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Research Article

Effect of antibiotics on the expression of pyocyanin synthetic genes in *Pseudomonas aeruginosa* isolated from different clinical sources of a few hospitals in Mosul, Iraq

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Abstract
Pyocyanin is a blue-green phenazine pigment and one of the most virulent factors produced by the opportunistic pathogen *Pseudomonas aeruginosa*. It has a redox activity and a toxic impact on living cells, as it interacts with oxygen to produce reactive oxygen species (ROS). Using antibiotics at a sub-lethal dose has an unexpected influence on the expression of pyocyanin-producing genes. In this study, qPCR technique was performed to identify the effect of eight antibiotics (cefotaxime, ampicillin, amoxiclav, ceftazidime, ceftriaxone, chloramphenicol, kanamycin and tetracycline) on the gene expression level of pyocyanin synthetic genes in *P. aeruginosa* isolated from different clinical sources of a few hospitals in Mosul, Iraq using qPCR technique. It was found that when *P. aeruginosa* was grown in media containing cefotaxime (CTX 30 µg/mL), ampicillin (AM 25 µg/mL) or amoxiclav (AMC 30 µg/mL), up-regulated the expression of pyocyanin producing genes belonging to different operons thereby increased pyocyanin production. Overexpression occurred in (CTX) treatment in *PhzA1* operon with 235.56 fold change and *phzM* and *phzS* genes with 340.14, 280.13 fold change, respectively. Lower expression levels showed in tetracycline (TE 30 µg/mL) treatment, which was a (1.44) fold change for *phzA1* and a (1.64, 1.08) fold change for *phzM* and *phzS* genes. More caution should be considered when delivering antibiotics to treat *P. aeruginosa* infections, as using drugs that the bacteria resists or at sub-lethal concentrations may trigger up-regulation of virulence factors, aiding in the spread of the disease.

Keywords: Antibiotic, Cefotaxime , Phenazine, *Psuedomonas aeruginosa*, Pyocyanin, qPCR

INTRODUCTION

Pseudomonas aeruginosa is a widespread microorganism isolated from various environments, including water, soil, plants, animals and humans (Batrich *et al*., 2019; Mesquita *et al*., 2016). This is a well-known causative agent of multiple nosocomial and life-threatening infections such as cystic fibrosis, burn and wound infections, urinary tract infections (UTIs), and pulmonary infections. Infection sources are usually from medical equipment, such as inhalers, dialysis equipment, respirators, anesthesiology equipment, vaporizers, and toilets and sinks (Azam and Khan, 2019). It is considered a crucial clinical agent, as this bacterium is an opportunistic pathogen that can trigger a wide range of acute and chronic human injuries and diseases (Weiner *et al*., 2016). *P. aeruginosa* has many virulence factors that aid in causing both acute and chronic disorders that lead to inflammation, particularly when they are transmitted from the external environment into the host body or from one location to another in the same host (Shaan and Robert, 2013). These factors could be associated with cells such as flagella, pili, and lipopolysaccharide (LPS). The secretion factors found in *P. aeruginosa* are responsible for secreting a number of extra-cellular enzymes and some cellular toxins which play a role in tissue damage, resistance to phagocytosis and the production of many pigments such as pyocyanin (Laxmi and Sarita, 2014). Pyocyanin is a redox-active phenazine blue pigment that is excreted by 90–95% of *P. aeruginosa* strains (Saleem *et al*.,2021). Phenazines provide a large group of nitrogen-containing heterocyclic compounds, regarded as electron shuttles to substitute terminal acceptors and revise

redox states in bacterial cells (Al-Shamary, 2018). Phenazines react with molecular oxygen to produce reactive oxygen species (ROS), such as superoxide (O2-1), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO·) (Montelongo□Martínez *et al*., 2022). Pyocyanin has a valuable implication on biofilm formation and iron uptake in low oxygen conditions and governs the genes involved in efflux pumps, which raise the metal's resistance, especially silver (Abdelaziz *et al*., 2023). Pyocyanin is made by two surplus operons, *phzA1-G1* (*phzA1*) and *phzA2-G2* (*phzA2*), which are similar to 98% in their sequence of nucleotide, along with the genes *phzH*, *phzM* (phenazine-specific methyl transferase) and *phzS* (flavin-dependent monooxygenase) (Hirakawa *et al*., 2021). Both phzM and phzS genes shroud the phzA1 operon, as the *phzA2* operon is shrouded by *qscR*, which encodes for the transcription factor of orphan quorum-sensing and gene coding to hypothetical protein (Cui *et al*., 2016).

