The Construction of Triple-Deleted Mutant M18ΔUMS as Cell Factory in Order to Improve PCA Production

Da Yang*, Yawen He [a]

School of Life Sciences and Biotechnology, Shanhghai Jiao Tong University, Shanghai 200240, China

Abstract: Phenazines are secondary metabolites chiefly known for their broad-spectrum anti-microbial property. Phenazine-1-carboxylic acid (PCA) produced by Pseudomonas aeruginosa, a gram-negative bacterium, is an effective biocontrol agent against a number of plant-related pathogens. This experiment aimed to increase the production of PCA by deleting genes UTR, phzM and phzS in Pseudomonas aeruginosa M18, thus rendering the new strain more suitable for commercial use. The triple-deleted mutant M18ΔUMS shows significantly increased performance in PCA production compared to wild-type M18 strain. The new strain should be more applicable for commercial usage. However, M18ΔUMS exhibits a decreased growth rate compared to M18, indicating a inhibitory effect caused by the excess PCA.

1 INTRODUCTION

1.1 Pseudomonas Aeruginosa

Psedomonas aeruginosa is a gram-negative, rod-shaped and mono-flagellated bacterium. It can colonize a wild range of environment including soil, water, plants, animals and human. Its ubiquitous presence is partially due to its ability to catabolize a broad-spectrum of organic molecules. P. aeruginosa is also an opportunistic human pathogen that frequently infects individuals with compromised immune system. Infection commonly occurs among patients with cystic fibrosis, cancer, or AIDS.

1.2 Phenazines

Phenazines designates to a group of nitrogen-containing, secondary metabolites. They are chiefly known for their broad-spectrum capacity of suppressing various plant-related pathogens. Up to 2017, it has been identified that there are around 100 different phenazines derivatives and an addition of 6000 compounds that contain phenazines (Guttenberger, 2017). Biologically, they function as both antibiotics and signaling molecules within bacterials. In addition to their agricultural application as antibiotics for plants, phenazines also attract the attention of researchers from areas including microbial fuel cell production, environmental sensing and antitumoractivity (Du, 2013). In general, phenazines are secreted by a number of bacterial genera including, notably, Psedomonas strains (Mavrodi, 2010). While it is feasible to produce phenazines through chemical synthesis, the method is complicated by its low yield and toxic byproducts such as aniline, azobenzoate, lead oxide or o-phenylenediamine (Cheluvappa, 2014). Thus, biosynthesis is employed in many occasion instead. By uilitizing recombinant DNA technologies, several strains of psedomonas can be engineered into cell factories that yield phenazines in large quantity (Bilal, 2017). Among all phenazine-producing pseudomonads, phenazine biosynthesis is chiefly controlled by the gene cluster phzABCDEFG (Blankenfeldt, 2013). Further cross-species examinations uncover that phzB, phzD, phzE, phzF, and phzG are conserved in all phenazine-producing pseudomonads (Mavrodi, 2010). Accessory genes, including phzO, phzH, phzM and phzS, that flank the core biosynthetic genes are found within most phenazine-producing bacteria and these genes are responsible for many phenazine derivatives (Chin-A-Woeng, 2001).

1.2.1.As antibiotics

Phenazines are known for their anti-microbial property. They have excellent antifungal capacity. For instance, phenazine antibiotics produced by Pseudomonas are proven to be an effective biocontrol against Fusarium wilt, a lethal fungal infection of planets, on a wild range of crops (Anjaiah, 1998). Other than fungus, phenazines are known to control a variety of plant pathogens including Streptomyces scabies, a bacterial that causes corky lesions (Arnau, 2015), Pythium spp., which causes infection among germinating tomatoes (Gurusiddaiah, 1986), Phytophthora infestans, an oomycete that causes serious potato loss worldwide (Morrison, 2016) as well as many other pathogens.

