Original Article

Expression of rhlR gene in Pseudomonas aeruginosa affected by Lactobacillus spp

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Abstract

P. aeruginosa is an opportunistic pathogen causes severe infections due to distinct virulence factors such as pyocyanin. This pigment confers the microbe severity during the infections. This study aimed to evaluate metabolites from Lactobacillus on pyocyanin production. Seven isolates of P. aeruginosa were screened for pyocyanin. All the isolates were produced pyocyanin in rate ranged between 0.47 to 0.82 µg/ml. The isolates were AHL producers with absorbance at 630 nm ranged between 2.26 to 4.88. The culture filtrate from Lactobacillus spp. showed inhibition activity against pyocyanin production. The clinical Lactobacillus spp. was characterised by their higher ability to inhibit pyocyanin production compared to the environmental isolate. There was a clear variation in folding change values in expression of rhlR gene after treatment with Lactobacillus filtrate. Treatment with CF of Lac9 was up-regulated 1.89-Fold the transcription of rhlR gene. The results of this study suggest that there is a clear interaction between P. aeruginosa and Lactobacillus regarding the pyocyanin production.

Keywords: Pseudomonas aeruginosa, Lactobacillus, rhlR gene, gene expression.

Introduction

P. aeruginosa is Gram-negative bacilli, opportunistic pathogen inhabits diverse environmental conditions. P. aeruginosa involved in severe human infections such as respiratory infections, urinary tract infections, hospital acquired pneumonia, wound and soft tissue infections (Moradali et al., 2017; Pachori et al., 2019). P. aeruginosa have improved resistance against broad spectrum of antibiotics that widely used for curing the infections in hospitals. It is well known that this bacterium is capable of acquiring resistance during antibiotic therapy (Pang et al., 2018). This aggressive behaviour is due to many reasons included several mechanisms for virulence factors, adaptation, survival in low nutrient environments and resistance of different antibiotics classes (Moradali, 2017). P. aeruginosa formed biofilm on biotic and abiotic surfaces. The biofilm matrix provides suitable environments for survive and maintain the cells, while shielding them from unfavorable conditions such antibiotics (Chan et al., 2021).

P. aeruginosa, like other bacteria, produce colored substances as soluble extracellular nitrogenous heterocyclic compounds called phenazines. Pyocyanin, a redox-active blue-green compound, is one of the most important among phenazines pigments. Most of P. aeruginosa strains produce one or more of these pigments (Riedel et al., 2019). Synthesis and secretion of pyocyanin contributes significantly to bacterial infection and makes P. aeruginosa, more aggressive (Zhou et al., 2022).

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Secretion of virulence factors by this bacterium is controlled by a mechanisms called Quorum sensing (QS) by which bacteria can communicate with the same species or other microbes in order to achieve a specific process associated with the virulence. QS network produced by P. aeruginosa is responsible for pyocyanin production and other virulence factors, which makes the bacteria highly drug-resistant (Tang et al., 2021; Lim et al., 2022).

Biosynthesis of pyocyanin is genetically controlled by several genes included phzM, phzS, and rhlR, and two identically organized operons that encode the enzymes that make pyocyanin (Askitosari et al., 2019).

To overcome the pathogenicity of P. aeruginosa, there are some strategies can be applied act as anti-virulence agents. These agents could be work synergistically with antibiotics to treat the infection by this bacterium. For example, nanoparticles, medicinal plant extract and the secondary metabolite derived from microorganisms. In this regard, the metabolites produced from probiotic bacteria, especially Lactobacillus spp. can be used to suppress some virulence factors formation like pyocyanin. Lactobacilli have received the greatest attention among the various microbial species in terms of potential therapies for human health (Pradhan et al., 2020). Many members of probiotics can also create a wide range of bioactive compounds that are comparable to the standard antibiotic. Bacteriocins, organic acid, hydrogen peroxide, diacetyl, acetoin is an example of these materials (Santacroce et al., 2019). The goal of this study the expression of rhlR gene in P. aeruginosa treated with Lactobacillus metabolites on pyocyanin production.