Chorismic acid, which is synthesized from shikimic acid via the *aro* pathway, is the precursor molecule in the biosynthetic pathway of pyocyanin (Hirakawa *et al*., 2021). Chorismic acid is converted to phenazine-1-carboxylic acid by the PhzA-G proteins and then to 5-methyl phenazine1-carboxylic acid betaine (MPCBA) via PhzM. Subsequently, MPCBA is converted to pyocyanin through hydroxylation catalyzed by PhzS (Dong *et al*., 2020; Abdelaziz *et al*., 2023). The three quorum□sensing systems (Las, Rhl, and Pqs) are in charge of the positive regulation of pyocyanin biosynthesis at the transcriptional level (Mukherjee *et al*., 2017; Montelongo-Martínez *et al*., 2022). On another note, RsaL, RpoS, and MvaU regulatory proteins are negatively regulated in pyocyanin synthesis. The expression of *phzA1* operon is regulated negatively by RsaL regulatory protein, which binds directly to promoter region of the *phzA1*, while causes activation of the *phzA2* operon indirectly (He *et al*., 2019; Fang *et al*., 2021). The antibiotic suitability profile of pathogenic bacteria equips high -value information to prescribe the most effective antibiotic therapy (Hata *et al*., 2019; Mojsoska *et al*., 2021). Currently, the most effective antibiotics used in the treatment of *P. aeruginosa* infections belong to fluoroquinolones, beta-lactams, and the aminoglycosides, with polymyxins being used as a late choice for treatment. Studies have noticed that these antibiotics could affect the production of several agents that have an essential role in establishing pathogenicity in the early stages of infection (Su *et al*., 2010). One of these studies revealed that sub-inhibitory concentrations of ciprofloxacin, meropenem, and tobramycin-induced pyocyanin secretion from *P. aeruginosa* (Alatraktchi *et al*., 2020; Mojsoska *et al*., 2021).

Another study confirmed that subinhibitory cefotaxime and levofloxacin concentrations promoted pyocyanin production in *P. aeruginosa*, even on plates containing higher concentrations of such antibiotics (Zhao *et al*., 2022). In contrast, Kumar *et al*. 2021demonstrated that pyocyanin production was inhibited in *P. aeruginosa* treated with cephalosporins: cefepime, ceftazidime, and ceftriaxone. Studies performed using microarray, proteomic and promoter-reporter fusion library technologies demonstrated that subinhibitory concertation of antibiotics can transcriptionally modulate a large number of genes (Davies *et al*., 2006; Skindersoe *et al*., 2008). However, no previous work has shown how these antibiotics affect the expression levels of the genes involved in the biosynthetic pathway of pyocyanin, thereby this study aimed to detect the impacts of different antibiotics on the expression of five genes located on three different operons that contribute in pyocyanin production using qPCR.

MATERIALS AND METHODS

Bacterial collection and growth media

Three hundred and twenty-five clinical specimens were collected from different infections and age groups patients were referred to Ibn-Sinna Hospital, Al-jamhori Hospital, Mosul General Hospital, Al-salam Teaching Hospital, and Al-Mosul Center for Burns and Plastic Surgery from January to May 2023. All specimens were cultured in MacConkey agar and incubated at 37˚C for 24 h. Morphological features of *Pseudomonas aeruginosa* were observed and pale colonies were transferred to cetrimide agar. Gram stain was used to determine the type and arrangement of bacteria. Biochemical tests (oxidase, catalase and citrate) and API20E were used to confirm identification (Winn *et al.,* 2006; Reiner, 2010). The isolate that was used in estimating the effects of different antibiotics on the expression levels of pyocyanin production was identified by 16S rRNA sequencing (Khaleel *et al*., 2023; Abdulrazzaq and Faisal, 2022).