*Corresponding author: Yangmike24@gmail.com
[a]yawenhe@sjtu.edu.cn

1.2.2.As signaling molecules

Two categories of Psedomonas can be established according to the copy number of the core phz gene clusters. Strains such as P. fluorescence 2–79, P. aureofaciens 30–84 and P. chlororaphis PCL1391 belong to Psedomonas that possess only one set of core phz gene cluster. These strains prove to be excellent Plant growth-promoting rhizobacteria. Another category of Psedomonas, on the other hand, possess two sets of nearly identical core phz gene clusters: phzA1-G1 and phzA2-G2. P. aeruginosa PAO1 and P. aeruginosa M18, belong to this group. It is discovered that among Pseudomonas sp. M18, phzA2-G2 only produces fairly small amount of PCA while the majority of PCA is produced from phzA1-G1. Moreover, the transcription of phzA1-G1 is in fact induced by the small amount of PCA produced from phzA2-G2. In this case, phenazines act as both the product and the signaling molecule (Li, 2011).

1.2.3.Phenazine Derivates

Phenazine-1-carboxylic acid (PCA) designates to an aromatic carboxylic acid. It substitutes a carboxy group at C1 position of phenazine molecule. PCA is an effective antibiotic agent and it has been used as the main ingredient in an environment friendly fungicide named Shenqinmycin, which is registered by the ministry of Agriculture of China in 2011 (Du, 2015). The 1% Shenqinmycin solution has been proven efficacious against Rhizoctonia solani and Fusarium oxysporum among a variety of rice, wheat and vegetable (Du, 2015).

Pyocyanin (PYO) is a metabolite produced from Pseudomonas. It is involved with a variety of important cellular activities. In P. aeruginosa, it is a virulence factor ad well as a signaling molecule for quorum sensing. It is also responsible for the blue-green colouration in P. aeruginosa. Moreover, it sometimes functions as a antimicrobial agent.

Phenazine-1-carboxamide (PCN) is an aromatic amide. It substitutes a carbamoyl group at C1 position of phenazine molecule. Like PCA, it is a strong antibiotic against fungal pathogens like Fusarium oxysporum (Chin-A-Woeng, 1998). It is also know to lessen the effect of root disease caused by Gaeumannomyces graminis var. tritici (Daval, 2011).

1.3 Phenazine Biosynthetic Pathway in M18 Phz Cluster

A comprehensive graph of Phenazine biosynthetic pathway from “Engineering Pseudomonas for phenazine biosynthesis, regulation, and biotechnological applications: a review” is presented below (Bilal, 2017).

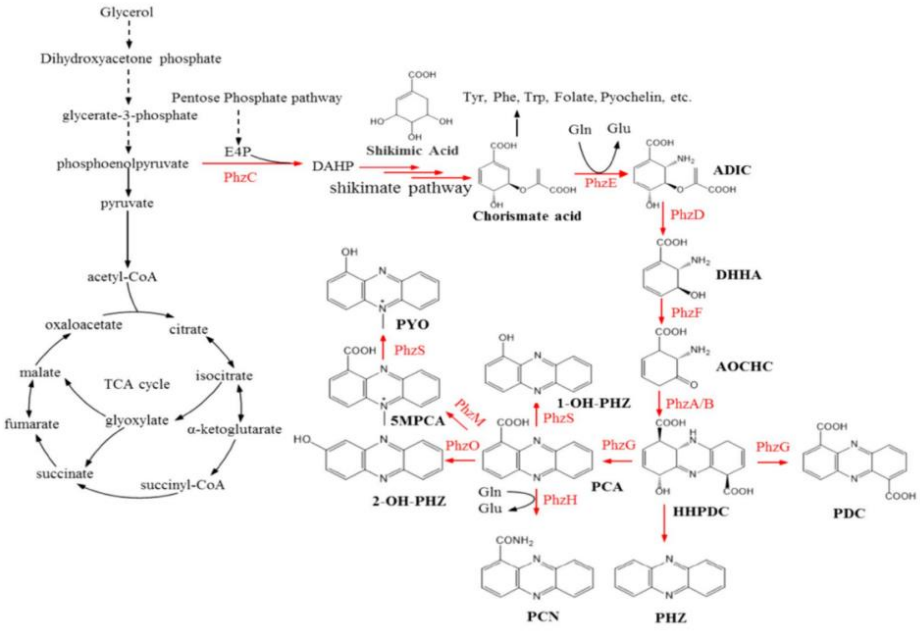

Figure 1: The proposed PCA biosynthetic pathway (Bilal, 2017)

As illustrated in Figure. 1, Phenazines biosynthesis starts with Shikimate pathway. At first, phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) is condensed in order to form 3-deoxy-d-ara-bino-heptulosonate-7-phosphate (DAHP). A three-step enzymatically catalyzed reactions took place and transform DAHP into chorismate, which can be transform into a wild variety of three-ringed aromatic core phenazine structures. Through a series of phz genes (A1-G1, or A2-G2), chorismate is eventually transformed into PCA. Then a number of accessory genes that flank the phz genes transform PCA into other phenazine derivates in accordance to a number of factors. These accessory genes includes phzO, phzH, phzM and phzS. PhzM can transform PCA into 5MPCA, which in turn transformed into PYO by phzS. PhzS alone can change PCA into 1-OH-PHZ while phzH can produce PCN from PCA.