Material and methods

Microorganisms

Seven isolates of P. aeruginosa were used in this study were obtained from samples of burns. The isolates were confirmed by API20E kit and VITEK2 compact system (bioMérieux, Franc) (Hernández-Durán et al., 2017). Two identified isolates of Lactobacillus spp. were kindly supplied by Microbiology Laboratory, Department of Biotechnology, University of Diyala. A clinical Lactobacillus Lac2 isolated from lateral vaginal wall sample and one environmentally bacteria (Lac9) isolated from local dairy sample.

Pyocyanin production assay

The amount of pyocyanin from seven isolates of P. aeruginosa was measured using a quantitative chemical assay in acidic solution as described by (Essar et al., 2012). P. aeruginosa Pa7 was selected as potent isolates for the next study.

Detection of N-acylhomoserine lactones (AHL)

AHL activity was extracted and quantified by colorimetric method according to (Rosa et al., 2016). Briefly, aliquot of 1.5 ml of P. aeruginosa Pa7 liquid culture was centrifuged at 10.000 rpm for 15 minutes and the supernatant was transferred into a new tube. The organic phase (top layer) was then extracted with ethyl acetate then the samples were left at 40 °C to dry. Forty microliters of dried samples were transferred to a microplate and 50 µl of 1:1 solution of (hydroxylamine 2 M: NaOH 3.5 M) and 50 µl of 1:1 solution of (FeCl3 10% in HCl 4 M: Ethanol 95%) were added. The absorbance of dark brown colored ferric hydroxamate complexes was measured at 520 nm by ELISA reader (HS-Human Reader, Germany).

Evaluate Lactobacillus spp. culture filtrates for inhibition of pyocyanin production

The ability of Lactobacillus spp. isolates to inhibit pyocyanin was evaluated according to (Valdez et al., 2005). An active inoculum of Lactobacillus spp. and P. aeruginosa Pa7 were used to inoculate a sterile tubes containing a liquid media of MRS and LB (Oxoid, England), respectively. Tubes were incubated at 37 oC for 24 hours. The culture filtrate (CF) (acid filtrate) recovered by centrifugation at 4000 rpm for 20 minutes was neutralised with 8 M NaOH to pH 7. The CF was sterilised by 0.22 µm Millipore® Membrane Filter (Sartorius, Germany) and used immediately for inhibition of pyocyanin production test.

The assay

Aliquots of 1 ml of Nutrient broth (Oxoid, England) inoculated with 2% (v/v) of P. aeruginosa in the presence of different volumes of neutralised CF included (250, 500, 750 and 1000) ul. The same volumes of MRS were added to P. aeruginosa as negative controls. After 48 hours of incubation at 37 C, the tubes were centrifuged at 4000 rpm for 20 minutes. The supernatants were filtered using a 0.45 µm Millipore filter and 200 µl of each sample was transferred to 96-well microplates. The absorbance intensity was measured at 630 nm which is proportional to the pigment concentration in the solution expressed as percentage (%) of inhibition.

Expression of rhlR in P. aeruginosa Pa7

The molecular study was achieved according to the kits manufacturer's instructions. All the molecular techniques in the experiments were carried out under biosafety guidelines.

Molecular detection of rhlR in P. aeruginosa Pa7

Gene detection of rhlR in P. aeruginosa Pa7 using conventional PCR (Thermo Fisher Scientific, USA). Genomic DNA was isolated from cells of P. aeruginosa according to the protocol of Geneaid™ DNA Isolation Kit (Geneaid, Taiwan). Quantus™ fluorometer (Promega, USA) assay was used to measure the concentration and quality of the extracted DNA. The amplification reaction was carried out in 30 cycles in conventional PCR included initial denaturation at 95 oC/5 second, annealing 60 oC/30 second and extension at 72 oC/30 second. The conventional PCR products were analysed on agarose gel (Promega, USA) (1.5%, w/v) stained with ethidium bromide to confirm the presence of PCR amplicons (Green and Sambrook, 2012). Electrical power was performed at 100 v/mAmp for 60 minutes. The bands visualized with ethidium bromide were examined in UV Transilluminator (320 nm/360w) using Gel Imaging System (Major Science, Taiwan) supplied with a digital camera.