Effect of antibiotics on pyocyanin production

To study the effect of antibiotics on pyocyanin production, four *P. aeruginosa* isolates (PA1, PA2, PA3 and PA4) were inoculated in Luria Bertani broth (LB) at 37˚C till the optical density reached 0.4-0.6 at 600 nm (Aleanizy *et al*., 2021). After that, 0.1 mL of bacterial culture was used to inoculate nine reagent bottles containing 10 mL of LB broth. Each bottle was supplied with a different antibiotic at a non-lethal concentration as follows: ampicillin (AM 25 µg/mL), chloramphenicol (C 30 µg/mL), tetracycline (TE 30 µg/mL), kanamycin (K 30 µg/mL), ceftazidime (CAZ 30 µg/mL), ceftriaxone (CRO 30 µg/mL), cefotaxime (CTX 30 µg/mL), amoxiclav (AMC 30 µg/mL), and one as control without antibiotic (inoculated with bacteria only). After that, they were incubated at 37˚C for (18-20) h under continuous shaking (180rpm). The supernatants were collected to

quantify the pyocyanin produced. This experiment was performed in triplicate on different days for each isolate. Pyocyanin concentration was determined by adding 4 mL of the supernatant to 3 mL of chloroform, mixed with a vortex till the color turned green-blue. Samples were centrifuged (10,000×g for 10 min) and 3 mL of the blue-colored product was conveyed to a tube including 1 mL of 0.2 M HCl and shaking till the color changed to pink. The pink layer was transported to a cuvette to measure absorbance at 520 nm. The absorbance was multiplied by factor 17.072 to obtain pyocyanin concentration per Essar *et al*. (1990).

Expression of pyocyanin genes

P. aeruginosa (PA2) isolate was selected and grown in LB broth at 37°C for (18-20) h with shaking (180 rpm), and then 1.5 mL of culture was centrifugated at 4°C (8000 rpm for 10 minutes) to harvest cells. RNA was extracted using (*TransZol* Up Plus RNA Kit /TransGen Biotech / China) kit. All isolation protocols were carried out based on the manufacturer's instructions without further modifications. NanoDrop spectrophotometer (BioDrop/UK) was used to estimate concentrations and purities of RNA, and values of ~2.0 were regarded as indicative of relatively pure RNA. The expression of the pyocyanin gene was estimated using a two-step reverse transcriptase (RT)-PCR assay. The RNA samples were purified from remaining DNA contaminants and then transcribed into complementary DNA (cDNA) using *EasyScript*® One-Step gDNA Removal and cDNA Synthesis SuperMix/ TransGen Biotech/ China kit. Then, cDNAs were amplified with qPCR primers targeting the genes (Table 1), and TransStar® Top Green qPCR SuperMix (*TransStar*® Top Green qPCR SuperMix/ TransGen Biotech/ China) kit. Expressions of pyocyanin were analyzed using the Step One Plus real-time PCR system (Analytikjena/ Germany). The relative expression levels of pyocyanin were calculated using the $2^{-\Delta\Delta CT}$ method.

Ethical approval

The study was conducted following the ethical principles of the Declaration of Helsinki. It was carried out with patients' verbal and analytical approval before the sample was taken. The study protocol, subject information, and consent form were reviewed and approved by a local ethics committee according to document number 15945 (9/1/2023).

Statistical analysis

All statistical analysis was conducted using the GraphPad prism virsion10.

RESULTS AND DISCUSSION

Isolation and identification of *Psuedomonas aeruginosa*

The percentage for isolation of *P. aeruginosa* on MacConkey agar and cetrimide agar from all clinical specimens collected was 33.8% (110/325). This ratio was distributed as follows: 41.8% (46/110) burns, 37.3% (41/110) urine and 20.9 % (23/110) swab and pus. The *P. aeruginosa* colonies on MacConkey agar were small and pale due to their disability to ferment lactose. When grown on cetrimide agar colonies, yellow-greenish mucoid colonies with flat edges and a fruity odor appeared. When stained with Gram stain, *P. aeruginosa* appeared as pink single rods. All *P. aeruginosa* isolates were positive to (oxidase, catalase, and citrate). API 20E test was used to identify *P. aeruginosa* with (95-99) % identity. The identification of the PA2 isolate used to study pyocyanin gene expression was confirmed by 16S rRNA sequencing, then submitted to the National Center for Biotechnology Information (NCBI) and was given the accession number (PP329816).

