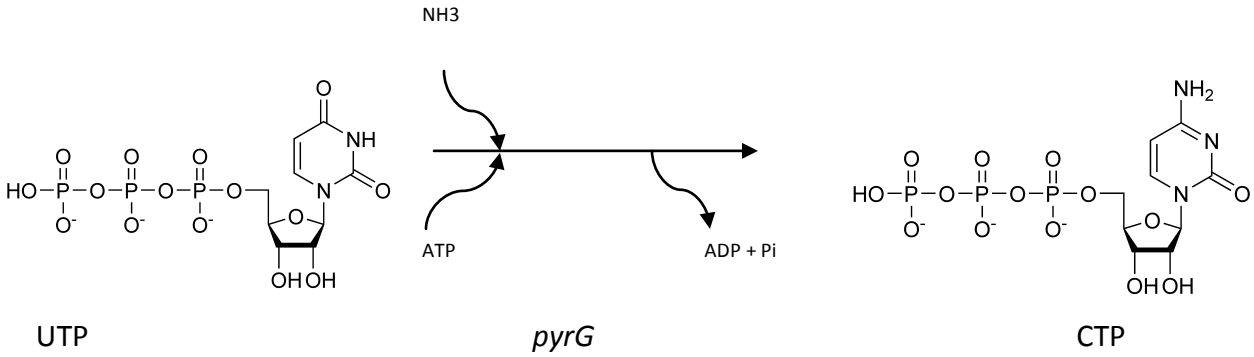


FIG. 5. Showing the conversion of UTP into CTP by the *pyr*G.

MATERIALS AND METHODS

The bacteria used in our experiments were *Pseudomonas aeruginosa* strain PAO1 wild type obtained from ATCC and the PAO0483 *pyrE* mutant obtained from Pseudomonas Stock Center.

Pseudomonas Minimal Medium

The *Pseudomonas* minimal medium (Psmm) used is a modification of the Ornston and Stanier (1966) Psmm(16). It contains 25 ml of 0.5 M KH_2PO_4, 25ml of 0.5 M K_2HPO_4 instead of the Na_2HPO_4 from the original composition. It also contains 10ml of 10% $(NH_4)_2SO_4$, 10ml of concentrated base, and 930ml of ddH_2O to form one liter of the media solution. To make the concentrated base, a few more ingredients were added to 600ml of ddH_2O in order: 7.3g of KOH, 10g of Nitriloacetic acid, 14.45g of anhydrous $MgSO_4$, 3.335g of $CaCl_2$ $2H_2O$, 0.00925g of $(NH_4^+)_6Mo_7O_{24}*4H_2O$, 0.099g of $FeSO_4*7H_2O$ and finally 50 ml of Hutner's Metals 44. The pH was fixed at 6.8 and then ddH_2O was added to get the final solution to a 1000ml volume. The solution was kept at 4^oC after the addition of 100ml toluene to prevent contamination.

The next step was to sterilize the solution in the autoclave at 121^oC and 15 pounds per square inch pressure for 20 minutes. In order to make a plate of Psmm, 430ml of ddH_2O together with 20 grams of the salts(total of 500ml) where autoclaved with 20g of 2% Difco Bacto agar in 500ml of ddH_2O in two separate flasks. After cooling to 65^oC, the two flasks were mixed and the supplements were added, both the

pyrimidine and carbon sources. The plates were then allowed to cool at room temperature overnight, and then refrigerated until used.

King's A and King's B Media

King's A medium is used to enhance the production of pyocyanin in a liquid medium to quantify the amount of pyocyanin produced. This medium contains 2%Bacto peptone (Difco), 0.14% $MgCl_2$, 1%K_2SO_4, and 1% glycerol. King's B on the other hand, is used to enhance the production of pyoverdin(12). It is consisted of 2% protease peptone No. 3, 0.15% K_2HPO_4 (anhydrous), 0.15% $MgSO_4*7H_2O$, and 1% glycerol.

Blood Agar Plates

Blood agar containing soy agar base was provided by BBL. The plates were used to detect the wild type *Pseudomonas* and the mutant strain's ability to breakdown RBCs.

Motility Agar Plates

These are plates designed to test the ability of *Pseudomonas aeruginosa* wild type and mutant to mobilize. It tests their ability to swarm, swim, and twitch as all three methods are considered pathogenic factors. The method used was adapted from Rashid and Kornberg (2000)(18). Swarming plates contained 8g of Difco nutrient broth, 5 grams of dextrose, and 5 grams of Difco Bacto agar in one liter ddH_2O.The medium was then autoclaved and left to cool to 65^{o}C. Then the pyrimidine source of orotate or uracil (40μg/ml) was added. Plates were left to solidify overnight and then used next day.

Swimming medium, on the other hand, contained 10g trypyone, 5g NaCl, and 3g

of Fisher Biotech agarose in a total volume of 1 liter. The plates were then autoclaved and left to cool to 65^{o}C. The pyrimidine sources were then added and plates were left overnight and used the next day.

Twitching plates, however, were made by adding 25g of Difco Luria-Bertani (LB) Miller broth and 10g of Difco Bacto granulated agar in one liter. The plates were then autoclaved and left to cool to 65^{o}C. The pyrimidine sources were then added and plates were left to solidify overnight and used next day.