1.4 Aim of the Study

The aim of the study is to increase the PCA production of Pseudomonas aeruginosa M18. Firstly, the object is to knock out UTR gene, which help regulating the production of PCA. PCA production is expected to increase without functioning regulatory system. Then, the study aim to delete phzS and phzM. These accessory genes are responsible for further conversion of PCA into PYO and other phenazine derivates. Their deletion can result in PCA accumulation. Thus, the goal is to construct the triple-deleted mutant M18ΔUMS that yield greater amount of PCA than the wild type. As mentioned before, the antibiotic quality against plant pathogens make PCA an ideal biocontrol agent for commercial use in agriculture. This study aims to further improve the efficiency of PCA production and explore more cost-effective, safer methods of biosynthesis.

2 MATERIALS & METHODS

2.1 Strains and Plasmids used in this Study

Pseudomonas aeruginosa M18 (M18) is a strain isolated from the rhizosphere soil of sweet melon in Songjiang, Shanghai, China.

1-aminocyclopropane-1-carboxylate (ACC) is used as a sole nitrogen source.

E.coli DH5a is a strain of E.coli commonly used for cloning and related application. It is quite versatile and have a wild range of application.

E. coli S17-1lpir contain the pir gene. It is commonly used as the host strain that houses the transposon vector DNA through biparental mating.

pk18 mobsacB Plasmid pk18 mobsacB is a cloning vector that can mobilize into a variety of gram negative and gram positive bacteria. It can integrate into host chromosome via homologous recombination. Medium containing 10% sucrose can facilitate selection against bacterial containing this plasmid.

2.2 Bacterial Growth

M18 is incubated on LB nutrient medium with Spectinomycin antibiotic at 28ºC. LB nutrient medium is created by a mixture of 5g yeast extract, 10g tryptone, 5g NaCI and a 1 liter of distilled water.

2.3 DNA Manipulation

M18 is incubated on LB nutrient medium with Spectinomycin antibiotic at 28ºC. LB nutrient medium is created by a mixture of 5g yeast extract, 10g tryptone, 5g NaCI and a 1 liter of distilled water.

2.3.1. The Construction of M18ΔUTR

A. Design Primers

Firstly, two pairs of primers, UTR F1/R1, UTR F2/R2 are designed to knock the UTR sequence. Then, genome is extract from the strain Pseudomonas aeruginosa M18.

B. PCR amplification

UTR-1 and UTR-2 are amplified through polymerase chain reaction (PCR) according to the following table and procedure. DNA is purified afterward.

PCR Sequence: 95˚C 5min, (95˚C 30s, 55˚C 40s, 72˚C 40s)*25, 72˚C 5min, 12˚C infinity

Table.1: Materials for PCR amplification

Materials	ul
10x Taq buffer with Mg2+	2.5*8=20
Taq	0.15*8=1.2
dNTP (2.5mM)	5*8=40
UTR F1/F2	1
UTR R1/R2	1
M18 delta u geomic DNA	0.5*8=4
ddH2O	16*8=128