Expression of rhlR

Extraction and purification of RNA from the sample was carried out by using the TRIzol™ Reagent technique. Quantus™ Fluorometer (Promega, USA) was used to determine the concentration of the extracted RNA according to manual instructions of the supplier. Real-time PCR run was performed with Gene 9600 Quantitative PCR Instrument (Bori, Hangzhou, China). The primers that designed for rhlR (Al-Kubaisy, 2018),

F-5′-CTCAGGATGATGGCGATTTC-3 ′and R-5′-AATTTGCTCAGCGTGCTTTC-3 ′and for the internal reference gene fbp F-5′ -CCTACCTGTTGGTCTTCGACCCG-3 ′ and R-5′-GCTGATGTTGTCGTGGGTGAGG-3′. The amplification step was carried out in 40 cycles in real time PCR included denaturation at 95 oC/20 second, annealing 59 oC/20 second and extension at 72 oC/20 second. The primers were designed according to ProbeGene (Shenyang, China), synthesized by Sangon Biotech (Shanghai, China). The differences in gene expression levels were calculated by ΔΔCT method according to (Parai et al., 2018).

Statistical Analysis

Data were analyzed using the statistical analysis application SPSS version 23 (Statistical Package for Social Science, Chicago, IL, USA). Data analysis was done using Chi-square for the comparison of categorical data. Data were expressed as means ± standard deviation and considered significant different when p value ≤ 0.05.

RESULTS AND DISCUSSION

Detection of N-acylhomoserine lactones (AHL) in P. aeruginosa isolates

According to the absorbance values recorded by microplate reader, all the isolates showed high levels of AHL molecules (Figure 1). The finding of this study is similar to those from a study, in Iraq, conducted by Al-Kubaisy, (2018). Modarresi and co-workers (2015) stated that AHLs formed by various bacteria differ in the length of the side-chain of the R-group. Both the production of AHL and the formation of biofilms were dose-dependent regulation of iron concentration.

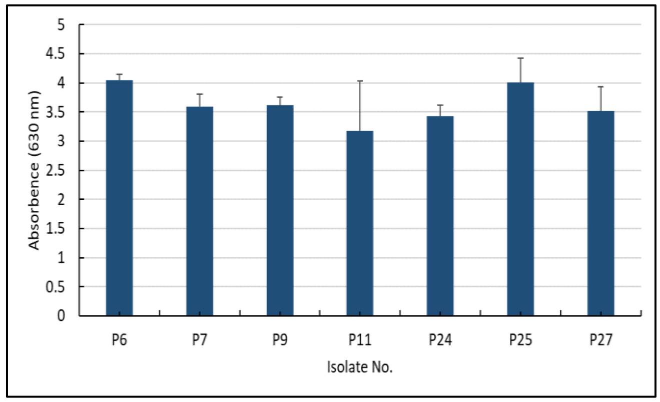

Figure 1: Determination the levels of N-acyl homoserine lactone (AHL) signal molecules in P. aeruginosa isolates by colorimetric method.

Screening of P. aeruginosa isolates for pyocyanin production

The results pointed out that different P. aeruginosa isolates produce varied levels of pyocyanin (Figure 2). The concentration of the produced pigment was ranged between 23.43 µg/ml (Pa9) to 31.5 µg/ml (Pa7). Generally, approximately 90-95% of all P. aeruginosa isolates produce pyocyanin as deep blue in colour (referred to as "blue pus" from pyocyaneus) (Jayaseelan et al., 2014). From clinical prospective, pyocyanin is a toxic secondary metabolite compound that causes cell lysis and release the cellular DNA (eDNA) to the surrounding space as part of the biofilm matrix. Once the eDNA is released, it binds to the pyocyanin making the solution more viscous. This action will enhance the physicochemical interactions of the biofilm structures with surrounding area causes the cells to aggregate (Thi et al., 2020).