Chrome Azurol Sulfate (CAS) Agar Plates

CAS is a minimal medium used specifically to study the iron chelating capabilities of the bacteria(21). *P. aeruginosa* uses siderophores to uptake iron into the cell. Once the iron is taken up, the medium changes color from blue to orange. CAS has three main components in which each has a separate preparation process. CAS-HDTMA iron-dye is prepared by adding 0.605g of CAS to 500 ml of ddH_2O and dissolving it completely. The next step is adding 100 ml of Iron (III) solution while still stirring which contains 1mM $FeCl_3$*$6H_2O$ in 10 mM HCl slowly. HDTMA solution was prepared by the addition of 0.729g of HDTMA to 400 ml of ddH_2O. A total of one litter was prepared and sterilized in the autoclave. The medium then has a blue color to it. The next solution to be prepared is the 10X MM9 salt solution. The 10X MM9 salt solution is formed by adding 60g of Na_2HPO4 to 3g of KH_2PO_4 and 10g of NH_4Cl forming a total volume of 1 liter. The solution is then autoclaved for sterilization. To prepare the deferrated acids, 10g of casamino acids were added to 100ml of ddH_2O. To remove any contaminating iron, an

equal volume of 3% 8-hydroxuquinoline in chloroform was extracted. Then the 8-hydroxyquinoline was subsequently extracted with an equal volume of chloroform. Finally the solution was sterilized by using a 0.2 µm filter. The next step was to prepare one liter of CAS agar plates. 30.24 grams of piperazinediethanesulfonic acid (PIPES) was mixed with 100ml of 10X MM9 in 750 ml of ddH_2O. NaOH crystals were then added to get the pH level between 6.15 and 6.18. For the next step, 15g of Difco Bacto were added and then the entire mixture was autoclaved. The solution was left to cool to 65^{o}C and then 1ml of 1 M $MgSO^{4}*7H_2O$, 1ml of 100mM $CaCl_2$, 30ml of 10% deferrated-casamino acids and 100ml of CAS-HDTMA iron-dye complex were added to the solution. The mixture was then gently stirred not allowing any foams to occur and making sure that all the ingredients were dissolved. Finally the carbon and pyrimidine sources were added. Glucose 0.2% or deferrated-succinate 0.4% as a carbon source and orotate 40µg/ml or uracil 40µg/ml as a pyrimidine source before the the solution was poured into plates and left to solidify overnight. The plates were kept at 4^{o}C until used.

Growth Curves

Both peptone tryptic soy broth (PTSB) as rich medium and minimal medium were used to study the growth rate in both wild type and mutant strain. 5ml of bacteria obtained from an isolated culture was used to inoculate the medium. The 5ml was left overnight in a shaker before inoculating the 50ml of the desired medium. The 50ml media were put into 250ml flasks. All media had the same initial optical density and were incubated at 37^{o}C and shaken at 250rpm. At each time point, 100µl of the culture

was removed aseptically and mixed with 900µl of the same medium and read at 600nm. Results were recorded accordingly.

Virulence Factor Analyses

Pyocyanin Quantification Assay

The method of Essar *et al*(1990) was used(7). The bacteria were cultured in King's A agar overnight and then cultured into a Kings'A liquid broth of 50ml the next day. The cultures were then kept in a 37^{o}C incubator and left to grow overnight while shaking at 250rpm. 5ml of the culture was removed and put into a 15ml polystyrene tube and centrifuged at 1300 x g rpm for 25 minutes at 4^{o}C. 1ml of the supernatant was then removed and measured at 600nm. It was then filtered by a 0.45 µm Ambion syringe filter disc and placed in a 15ml polypropylene conical tube. 3ml of chloroform was then added to the supernatant to extract the pyocyanin. After mixing the sample on a vortex, the sample was centrifuged at 1300 x g for 10 minutes. The pyocyanin found in the bottom layer was then transferred into a new 15ml polypropylene tube. To extract the pyocyanin from the chloroform, 1ml of 0.2N HCl was added. After mixing, the sample was then centrifuged at 1300 x g for another 10 minutes. This leads to the formation of a pink color solution which contains the pyocyanin. This top layer is then extracted and transferred into a cuvette and absorbance at 520nm is measured. To calculate the microgram amount of pyocyanin per 5ml, the absorbance reading was multiplied by 17.072 (Kurachi 1958) (13).

Pyoverdin Quantification Assay

The bacteria were grown in Kings'A medium overnight and cultured into a 50ml King'sA lquid medium. The liquid medium was then left overnight at 250rpm and 37^{o}C. 1ml of the medium was then removed and put into a microcentrifuge tube and centrifuged at 10,000 x g for 3-5 minutes at 4^{o}C. The supernatant was then removed and put into a cuvette which was then read at 405nm. Pyoverdin expression was measured at the ratio of A_{405}/A_{600} (Stinzi et al. 2000)(22).

Hemolysin Assay

The bacteria were streaked and grown on blood agar (BA) while the plates were supplied with 40µg/ml uracil as a pyrimidine source. The plates were incubated at 37^{o}C overnight. Isolated colonies were then picked with a sterile tooth pick and transferred to the corresponding BA plate. Photographs were taken at 24 and 48 hours post inoculation.

Iron Chelating Assay

The bacteria were streaked and grown on CAS medium overnight at 37^{o}C while supplied with 40µg/ml uracil as a pyrimidine source. An isolated colony is then chosen and picked with a sterile toothpick. The chosen colony is then inoculated into the corresponding CAS plate and left to grow overnight at 37^{o}C. Photographs were then taken at 24 and 48 hours post inoculation.

Swimming Motility Assay

The bacteria were inoculated into a swim motility plate and incubated overnight

at 37^{o}C and supplied with 40µg/ml uracil as a pyrimidine source. The next day, using a sterile toothpick, colonies were picked and inoculated into the corresponding swimming plates. The plates were sealed with Saran wrap to prevent dehydration and left to grow overnight at 37^{o}C. Photographs were then taken at 24 and 48 hours as above.

Swarming Motility Assay

The bacteria were inoculated into a swarming motility plate and incubated overnight at 37^{o}C and supplied with 40µg/ml uracil as a pyrimidine source. The next day, using a sterile toothpick, colonies were picked and inoculated into the corresponding swarming plates. The plates were left to grow overnight at 37^{o}C. Photographs were then taken at 24 and 48 hours.

Twitching Motility Assay

The bacteria were inoculated into a twitching motility plate and incubated overnight at 37^{o}C and supplied with 40µg/ml uracil as a pyrimidine source. The next day, using a sterile toothpick, colonies were picked and inoculated into the corresponding swimming plates. The toothpicks were pressed until they hit the bottom of the plate to inoculate the bacteria deep into the agar. The plates were left to grow overnight at 37^{o}C. Photographs were then taken at 24 and 48 hours.