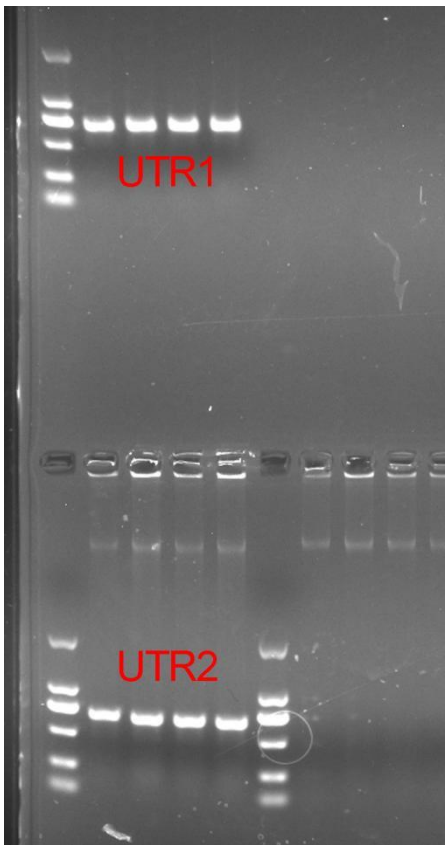

Figure 2: Agarose gel analysis to show the PCR products

As shown in Figure. 2, 4 liens above are UTR1 and 4 liens below are UTR2.

C. Fragment Alignment

UTR-1 and UTR-2 are aligned by performing another PCR. DNA is purified afterward.

PCR Sequence: 95˚C 5min, (95˚C 30s, 55˚C 40s, 72˚C 1min30s)*25, 72˚C 5min, 12˚C infinity

Table. 2 Materials for fragment alignment

Materials	ul
10x KOD buffer	5*8=40
KOD	0.25*8=2
MgSO4	4*8=32
dNTP (2.5mM)	5*8=40
UTR F1	2*8=16
UTR R2	2*8=16
UTR1 product	0.25*8=2
UTR2 product	0.25*8=2
ddH2O	33*8=264

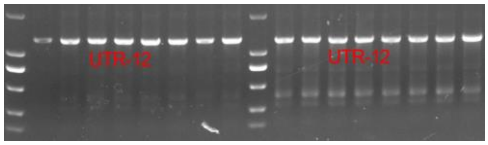

Figure 3: Agarose gel analysis to show the fusion PCR products

As shown in Figure.3, all 16 lines are UTR-12.

D. Enzyme digestion

The aligned UTR fragment are treated with enzyme EcoRI and XbaI, then placed in 37˚C water bath for 4 hours, followed by 65˚C water bath for 10 min.

E. Plasmid digestion

UTR fragment is digested with plasmid Pk18 mobsacB (Ecor-, Xba) and placed in 4˚C refrigerator over night.

F. Plasmid transformation and bacterial colony PCR

E.coli DH5a and the 5 ul product are mixed waited upon for 30 minutes while keeping E.coli DH5a on ice for all time. Then the mixture is placed in 42˚C water bath for 90s and return it to ice for 2 min. On Clean Bench, 800 ul liquid LB nutrient medium is added to the mixture. Then the mixture is put in 37˚C, 2000 rpm shaker for 1h. 100 ul liquid is extracted from the mixture and plastered on petri dish with LK nutrient medium. Then the petri dish is left in 37˚C shaker to incubate overnight. Bacterial colony PCR is performed according to the following table and procedure in order to confirm the the success of plasmid conjugation.

PCR Sequence: 95˚C 5min, (95˚C 30s, 55˚C 15s, 72˚C 1min30s)*25, 72˚C 5min, 12˚C infinity

Table. 3 Materials for bacterial colony PCR

Materials	ul
10x Taq buffer with Mg2+	2.5*32=80
Taq	0.2*32=6.4
dNTP (2.5mM)	2*32=64
M13F	0.5*32=16
M13R	0.5*32=16
ddH2O	19.3*32=617.6

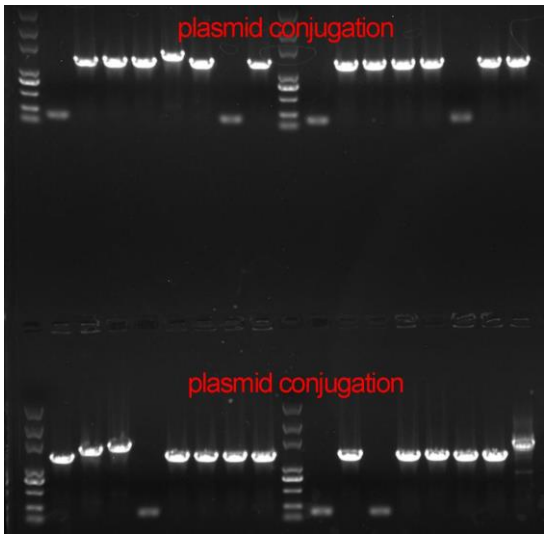

Figure 4: Agarose gel analysis to show the plasmid conjugation

As shown in Figure.4, bright lines in the middle represent successful plasmid conjugation.