Quantification of pyocyanin production

Pyocyanin production was quantified in 4 isolates of *P. aeruginosa* treated with antibiotics. Production varied between isolates as compared with the control (without antibiotic). Differences in pyocyanin concentration for all isolates ranged between 0.37 – 5.13 µg/ml. PA2 isolate showed a significant difference in pyocyanin production, where there was a noticeable increase in pyocyanin concentration that reached 5.13 and 4.22 µg/mL with cefotaxime (CTX) and ampicillin (AM), respectively.

Table 1. Primers used in amplification of pyocyanin-producing genes by Qpcr

Primer Name	Primer Sequence (5' – 3')	Reference
FabD-F	GCATCCCTCGCATTCGTCT	(Meng *et al.,* 2023)
FabD-R	GGCGCTCTTCAGGACCATT	
PhzA1-F	AACGGTCAGCGGTACAGGGAAAC	
PhzA1-R	ACGAACAGGCTGTGCCGCTGTAAC	
PhzA2-F	CTGTAACCGTTCGGCCCCCTTCATG	
PhzA2-R	ATGCGAGAGTACCAACGGTGAAAG	
PhzH-F	GCTCATCGACAATGCCGAACT	(Dong *et al.,* 2020)
PhzH-R	GCGGATCTCGCCGAACATCAG	
PhzM-F	AGCAACCTGGCATTCCACGAG	
PhzM-R	TGCAGGATGGCCTTGGTCAATT	
PhzS-F	CCGAAGGCAAGTCGCTGGTGA	
PhzS-R	GGTCCCAGTCGGCGAAGAACG	

Table 2. Quantification of pyocyanin in *Psuedomonas aeruginosa* (PA2)

Treatment	Pyocyanin Concentration µg/ml
Control (without antibiotic)	3.22
Cefotaxime (CTX)	5.13
Ampicillin (AM)	4.22
Chloramphenicol (C)	3.24
Tetracycline (TE)	2.81
Kanamycin (K)	2.90
Ceftazidime (CAZ)	3.61
Ceftriaxone (CRO)	3.31
Amoxiclav (AMC)	3.98

While, pyocyanin concentration decreased to 2.81 µg/mL with tetracycline (TE) and (2.90) µg/mL with kanamycin (K), as listed in Table 2.

Differential expression of pyocyanin related Genes:

Psuedomonas aeruginosa releases multiple virulence agents that permit occupation and cause host infections involving humans. Pyocyanin is an essential virulence factor, a phenazine carrying a redox activity that enables the interaction with oxygen to produce ROS. Pyocyanin synthesis and regulation in *P. aeruginosa* has been extensively studied (Higgins *et al*., 2018). To elucidate the change in the level of expression for pyocyanin production genes in *P. aeruginosa* isolate PA2, the expression levels of (*phzA1*, *phzA2*, *PhzH*, *PhzM* and *PhzS*) genes were compared in all antibiotic-treated cultures and the control. FabD was used as a housekeeping gene for normalization. Results showed a variation in the expression levels of tested genes according to different antibiotics tested; however, most of the pyocyanin production genes were up-regulated compared to the control. High expression was observed in *phzA1*, *phzM* and *phzS*, while lower expression was detected in *phzH*. The difference in gene expression between pyocyanin genes is probably due to the presence of these genes on different operons, thereby responding differently. In addition, it has been shown that antibiotics have two different and opposite effects; at high doses, they will be lethal, but at low doses, they may stimulate genetic variations and alter the expression of virulence genes. Continuous exposure of bacteria to antibiotics has been suggested to enhance mutations, which may affect gene expression in regulatory regions (Couce and Blazquez, 2009). It is well-known that subinhibitory concentrations (SIC) of many antibiotics can intertwine with some important aspects of bacterial physiology, involve alterations in the morphology, virulence, genome stability and cause genetic variations (Davies *et al*., 2006). Antibiotics exhibit a hormesis phenomenon, and their antimicrobial activity can impact alternative pathways at the molecular level in bacteria (Kumar *et al*., 2021).