RESULTS

It has been shown in our lab previously that *pyrB* and *pyrC* knockout mutations resulted in an inability of *Pseudomonas aeruginosa* to produce its virulence factors normally. This was also shown to be true in *Pseudomonas putida* as well. In *P. putida* , It was shown by Ralli, 2005 that a mutation in *pyrD* showed a pyrimidine auxotroph phenotype but produced normal amount of pigments when supplied with pyrimidine (17). It was important to investigate that in *P. aeruginosa* due to its important involvement in human and animal diseases. In addition to investigating the production of pyocyanin and pyoverdin, we also investigated the production of hemolysins, motility, and Iron binding capacity in *pyrE* mutants and compared them to wild type *P. aeruginosa*.

Growth Curve Analysis

In this study we compared the growth rate of wild type *P. aeruginosa* (PAO1) and *pyrE* mutant (PAO0483). We did growth rate analysis on minimal medium and rich medium. Fresh cultures were used and a spectrophotometer was used to measure the growth rate of the bacteria. On minimal media, when supplied with glucose or succinate as a carbon source and uracil as a pyrimidine source, both PAO1 and PAO0483 showed the same growth rate (Fig. 6).

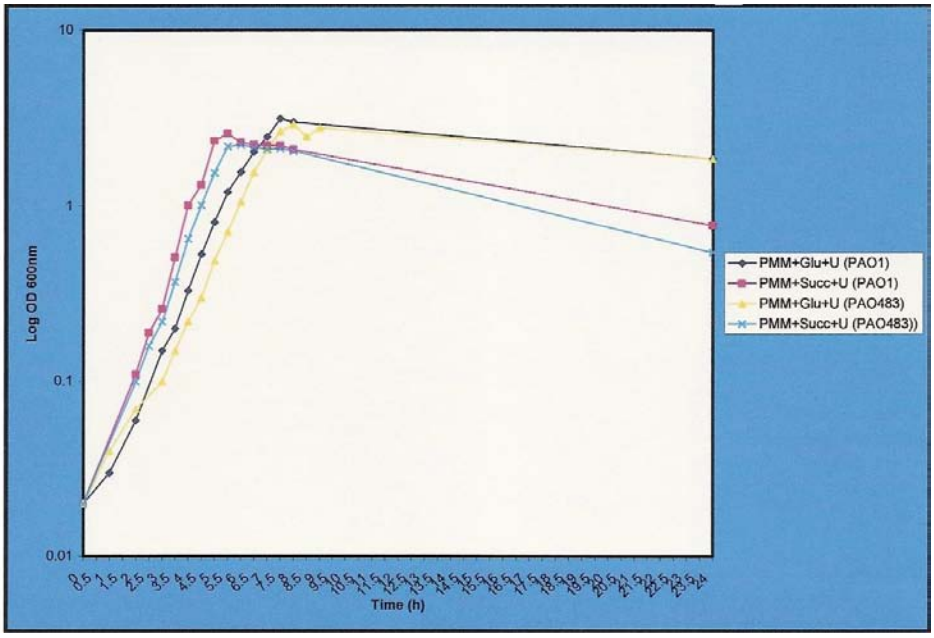


FIG. 6. Growth curves on Pmm for PAO1 and PAO0483 showing growth rates with glucose 0.2% with uracil and with 0.4% succinate and uracil.

When PAO1 and PAO0483 were grown in rich medium, there was no difference between wild type and *pyrE* mutant as well (Fig. 7).

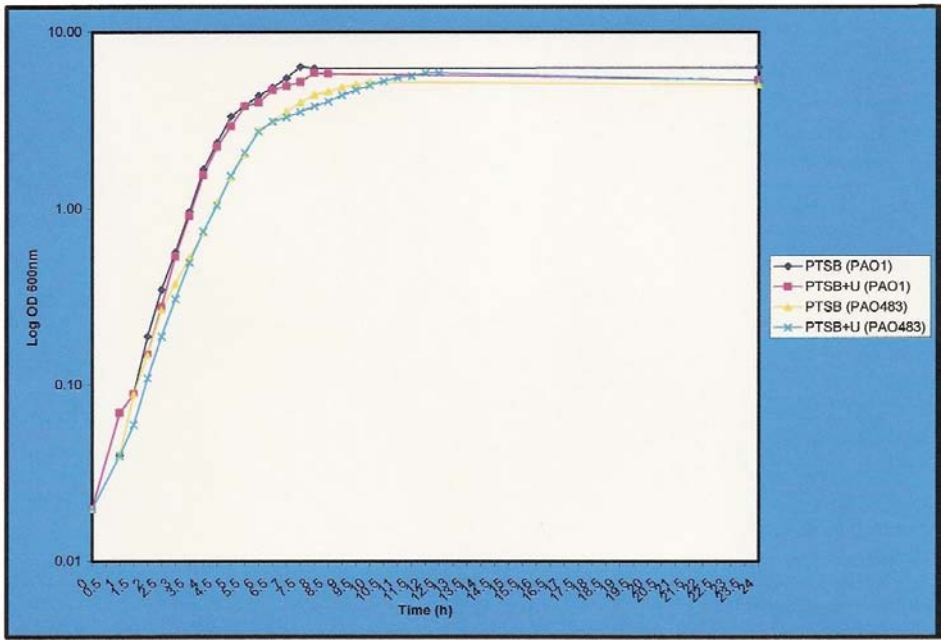


FIG.7. Growth curves showing the growth of PAO1 and PAO0483 on rich medium with uracil and without uracil.

Quantitation of Pigment Production for Both Pyocyanin and Pyoverdin

King's A and King's B media were used to enhance pigment production of both pyocyanin and pyoverdin. The method was described in detail in Materials and Methods. Samples were tested after 24 hours of incubation and shaking at 37^{o}C. When *pyrE* mutant was deprived of uracil, there was a sharp drop in the production of pyocyanin while the wild type showed normal production. When supplied with uracil, PAO0483 showed an increase in pyocyanin production but did not reach the levels of wild type pyocyanin production (Fig 8).