G. Gene sequencing

Then the samples are sent for gene sequencing. Plasmid is extracted and transformed into E. coli S17-1lpir.

H. Plasmid transformation into E. coli S17-1lpir

E. coli S17-1lpir and the 1 ul product are mixed and waited upon for 30 minutes while keeping E. coli S17-1lpir on ice for all time. Then the mixture is placed in 42˚C water bath for 90s and on ice for 2 min. On Clean Bench, 800 ul liquid LB nutrient medium is added to the mixture. Then put the mixture in 37˚C, 2000 rpm shaker for 1h. 100 ul bacterial liquid is extracted from the mixture and plastered on petri dish with LK nutrient medium. Then left the petri dish to incubate at 37˚C overnight. Next, 10 ml LB and 5 ul Kanamycin are added to conical flask, then one colony is selected on the petri dish and dipped in the flask using inoculating stick. Then the flask is left in 37˚C shaker to incubate overnight. 8 lines are draw with the bacterial fluid in conical flask on a new petri dish with LS nutrient medium. The petri dish is incubated at 37˚C overnight. 10 ml LB and 5 ul Kanamycin are added to the conical flask, then one line is scratched on the petri dish and dipped in the flask using inoculating stick. Then the flask is left in 37˚C shaker to incubate overnight.

I. Preparing M18

Firstly, Pseudomonas aeruginosa M18 is retrieved from refrigerator, then the bacterial fluid is plastered on petri dish with LS nutrient medium. It is incubated at 28˚C overnight. 8 bacterial colonies are selected on the incubated petri dish. 8 lines are draw on a new petri dish with LS nutrient medium, the dish is incubated at 28˚C overnight. 10 ml LB and 5 ul Kanamycin are added to the conical flask, then one line is scratched on the petri dish and dipped in the flask using inoculating stick. Then the flask is left in 28˚C shaker to incubate overnight.

J. Mating

1 ml of Pseudomonas aeruginosa M18 and 1 ml of E. coli S17-1lpir are extracted and placed in separate 1.5 ml plastic tubes. The tubes is centrifuged at 5000 rotation,

25˚C for 2 minutes. Then 1 mL LB is added and mixed after removing the liquid. The process is repeated once. 600 ul LB is added to E. coli S17-1lpir and 300 ul LB to Pseudomonas aeruginosa M18, they are mixed in one tube and the bacterial liquid is dipped on a petri dish with LK nutrient medium. The petri dish is dried and incubated at 28 ˚C for 6-12 hours.

K. 1st round of selection

1 ml of PBS buffer is mixed with 2 loops of colonies. Then two dilution (3* and 10*) are made. 100 ul liquid is took from each dilution and plastered on petri dishes with LSK nutrient medium. The dishes are incubated at 28˚C for 36 hours. 8 colonies are selected from the petri dish 3* dilution and 8 lines are draw on a new petri dish. Incubate the new petri dish at 28˚C for 1 day.

L. 2nd round of selection and bacterial colony PCR

2 loops are scooped from each lines and mixed with 1 ml PBS buffer. Then 2 dilutions (10^-2, 10^-3) are made. Each dilution is plastered on a petri dish with LBS nutrient medium. They are incubated at 28 ˚C for 36 hours. Two petri dishes are prepared with LK and LS nutrient medium and the following markings. Colonies are selected from the petri dish with 10^-3 dilution and draw a short line beneath each marks. Both dishes are incubated at 28˚C for 1 day. The picture below shows the result.

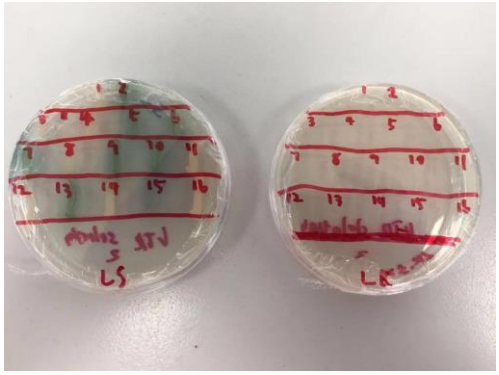

Figure 5: Colonies in the selection plates

As shown in Figure.5, number 2,5,6,7,9,10,13,15 show positive results. Colonies 1-15 on LS plate are chosen for bacterial colony PCR. Steps are performed according to the following table and procedure in order to confirm the the existence of M18ΔUTR.