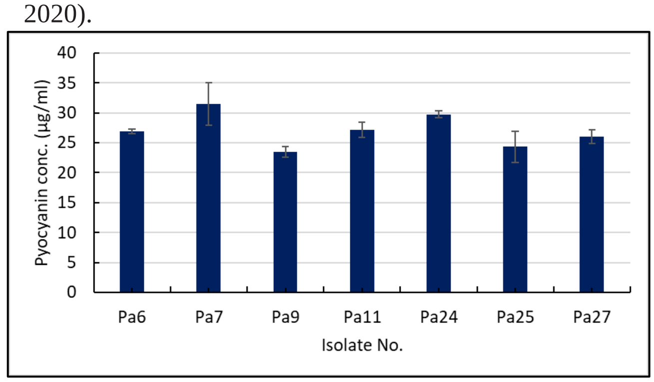

Figure 2: Screening of P. aeruginosa for pyocyanin production. The pigment concentration in liquid culture was measured as ug/ml (OD630 x 17.072).

In an Iraqi study, most of P. aeruginosa non-producer and producer pyocyanin isolates were obtained from burns sources by 34.92% and 23.81%, respectively. The concentration of the pigment in the burn isolates was 10.85 µg/ml and the highest level in wound isolates reached to 6.163 µg/ml (Khadim and Marjani, 2019).

The enhancement of pyocyanin production is clearly influenced by culture medium composition (Jayaseelan et al., 2014). In this study, Luria-Bertani broth medium was used in pyocyanin production test. DeBritto et al. (2020) investigated the effect of nutrients on pyocyanin production using King's medium and Nutrient medium. The enhancement rate of the pigment raised by 2.56 µg/ml in culture medium when supplemented with soya bean in comparison with 1.702µg/ml achieved nutrient medium with sweet potato.

Pseudomonas agar was developed and recommended for the production of pyocyanin by Pseudomonas species. This medium promotes the production of pyocyanin while inhibit production of other pigments. Different substances were included in culture media and confirmed to produce high levels of pyocyanin such as glycerol, alanine, sulphur, and iron.

Adding alanine and glycerol together in culture medium was extremely effective and served as a precursor in the pigment formation (Jayaseelan et al., 2014). Moreover, the differences in the amount of pyocyanin pigment within the isolates of P. aeruginosa can be due to presence of a regulators of the quorum sensing system named QteE. The overexpression in this regulator will reduce the accumulation of homoserine lactone signals affecting the production of pyocyanin (El-Fouly et al., 2015).

Ability of Lactobacillus spp. filtrate to inhibit of pyocyanin production

The effect of culture filtrate from Lactobacillus spp. bacteria was tested on P. aeruginosa Pa7. The culture filtrates did not show antibacterial activity against P. aeruginosa Pa7. As shown in Figure (3) there is a clear variation in the ability of Lactobacillus spp. isolates to inhibit the production of pyocyanin pigment from P. aeruginosa Pa7. The clinical isolate (Lac2) of Lactobacillus spp. is characterized by high ability to produce inhibitors substances for the pigment compared to the environmental strain (Lac9). In addition, it is clear that the ability of Lactobacillus isolates to inhibit pyocyanin production is not directly related to the volume of the culture filtrate. From therapeutic prospective, the importance of inhibition pyocyanin production came from their critical role in pathogenicity of P. aeruginosa as virulence factor. It is known that pyocyanin has negative impacts on host organs such central nervous system, cardiovascular system, respiratory system and urological system (Ismail et al., 2021).

Probiotic have shown different mode of action against pathogenic microbes in deferent ways that inhibit or kill the target, for example, destruction toxins production. However, not all probiotic bacteria can fellow these mechanisms which explain why some probiotics are effective against specific pathogen and not effective against other types of diseases (McFarland et al., 2018). According to Fuochi et al. (2019), Lactobacillus is well known produces a metabolites called postbiotics and many of these substances are partially or entirely characterized. However, it has been supposed that every Lactobacillus bacteria has the ability to produce its own distinctive postbiotics with beneficial effects to the host.