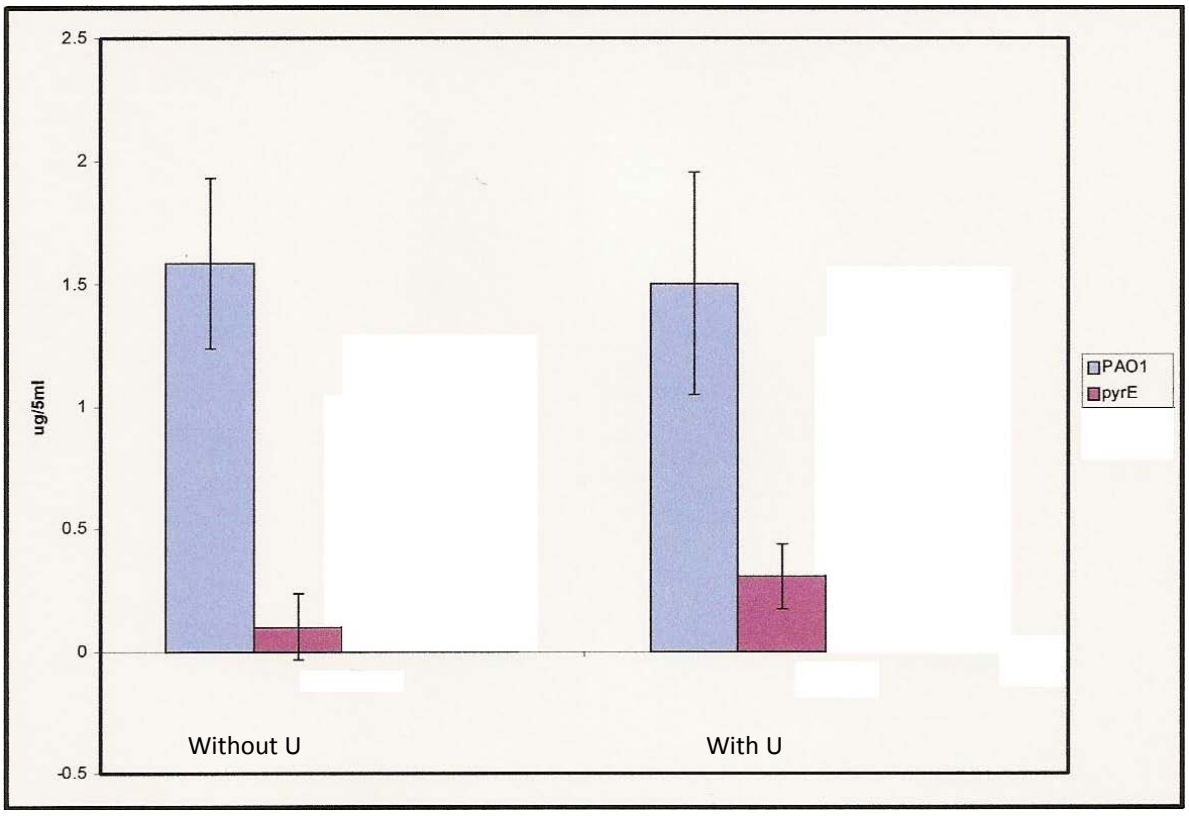


FIG. 8. Amount of pyocyanin produced by wild type PAO1 and *pyrE* mutant strain after 24 hours incubation in King's A medium reaching 600nm optical density.

Pyoverdin production was tested in King's B medium after incubation for 24 hours at 37^{o}C while shaking. PAO1 and PAO0483 showed almost the same amount of pyoverdin production in the absence of uracil. When uracil was added, there was a drop in pyoverdin production for both wild type and the *pyrE* mutant (Fig. 9).