In cefotaxime (CTX) treatment, generally all genes (*phzA1*, *phzA2*, *phzH*, *phzM* and *phzS*) were overexpressed compared to the housekeeping gene, the fold change was (235.56, 88.64, 97, 340.14 and 280.13), respectively as shown in Fig. 1.a . This result is in accordance with what has been achieved by Zhao *et al.* (2022) who showed that cefotaxime (CTX) promotes pyocyanin production in *P. aeruginosa* PA-COP2 and elevated *pqs* QS system when growing in plates containing sub-inhibitory concentrations of cefotaxime (CTX) or Levofloxacin. The increasing expression of *phzM* and *phzS* explains the high concentration of pyocyanin observed when quantified by spectrophotometer after extraction, as shown in Table 2. In the case of ampicillin (AM) treatment, there was a significant up-regulation in fold-change expression of all genes, especially with *phzA1, phzH* and *phzM* (108.38, 88.64, 79.89), respectively, Fig. 1.b. This result indicates the activation of *phzA1* operon and is consistent with the increase in pyocyanin concentration, as shown in Table 2.

Gene expression in amoxiclav (AMC) treatment was up-regulated, particularly in *phzA1* and *phzM* (24.42, 18.50) fold change, respectively, while (2.05) fold change for *phzH*, as shown in Fig. 1.c. Over expression of genes involved in pyocyanin synthesis with cefotaxime (CTX), ampicillin (AM) and amoxiclav (AMC) may be because the production of pyocyanin is changed as a result of the influence of exposure to non-lethal doses of antibiotics by the QS system and other regulatory genes. Increase in pyocyanin production might be related to the inactivation of *LasA*, an important component of the QS system (Aleanizy *et al*., 2021). Also, the inactivation of *LasR* led to higher expression in *phzA1* (Soto-Aceves *et al*., 2021). On the other hand, RsaL was also shown to act as a negative regulatory protein that activates *phzA1* operon expression (Rampioni *et al*., 2009), as the inactivation of *RhlR* was shown to cause a decrease in *rsaL* expression (Babić *et al*., 2010). Genes related to pyocyanin synthesis fall under the influence of *mvaT* and *mvaU* genes, which encode two transcriptional proteins, MvaT and MvaU. Transcriptional levels of genes related to pyocyanin synthesis (*phzM*, *phzS* and *phzH*) and *phzA1* operon are elevated by the inactivation of *mvaU,* which leads to the enhancement of pyocyanin production (Dong *et al.,* 2020). Expression of pyocyanin genes treated with chloramphenicol (C) was also up-regulated, *phzA1* showed the highest effect with (22.78) fold change; meanwhile, *phzH* and *phzM* genes were up-regulated with (19.02, 16.33) fold change as shown in Fig. 1.d. There was no difference in pyocyanin production of cultures treated with chloramphenicol (C) as compared with the control (Table 2); however, up-regulation in gene expression was still observed. That reflects the important role of the enzyme produced by *phzM* gene in converting phenazine-1-carboxylic acid to 5-methylphenazine-1-

carboxylic acid betaine, which is later converted to pyocyanin by *phzS*.

In ceftazidime (CAZ) and ceftriaxone (CRO) treatments, *phzA1* gene documented less expression levels compared to previous treatments with (7.56, and 7.36) fold change, respectively. High expression levels in ceftriaxone (CRO) treatment were detected in *phzM* with (13.26) fold change, while in ceftazidime (CAZ) treatment, both *phzM* and phzS showed (22.31) fold change as in Fig. (1. e and f). The present results contradict what has been previously detected by Kumar *et al*. (2021), who found that both ceftazidime (CAZ) and ceftriaxone (CRO) at sub-inhibitory concentrations suppress pyocyanin production in *P. aeruginosa* PAO1. They documented that these cephalosporins interact genetically with the pathway related to pyocyanin synthesis.