Natural products have recently emerged as a promising source for generating molecules that can potentially inhibit QS related anti-virulent activities (e.g. pyocyanin) (Fuochi et al., 2019). Amly and co-workers (2021) were investigated the effect of royal jelly on growth of P. aeruginosa and pyocyanin production. They found that royal jelly is effective in inhibition the production of pyocyanin pigment in dose-dependent manner. In contrast, at sub-MIC of royal jelly, the production pyocyanin was enhanced, suggesting use appropriate concentration of royal jelly in order to obtain beneficial virulence inhibiting activity (Amly et al., 2021). Plant extracts were also examined for their ability to inhibit pyocyanin production. For example, Callistemon citrinus leaf extract and their derived substance (Pulverulentone A) were used as source of inhibitors for production of pyocyanin in two strains of P. aeruginosa (Ismail et al., 2021). Jalli et al. (2019) also used a plant extracts from Phrynium capitatum and Dryptes indica and examined their potential anti-quorum sensing activity in P. aeruginosa PAO1.

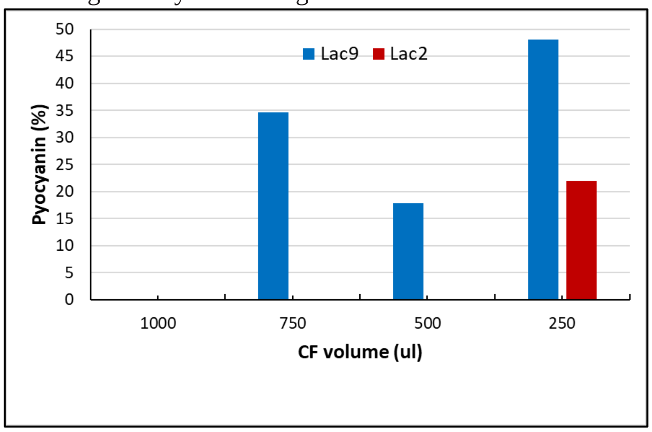

Figure 3: Assessment of fresh culture filtrate (48 hours) Lactobacillus spp. (Lac9, Lac2) for their ability to inhibit pyocyanin production of P. aeruginosa Pa7.

Molecular detection of rhlR gene mediated pyocyanin production

In this experiment, rhlR was detected in Pa7 isolate by conventional PCR technique. This isolate was showed high level of pyocyanin. As shown in Figure (4), rhlR gene was detected in using specific primer (233bp) according to other studies (Al-Kubaisy, 2018; Khadim and Marjani, 2019; Ali, 2021). The amplified products were resolved by gel electrophoresis with 1.5% agarose in the presence of molecular marker (DNA ladder size 100-1500bp). AL-Kubaisy (2018) showed that 98.41% of the tested P. aeruginosa were possessed rhlR gene.

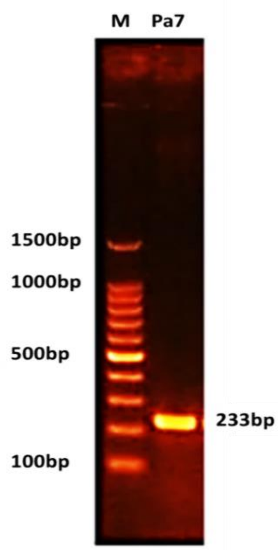

Figure 4: Gel electrophoresis image of the amplified rhlR gene P. aeruginosa Pa7. Lane 7: Pa7 for rhlR and Lane M: DNA ladder (100-1500bp).