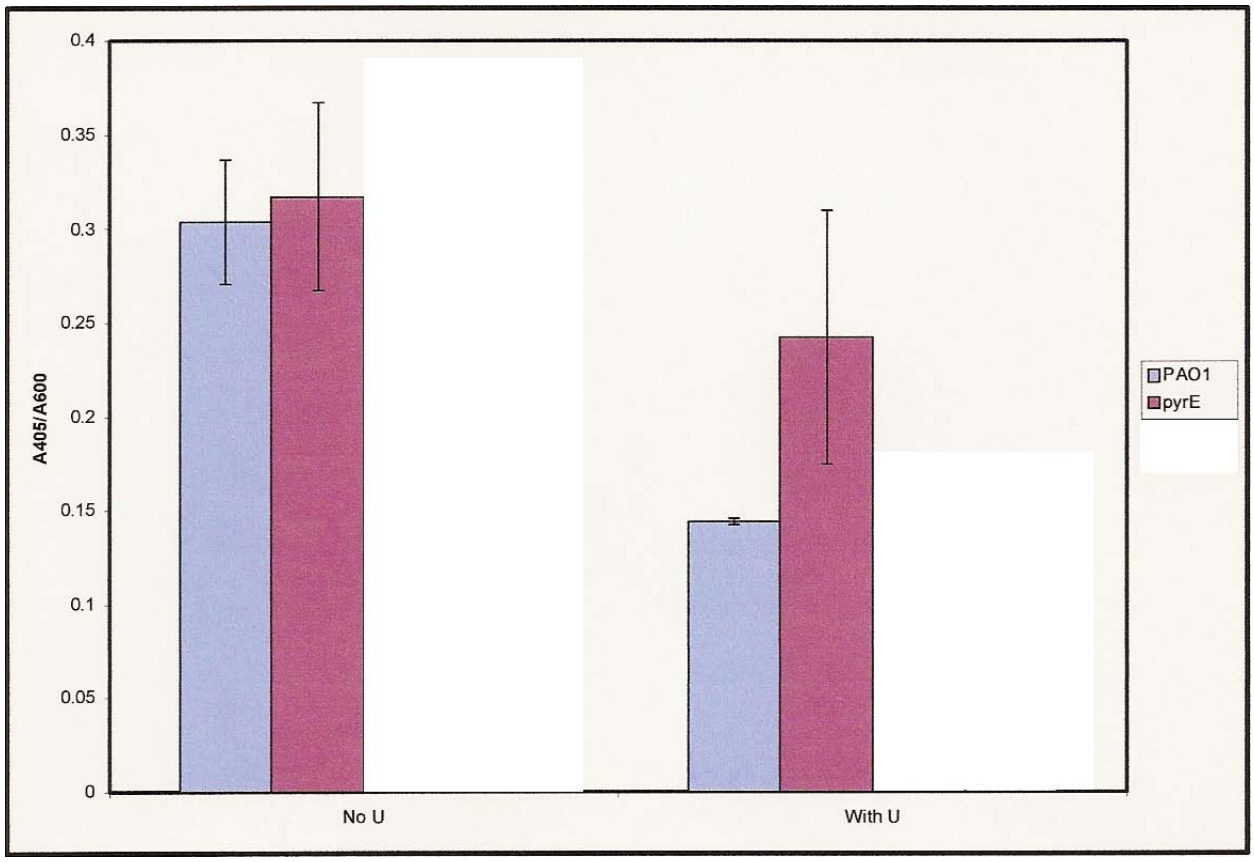


FIG. 9. Amount of pyoverdin produced in wild type PAO1 and *pyrE* mutant after 24 hours incubation on King's B medium and reaching 600nm optical density.

Iron Chelation Analysis

The CAS minimal medium plates were used to analyze the ability of wild type PAO1 and *pyrE* mutants to produce siderophores. Siderophores are the chemicals

produced by the *P. aeruginosa* to acquire iron from the medium. When a siderophore is released, it gives an orange color to the medium. Wild type *P. aeruginosa* is able to grow in the CAS minimal medum while *pyrE* needs uracil for growth. PAO0483 when supplied with uracil, and glucose 0.2% or succinate 0.4% produced larger amount of siderophores than wild type after 24 and 48 hours of incubation at 37^{o}C (Fig. 10, 11).

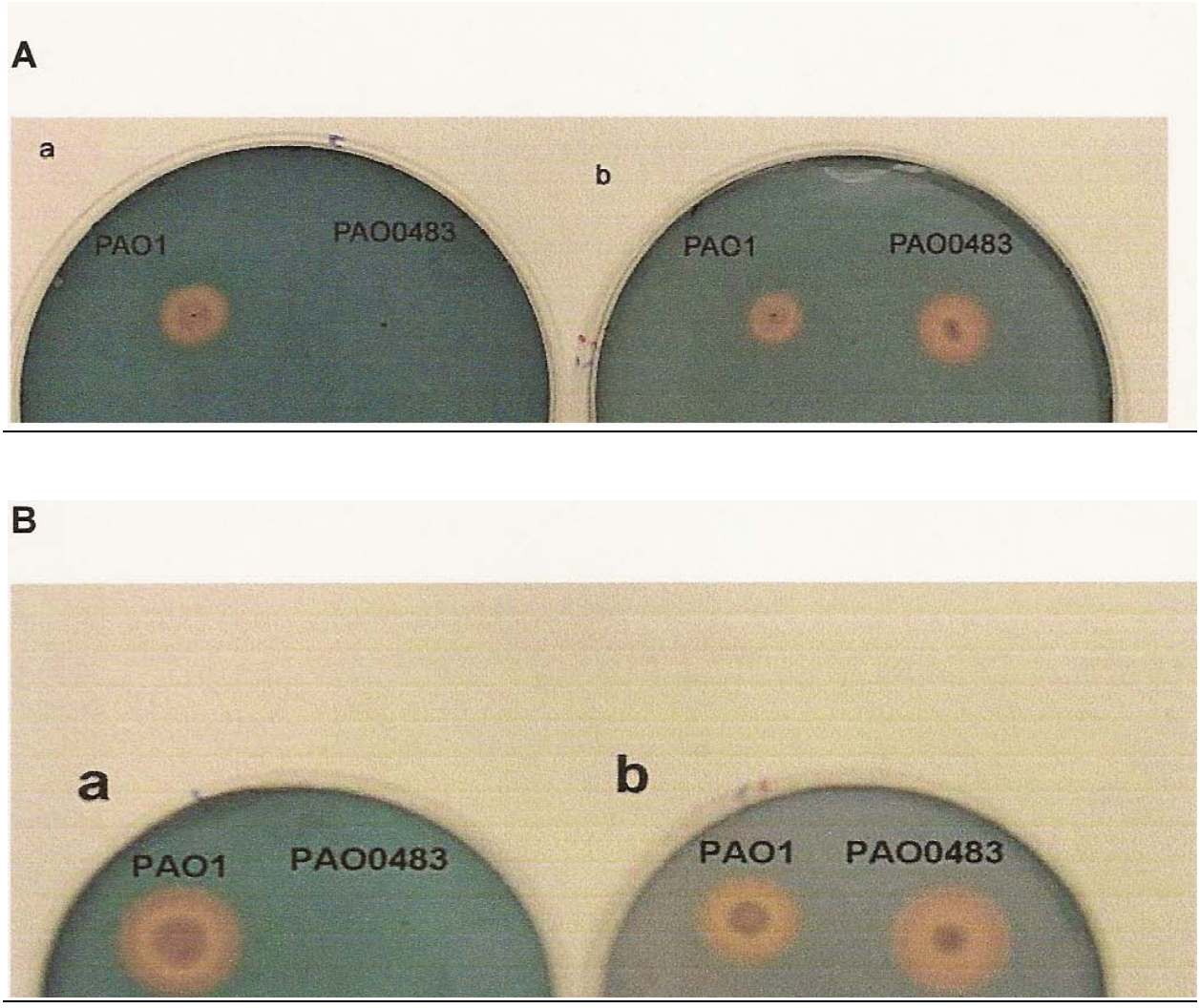


FIG. 10. A is the growth of PAO1 and PAO0483 on CAS (a) without glucose 0.2% and (b) was supplied with glucose 0.2% as a carbon source after 24 hours of incubation to. B is the growth PAO1 and PAO0483 on CAS (a) without glucose and (b) was supplied with glucose 0.2% as a carbon source 48 hours after incubation.