Treatment of kanamycin (K) showed upregulation in *phzS* (6.14) fold change and a slight rise in gene expression for *phzA1*, *phzM* with (2.77, 2.41) fold change,

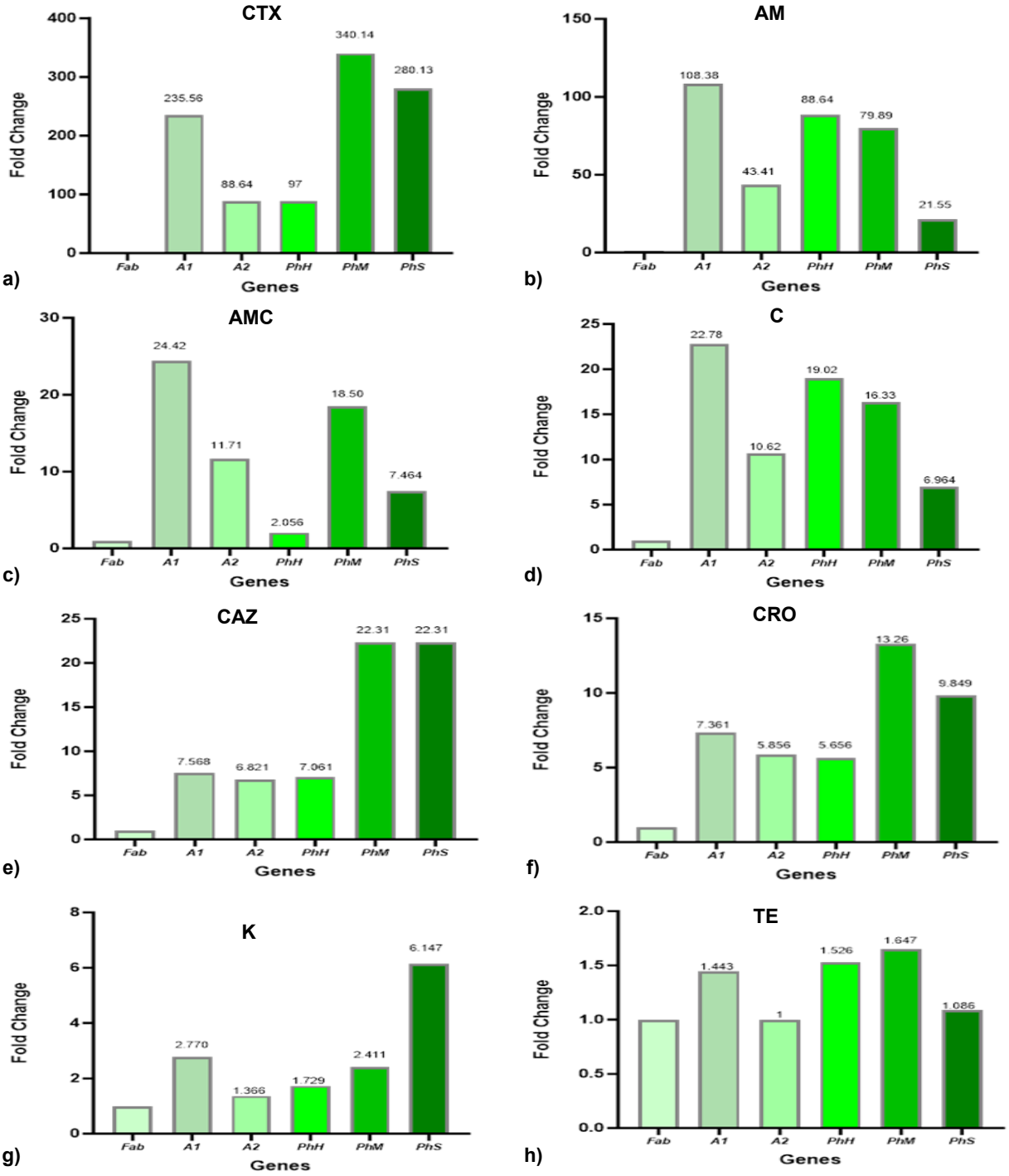

Fig. 1. *Effect of antibiotics on the expression levels of genes involved in pyocyanin production (phzA1, phzA2, phzH, phzM, phzS)*