PCR Sequence: 95˚C 5min, (95˚C 30s, 55˚C 40s, 72˚C 40s)*25, 72˚C 5min, 12˚C infinity

Table. 4 Materials for bacterial colony PCR

Materials	ul
10x Taq buffer with Mg2+	2.5*16=40
Taq	0.2*16=3.2
dNTP (2.5mM)	2*16=32
UTR-F1	0.5*16=8
UTR-F2	0.5*16=8
ddH2O	19.3*16=308.8

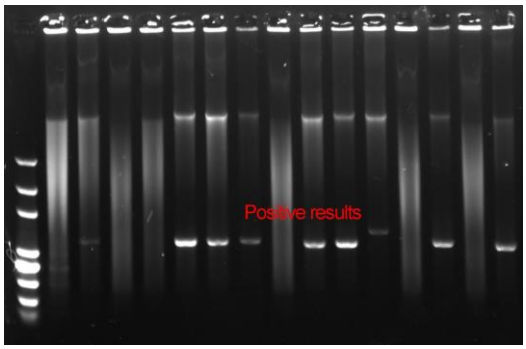

Figure 6: PCR analysis to identify the deletion mutants

As shown in Figure.6, lines at the lower positions are positive. Number 5,6,7,9,10,13,15 show positive results with the line at 2 being too faint.

The colonies with positive results (which are 5,6,7,9,10,13,15) are selected. 10 ml LB and 5 ul Kanamycin are added to the conical flask, then the selected colonies are scratched and dipped in the flask using inoculating stick. Then left the flask in 28˚C shaker to incubate overnight.

The constructed M18ΔUTR are preserved by adding 500 ul bacterial fluid and 500 ul glycerinum in a 1.5 ml test tube, briefly showered by liquid nitrogen. The frozen test tube is place within refrigerator.

2.3.2.phzM knockout

The general procedure is the same as UTR knockout. Two pairs of primers, phzM F1/R1, phzM F2/R2 are designed to knock the phzM sequence. Then, genome is extract from the strain M18ΔU. In the following steps, taq is used in PCR amplification, KOD is used for fragment alignment, and enzymes EcoRI and BamHI are used in enzyme digestion. Pk18 mobsacB (Ecor-, BamH-) is constructed for plasmid digestion. Lastly, phzM-F1 and phzM-F2 are used in bacterial colony PCR. The constructed M18ΔUM is preserved in a 1.5 ml test tube showered by liquid nitrogen. The frozen test tube is place within refrigerator.

2.3.3.phzS knockout

The general procedure is the same as UTR knockout. Two pairs of primers, phzS F1/R1, phzS F2/R2 are designed to knock the phzS sequence. Then, genome is extract from the strain M18ΔUM. In the following steps, taq is used in PCR amplification, KOD is used for fragment alignment, and enzymes EcoRI and BamHI are used in enzyme digestion. Pk18 mobsacB (Ecor-, BamH-) is constructed for plasmid digestion. Lastly, phzS-F1 and phzS-F2 are used in bacterial colony PCR. The constructed M18ΔUMS is preserved in a 1.5 ml test tube showered by liquid nitrogen. The frozen test tube is place within refrigerator.

3 RESULTS

3.1 M18 Colony phenotypes/colors

M18 appear to be yellow. M18ΔU was deep blue/green, PYO is responsible for this coloration. It can be inferred that a great amount of PYO is produced once UTR is deleted. M18ΔUM was also blue/green. M18ΔUMS appear to be gold, indicting PYO is no longer present once genes (phzM, phzS) are both knocked out.

3.1.1.Rates of Growth according to OD

In order to measure rate of growth for strain each strain, first 500 ul bacterial liquid is added to two 1.5 ml test tubes respectively. Then they are centrifuged at 12000 rotation for 5 minutes, the liquid is discarded and 500 ul water is added. Solution is mixed well. 100 ul solution is moved to a new 2 ml tube, then 900 ul water is added and the OD value is measured. This process is repeated for M18, M18ΔU, M18ΔUM, M18ΔUMS.