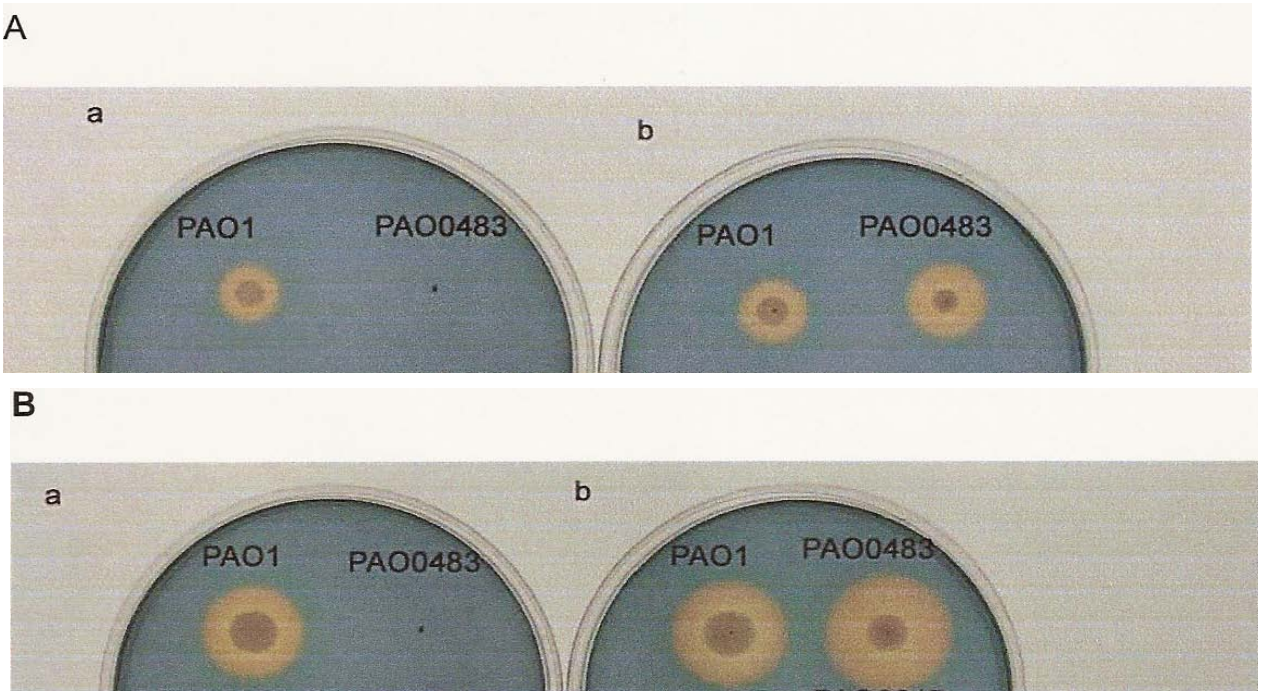


FIG. 11. A is the growth of PAO1 and PAO0483 on CAS (a) without succinate 0.4% (b) was supplied with succinate 0.4% as a carbon source after 24 hours of incubation. B is the growth PAO1 and PAO0483 on CAS (a) without succinate 0.4% (b) was supplied with succinate 0.4% as a carbon source 48 hours after incubation.

Hemolysin Production

Hemolysin is an enzyme produced by *P. aeruginosa* to breakdown red blood cells. The bacteria were grown on blood agar and incubated for 24 and 48 hours. Wild type showed normal hemolysis in the absence of uracil as a pyrimidine source. *pyrE* mutants, on the other hand, showed no hemolysis in the absence of uracil. When uracil was added to the medium, wild type showed normal amount of hemolysis while the *pyrE* mutant showed some hemolysis, but was less than that of the wild type (Fig. 12).