as seen in Fig. 1.g. Despite that increase, pyocyanin production decreased compared to the control, as shown in Table 2. In the case of tetracycline (TE) treatment, there was no effect on the expression of genes, and this result was in line with a decrease in pyocyanin production, as noticed in Fig. 1.h and Table 2. Inactivation of both *mvaT* and *mvaU* causes a reduction in the transcriptional levels of all genes except *phzH* (Dong *et al*., 2020). On the other hand, Higgins *et al*. (2018) explained that inactivation of both *mvaT* and *mvaU* led to the increase in *qsrO* expression, which in turn decreases gene coding signal molecules (*pqsH, rhlI* and *pqsE*) and transcriptional activators (*rhlR*, *lasR* and *pqsR*). Reduction of *rhlR* and *pqsE* expression causes a reduction in gene expression levels of *phzA1* and *phzA2*.

Regulation of pyocyanin production includes various systems and regulators embedded in the three quorum-sensing systems Las, Rhl, and Pqs, the regulators RsaL, MvaU, the alternative sigma factor RpoS and the posttranscriptional regulatory system Rsm (Montelongo-Martínez *et al*., 2022). High pyocyanin production in different treatments may be a concern to mutations that occur in *RpoS*, which encode for stationary phase sigma factor, executes to high expression of *phzA1* operon and *phzM* and then high pyocyanin production (negative regulation) (He *et al*., 2019, Chen *et al*., 2020, Wang *et al*., 2020). Variation in pyocyanin production might be clarified by the role of RsmA (a transcriptional regulatory factor) in controlling the operons and genes related to pyocyanin synthesis (Montelongo-Martínez *et al*., 2022). Generally, proteins of the Rsm system have the ability to regulate expression by binding to different sites within the un-translating leader region, either through overlapping with Shine-Dalgarno sequence or by destabilization of messenger RNA (mRNA) (Pourciau *et al*., 2020). Thus, the inactivation of *rsmA* causes a rise in the expression level of *phzA1* operon, whilst the case is reversed with the *phzA2* operon (Montelongo Martínez *et al*., 2022). Expression of *phzM* and *phzS* genes is elevated by inactivation of *rsmA*. Regulation of *phzM* and *phzS* by RsmA are directly affected because both of them have ANGGA pentanucleotide in their regulatory region that can be recognized by RsmA (Schulmeyer *et al*., 2016; Irie *et al.,* 2020). Because antibiotics induce pyocyanin and other virulent factors in *P. aeruginosa*, antibiotic administration is a significant factor to consider when treating infections caused by this pathogen, as the use of antibiotics that the bacteria resists may cause up-regulation of virulence factors and thus aid in the spread of the pathogen, particularly when taken at sub-lethal concentrations.

Conclusion

The present study on the impact of various antibiotics on the expression of five genes located on three separate operons involved in pyocyanin synthesis via qPCR experiments indicated that using different antibiotics at non-lethal concentrations stimulated the production of pyocyanin in *P. aeruginosa*, by inducing the expression of five major genes related to pyocyanin synthesis. The results showed that different genes were induced differentially according to the operon they were located on the influence of antibiotics at sub-inhibitory concentrations was previously shown to affect quorum system components, regulatory genes (*mvaU* and *mvaT*) and RsmA posttranscriptional regulatory system, which in turn regulate the production of pyocyanin. More care should be taken when administering antibiotics to treat infections caused by *P. aeruginosa*, as the use of antibiotics that the bacteria resists may cause up-regulation of virulence factors and, therefore, help spread the pathogen, especially when taken in sub-lethal concentrations.

Conflict of interest

The authors declare that they have no conflicts of interest.

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