Expression of rhlR gene RT-PCR

The total genomic RNA was extracted from P. aeruginosa Pa7 to measure the expression levels of rhlR. The assay was conducted before and after treatment with sterile culture filtrate (CF) produced from two isolates of Lactobacillus spp., Lac2 and Lac9, at volumes of 250 µl and 1000 µl under free-contamination conditions (particularly RNase). The expression level of rhlR was tested in comparison with the control group (untreated). The real-time PCR was performed to verify the effect of culture filtrate obtained from two Lactobacillus spp. bacteria, clinical (Lac2) and environmentally (Lac9) isolates, on expression levels of this gene. Both culture filtrates were used against the potent producer of pyocyanin, P. aeruginosa Pa7 with two volumes, 250 and 1000 µl of CF. The mRNA levels of the desired genes were normalized by using fbp gene to compare the variation between the samples in real-time PCR (TaqMan type). males and 80 females. The Data were analyzed with descriptive statistics to evaluate the correlation between CF obtained from Lactobacillus spp. Lac9 was inhibited synthesis of pyocyanin compared with that in the control group. The expression level of rhlR gene at dose 1000 µl was similar (1.19) to that with the control ($p \geq 0.05$). Treatment with 250 µl of CF is strongly up-regulated rhlR by 1.89-Fold ($p \leq 0.05$) (Figure 5). Regarding CF obtained from Lactobacillus spp. Lac2, the expression level of rhlR at dose 250 µl was similar (1.16-Fold) ($p \geq 0.05$) and dramatically decreased to o.46-Fold with increasing the dose to 1000 µl.

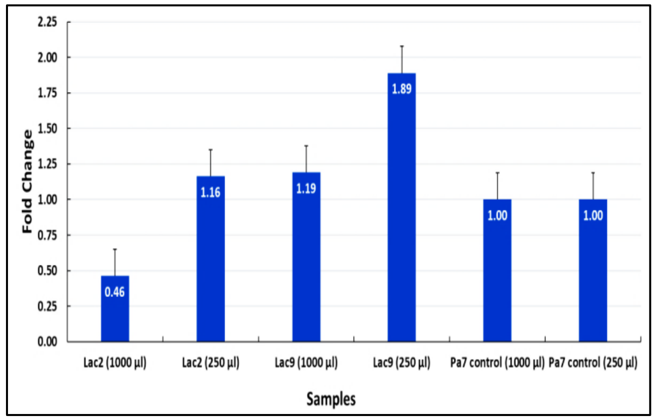

Figure (5): Comparison of folding change rate of rhlR gene between treated with Lac2 CF and untreated of Pa7 group. The asterisk indicates a significant difference between the treatment and the control group ($p \leq 0.05$).

The results of this study showed that the folding rates of genes that control pyocyanin production are differing from each another as shown in the following discussion. Lactobacillus is characterised as potent and important bacteria due to their advantages in controlling other microorganisms. The agents responsible for antimicrobial activity in Lactobacillus are varies, for example, production antimicrobial molecules such as H2O2, organic acids and bacteriocins (Ibrahim et al., 2021). Several mechanisms were proposed to explain the action of probiotic bacteria against other bacteria. Adhesion of probiotics cells to the intestinal mucosal surfaces and colonization in the intestinal cavity provide them preference in competition with the microorganisms that inhabit the same niche (Stavropoulou et al., 2020).

It is clearly that the culture filtrate of Lactobacillus affects the expression of some genes that influence the pyocyanin synthesis. The results showed that the inhibition by culture filtrate is dose-independent. The secondary metabolites in culture filtrate of Lactobacillus may contain small substances that act as antagonist/agonist for the tested genes which inhibits or stimulate pyocyanin production in P. aeruginosa suggesting that these genes could be a convenient target for antivirulence therapeutics strategy. Therefore, it is necessary to understand the mechanism of these molecules before applying the antivirulence strategy. rhlR is part of QS system genes in P. aeruginosa and it responsible on synthesis of N-(butanoyl)-L-homoserine lactone (C4-AHL) which interact with the cognate regulator RhlR (receptor) to form the complex AI-R and activate the target gene (Cruz et al., 2020).

In conclusion, Lactobacillus from environmental source showed a promising inhibitory activity which can be used as safe, ecofriendly and potent bacterial agent to overcome the growth and proliferation of pathogenic and food spoilage bacteria like P. aeruginosa.

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