Table. 5 Initial OD values of M18 strains

M18	M18ΔU	M18ΔUM	M18ΔUMS
0.571A	0.592A	0.567A	0.491A
0.562A	0.610A	0.564A	0.491A

Formula A=OD*(x+2) is used to calculate x, which is the volume of ppm. “OD” beng the average for each strain and times it by 10. “A” being the volume of bacterial liquid added, in this case, is 2 ml.

The calculated ppm value being:

Table. 6 ppm of M18 strains

M18	M18ΔU	M18ΔUM	M18ΔUMS
9.33 ml	10.02 ml	9.31 ml	7.82 ml

Then 50 ul Spectinomycin and 2 ml bacterial liquid are added, calculated volume of ppm is added into a 250 ml conical flask for each strain. The flasks are incubated in 28˚C shaker. The OD values of each strain are measured at 12 hour, 24 hour and 36 hour. The final average measurement is listed in the following table and a graph is plotted accordingly.

Table. 7 OD values of M18 strains at 12, 24, and 36h

	0h	12h	24h	36h
M18	0.02	0.2385	0.409	0.585
M18ΔU	0.02	0.2	0.398	0.542
M18ΔUM	0.02	0.207	0.3825	0.5255
M18ΔUMS	0.02	0.1745	0.373	0.5285

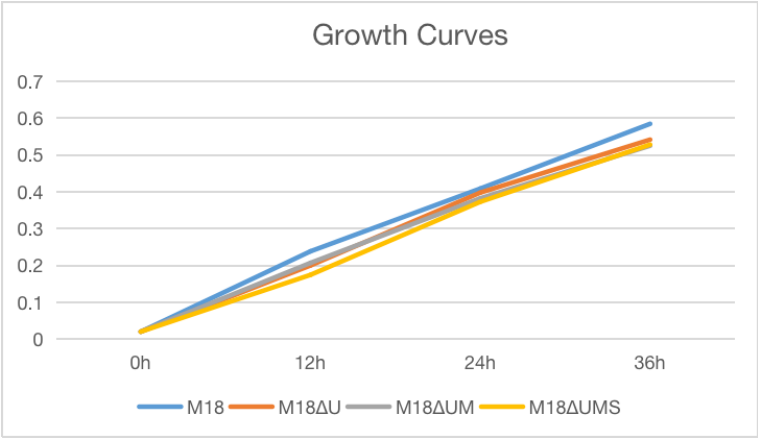

Figure 7: Growth Curves of M18 Strains

As shown on the graph, for all four strains, the growth rate remain approximately positive and steady throughout 36 hours of growth. This means all four strains are growing at a constant rate. The slope of M18 appears to be steepest among the four strains while the slope of M18ΔUMS seems to be the flattest. This indicates that M18 is growing faster than all other strains, and M18ΔUMS is the slowest.

3.1.2.PCA Yield

3 test tubes are prepared for each strain. Each tube is filled with 180 ul bacterial liquid and 20 ul hydrochloric acid, they are shaken vigorously for 10 seconds. 540 ul chloroform is added and shaken for another 2 minutes. The tubes are centrifuged at 12,000 rotation for 10 minutes. The lower clear fluid id moved into new tubes. This procedure is performed at 24 hour and 36 hour. Next, the tubes are placed in rotating spiral heater at 30˚C for 30 minutes. 100 ul of methyl alcohol is added and mixed. Then 70 ul solution is moved into designated glass tubes. HPLC is performed. The measured PCA production is listed below and a graph is plotted accordingly.

Table. 8 PCA yield at 24h and 36h

	M18	M18ΔU	M18ΔUM	M18ΔUMS
24h	24.2580568	117.114612	176.8403066	242.098137
36h	35.77176314	205.0600281	262.2362896	294.745135

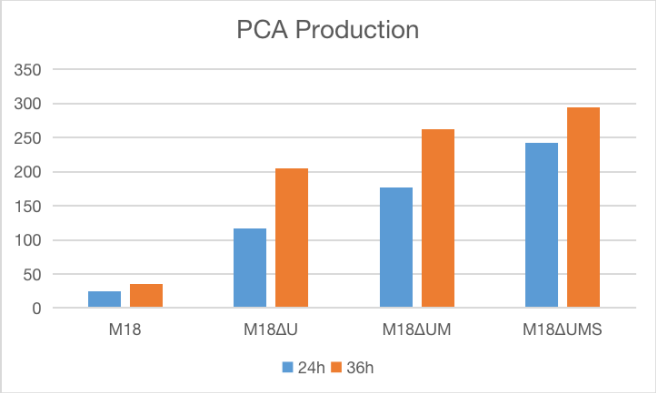

Figure 8: PCA Production of M18 Strains

From this graph, we can see that at both 24h and 36h, the production of PCA increases as more targeted genes are knocked out. M18 produce the least amount of PCA while M18ΔUMS produce the most amount of PCA.