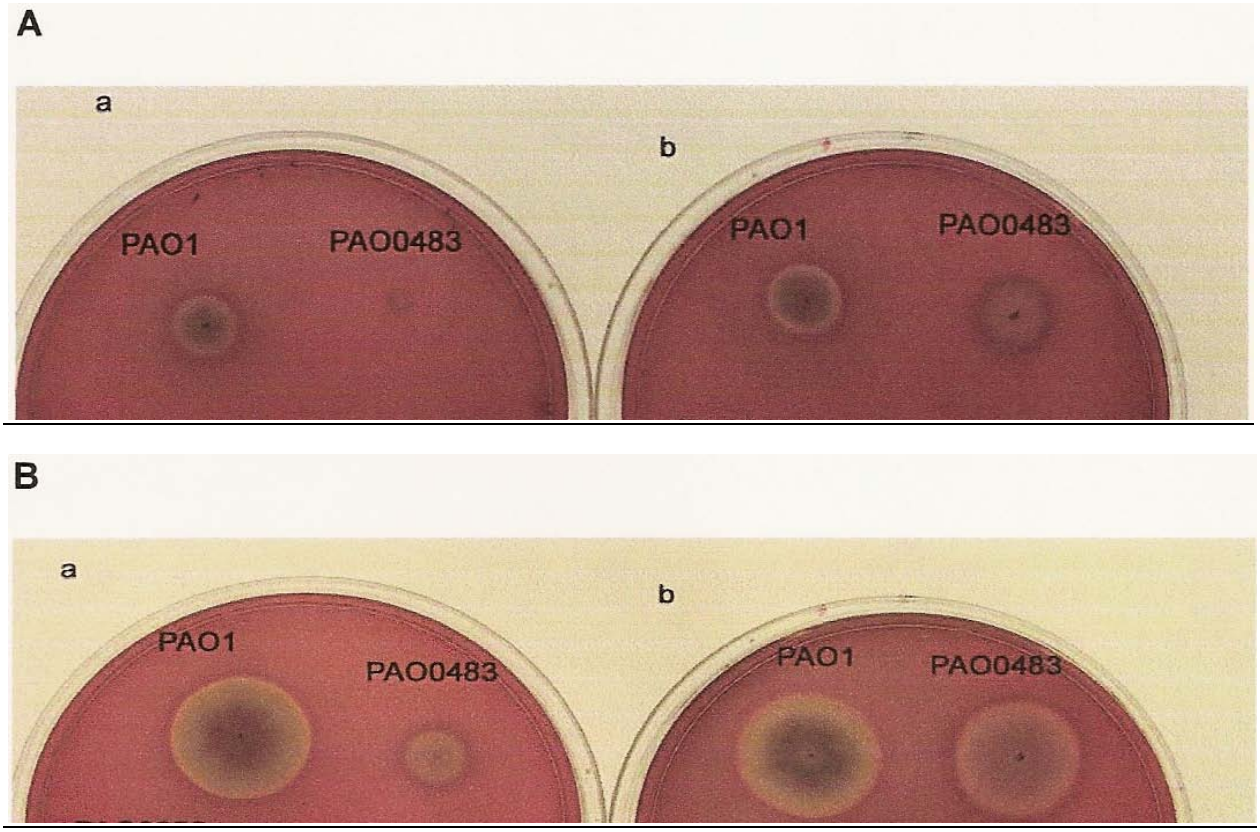


FIG. 12. Ability of PAO1 and PAO0483 to breakdown RBCs with the production of hemolysin on BA plates. The a plate was not supplied with uracil while plate b was supplied with uracil as a pyrimidine source. “A” photo was taken 24 after incubation while “B” was taken 48 after incubation.

Motility Studies

There are three modes of motility in *P. aeruginosa*. All three modes of motility are considered virulence factors because the bacteria need to mobilize in order to reach the target. Swimming and swarming require flagella while twitching requires type IV pili. Pili are not only involved in twitching, but they are also needed for the bacterium to attach to its target host. It is clear that all these factors play a huge role in the pathogenicity of *P. aeruginosa*.

Swimming

In the swimming studies, PAO1 showed normal swimming abilities after 14 hours of incubation with or without uracil. PAO0483 was unable to swim after 14 hours of incubation without uracil. When uracil was added, the *pyrE* mutants were able to swim but not to the extent of the wild type (Fig. 13).

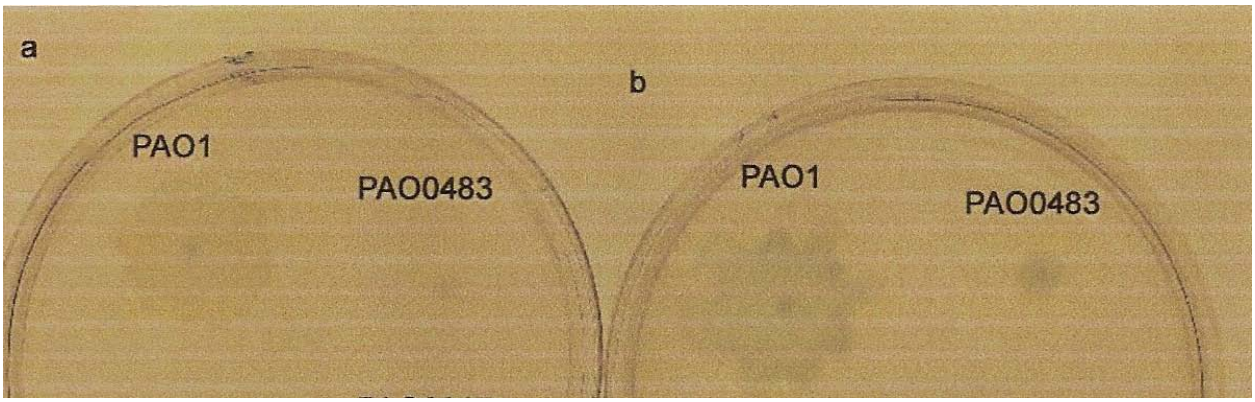


FIG. 13. Swimming ability of both PAO1 and PAO0483 in the absence of uracil (a) and with the presence of uracil (b).

Swarming

PAO1 was able to swarm normally in both the presence and absence of a uracil supplement. However, the POA0483 was unable to swarm after 14 hours of incubation without urcail. When uracil was added, PAO0483 was able to swarm, but less effectively than the wild type (Fig. 14).

Twitching

The twitching ability was studied after 24 hours of incubation at 37^{o}C after inoculation of the bacteria in twitching medium. Wild type showed normal ability to twitch in the presence or absence of uracil. PAO0483 was unable to twitch in the

absence of a uracil supply. When uracil was added to the *pyrE* mutant twitch plate, it was able to twitch but not as efficient as the wild type (Fig. 15)

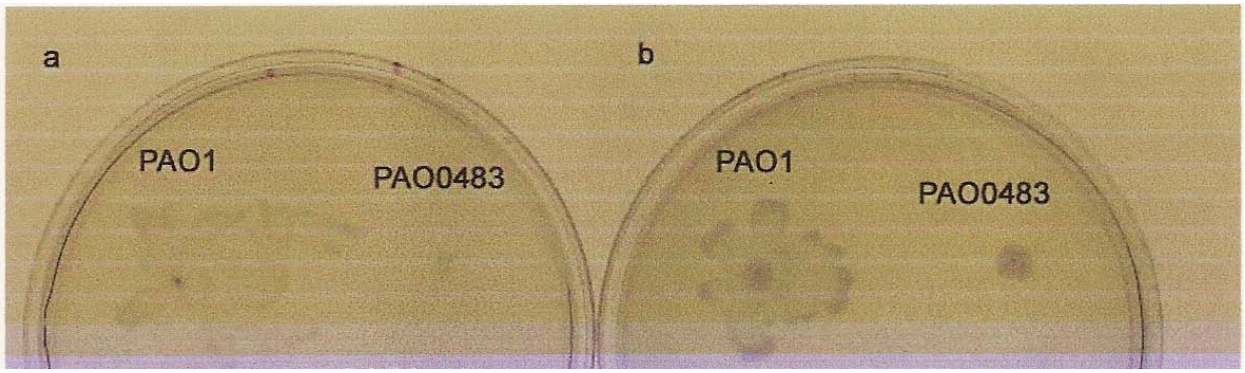


FIG. 14. Swarming ability of both PAO1 and PAO0483 in the absence of uracil(a) and in the presence of uracil(b).