4 DISCUSSIONS

4.1 Increased PCA production of M18ΔUMS

As expected, the triple-deleted mutant produce the highest amount of PCA, while the wild type produce the lowest amount. The construction of triple-deleted mutant successfully improve the production of PCA.

4.2 Toxic Inhibition Effect of PCA

It can be noticed that M18ΔUM produce the highest amount of PCA and the least amount of growth. Conversely, the wild type M18 produce the least amount of PCA and the highest amount of growth. It can be inferred that the production of PCA might be negatively correlated with the growth rate of M18 strain. Another study also discovers that the when phz genes become inactivated, mutant stains of M18 produce less PCA and show a greater amount of growth compared to the wild type (Li, 2011). The researchers suggest that a potential toxic inhibition effect on bacterial might emerge under certain PCA concentration (Li, 2011). Thus it is reasonable to infer that the excessive concentration of PCA caused by deletions might have negatively impact the growth of bacterials in M18ΔUMS, M18ΔUM, and M18ΔU. Although it is possible that the deletion of genes might have affect other mechanisms. PYO has a range of biological functions including being electron shuttle for cell respiration.

4.3 The Regulatory Function of UTR Genes

The PCA production increases sharply after the deletion of UTR genes. This elucidates that UTR genes play a negative regulatory role in the production of PCA. The regulation of phenazine production is maintained by a complex network of QS system, GacS/GacA two-component signal transduction, small non-coding RNAs including RsmX, RsmY, and RsmZ as well as some other regulators in response to the immediate environmental condition (Morrison, 2016). In P. aeruginosa, GacS/GacA signal transduction system performs its function exclusively by controlling the transcription of the these small RNAs (Brencic, 2009). These RNAs bind to the mRNA-repressor proteins of the RsmA/CsrA family, which themselves bind to UTR regions (Karine, 2008). The precise mechanism of entire regulatory system and its connection to UTR are still wanting, but it is definite that UTR inhabit the production of PCA.

4.4 Expected PYO Production

Both M18ΔU and M18ΔUM show deep blue/green coloration, indicating the presence of PYO. The deep blue/green coloration of M18ΔU suggests an overabundance of PCA will lead to an overproduction of PYO as well. M18ΔUM's color indicates that there are two pathways that can convert PCA into PYO. As mentioned before, phzM can transform PCA into 5MPCA, which I can be converted into PYO by phzS. Another pathway seems to be functioning when phzM is knocked out. It converts PCA into 2-OH-PHZ through phzO, then 2-OH-PHZ changes into 5MPCA, which can be covered into PYO in the presence of phzS. Thus, the deletion of phzM and phzS should eliminate the

production of PYO altogether. Since these genes are primely involved in the conversion from PCA to PYO. PYO is a major virulence factor, thus the reduction of PYO should decrease the pathogenicity against human and animals (Du, 2013). The wild type M18 already produces less PYO than other P. aeruginosa strains (Huang, 2009). However, mutant M18ΔUMS should not produce any PYO at all. This would render M18ΔUMS a safer agent than M18 and more suitable for commercial use.

4.5 Expected PCN Production

PhzS also promote the conversion from PCA to 1-OH-PHZ. So, ideally a decrease or elimination of 1-OH-PHZ is expected after the deletion of phzM. The conversion from PCA to PCN is mostly depended on phzH, which remain intact. So, the production of PCN is expected to stay unchanged. Although the deletion of phzS and phzM will result in the accumulation of extra PCA, it is unknown whether the excess will result in increased PCN production.

5 CONCLUSION

To conclude, the construction of triple-deleted mutant M18ΔUMS is a success. The mutant strain produces more PCA than the wild type, presumably cost-effective. Thus, it can be a potential candidate for commercial use. However, more improvements might be made if a better understanding of the complex interaction between various regulatory systems is gained. So further studies should focus on the regulatory mechanisms go phenazine production.

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