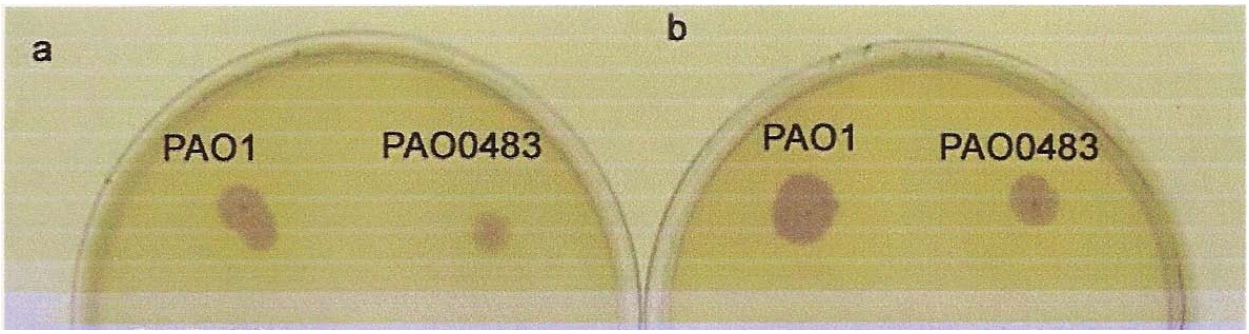


FIG. 15. Twitching ability of both PAO1 and PAO0483 in the absence of uracil (a) and in the presence of uracil (b).

Adherence

On the twitching plate, the agar was washed off with a gentle stream of sterile water. This shows the ability of the bacteria to adhere to the surface of the plate. Adherence is also considered a virulent factor for *P. aeruginosa*. The plate is then stained with crystal violet to show the ability of the bacteria to adhere. Wild type showed a significant amount of adherence on to the plate. PAO0483 was able to adhere

slightly to the plate with both the presence of uracil and without the presence of uracil.

The adherence for the mutant was significantly less than the wild type adherence (Fig. 16).

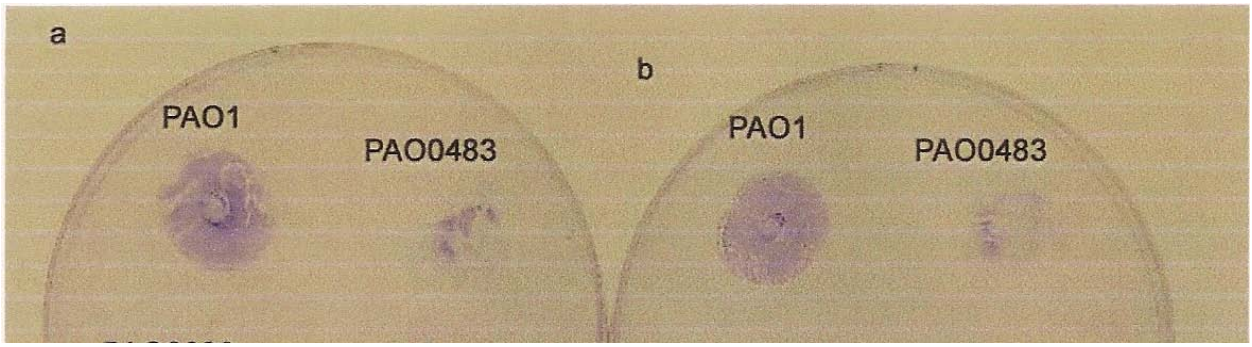


FIG. 16. Adherence ability of both PAO1 and PAO0483 in the presence of uracil (a) and in the absence of uracil. (b) after washing the twitch plate gently with streaming water and staining with crystal violet.

DISCUSSION

I wish to make the following five points as a result of my study:

1) Both wild type and *pyrE* mutant showed the same growth rate when grown in PTSB rich medium. They also showed the same growth rate in minimal medium when the medium was supplied with an external carbon source of succinate 0.4% or glucose 0.2% and uracil as a pyrimidine source.

2) When testing for pigment production, the *pyrE* mutant showed a reduced amount of pyocyanin production. The mutant was able to produce more pyocyanin when supplied with uracil as a pyrimidine source but was less than the amount produced by the wild type. The pyoverdin production, on the other hand, was increased in the *pyrE* mutant when compared to the wild type. Both wild type and *pyrE* mutant had a drop in pyoverdin production when supplied with uracil.

3) Comparing wild type and *pyrE* muntant's iron chelation ability showed that the addition of uracil did restore some the *pyrE* mutant's ability to produce siderophores, but it was not restored to the wild type's level.

4) When testing hemolysin production for both wild type and *pyrE* on BA, wild type is normal while *pyrE* mutant showed a decrease in hemolysin production. Adding uracil to the BA medium increased the amount of hemolysin production in the *pyrE* mutant, but did not restore it to the wild type level.

5) Testing the ability to move in both wild type and *pyrE* mutant showed the *pyrE* mutant to be deficient in its ability to mobilize. All three motility methods, swimming, swarming, and twitching, were impaired in the *pyrE* mutant. The addition of uracil as a pyrimdine source did restore some of the mutant's motility, but was not to the level of the wild type. Interestingly, when testing for adherence on the twitch plate, the *pyrE* mutant showed some degree of adherence at a lesser level than wild type. The addition of uracil did not change the level of adgerence.

These finding are in agreement with those of Isaac and Holloway (1968) where they showed that the addition of an external source of pyrimidine can satisfy the pyrimidine requirement of the mutants(11). These findings also agree with our lab's previous findings. Hammerstein in 2004 knocked out the *pyrB* and showed that there was a decrease in the virulence factor production(8) . Brichta *et al* in 2003 were also able to show the same thing after knocking out *pyrC* from the pyrimidine biosynthesis pathway(3). In 2005, Ralli knocked out the *pyrD* and also showed a decrease in virulence factor production when compared to wild type.

To summarize: mutations in the pyrimidine pathway have been shown to decrease the ability of *P. aeruginosa* to produce virulence factors. Mutations to the *pyrE* gene gave similar results when other genes were mutated in the pyrimidine pathway.

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