The expression of type II TA system genes following persister cell formation in *Pseudomonas aeruginosa* isolates in the exponential and stationary phases

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Abstract

Failure of infection therapy in the presence of antibiotics has become a major problem which has been mostly attributed to the ability of bacterial persister cell formation. Bacteria use various mechanisms to form persister cells in different phases, among which is the toxin–antitoxin (TA) systems. This study aimed at investigating the expression of type II TA system genes under the stress of ciprofloxacin and colistin antibiotics in the exponential and stationary phases. To determine the effects of ciprofloxacin and colistin on persister cell formation in the exponential and stationary phases of *Pseudomonas aeruginosa* strains, colony counting was performed at different time intervals in the presence of fivefold MIC of ciprofloxacin and colistin. In addition, the expression of *relBE*, *Xre-COG5654*, *vapBC*, and *Xre-GNAT* genes in *P. aeruginosa* isolates was assessed 3.5 h after antibiotic treatment in the exponential and stationary phases using qRT-PCR. Our results indicated the presence of persister phenotype of *P. aeruginosa* strains in the presence of fivefold MIC of ciprofloxacin and colistin compared to the control after 3.5 h of incubation in the exponential and stationary phases. Also, the number of persister cells in the stationary phase was higher than that of the exponential phase. According to the results of qRT-PCR, ciprofloxacin and colistin may induce persister cells by increasing the expression of type II TA systems in stationary and exponential phases. Ciprofloxacin and colistin may increase the formation of persister cells by affecting the expression of type II TA systems.

Keywords Persister cell · Antibiotic ciprofloxacin and colistin · *P. aeruginosa* · TA systems · Real-time PCR · Exponential and stationary phases

Introduction

Chronic wound infections are mostly due to the formation of a subpopulation of bacteria called “persister cells” which can survive in the presence antibiotics (Sultana et al. 2016). Formation of these cells can cause recurrent infections, often resulting in treatment failure. These cells, with genetic and antibiotic minimal inhibitory concentration (MIC) values, are similar to antibiotic susceptible cells in the exposure of antibiotics by phenotypic switching which cause inactivation of physiological state, slow growing, and dormancy, and can tolerate high concentrations of antibiotics. However, after the antibiotic removal, the persister cells regrow and form a heterogeneous population, including tolerant and susceptible subpopulations equivalent to the parental culture. Most pathogenic bacteria can form persister cells, providing an opportunity for bacteria to become resistant and making infection treatment more challenging (Dzidic...
and Bedeković 2003; Narimisa et al. 2020). Several mechanisms contribute to the formation of persister cells, with TA systems being one of them, and in particular type II TA systems. TA systems are categorized into five groups according to their gene-encoded products. Meanwhile, toxins have an effect on RNA, ribosomes, and DNA gyrase in the cell and cause bactericidal and bacteriostatic responses. An antitoxin is an RNA sequence or protein which binds to the toxin and blocks its activity. Toxin and antitoxin of type II TA systems are both proteins with a wide distribution in bacteria (Mohammadzadeh et al. 2020; Narimisa et al. 2020a, b; Rocker and Meinhart 2016). The mechanism of TA systems is based on a stable toxin as well as a labile antitoxin located on a plasmid or chromosome. Under stress conditions such as antibiotic exposure, cellular proteases degrade the antitoxins, thereby liberating the toxins to disrupt essential cellular processes (replication, translation, etc.) (Gerdes et al. 1986; Masuda and Inouye 2017). Also, TA systems have the ability of controlling the secondary messenger 3′,5′-cyclic diguanylic acid in response to stress and might play a key role in formation of persister cells (Wang and Wood 2011). For example, in the model organism Escherichia coli, quorum sensing or general stress responses plus TA modules including HipBA as global regulators have been associated with the persister phenotype regulation (Lewis 2010; Wang and Wood 2011). Pseudomonas aeruginosa, as a Gram-negative pathogen, is a key cause of nosocomial infections which can form a specific biofilm containing alginate. This pathogen has been described as a major cause of mortality in patients suffering from cystic fibrosis due to biofilm formation following colonization in the lungs. The patients suffering from chronic diseases or immune system defects, such as immunodeficiency or burns, are more susceptible to infections. These infections often become chronic and show high mortality (Neidig et al. 2013). For instance, P. aeruginosa colonizes the lungs of cystic fibrosis patients and is considered as a major cause of death in these patients (Oliver et al. 2000). Apart from intrinsic tolerance of P. aeruginosa against a broad spectrum of antibiotics, the presence of specialized persister cells makes it virtually impossible to fully eradicate these bacterial populations (Hemati et al. 2014). Therefore, this study aimed to evaluate persister cell formation of P. aeruginosa PA01 plus clinical P. aeruginosa in the exponential and stationary phases after exposure to ciprofloxacin and colistin antibiotics. Moreover, due to the implication of TA systems in persister cell formation, the impacts of lethal concentrations of selected antibiotics were analyzed on the expression levels of type II TA systems in the examined isolates.

### Materials and methods

#### Bacterial strain and growth conditions

In this research, P. aeruginosa PA01 and clinical P. aeruginosa isolates were used. The clinical P. aeruginosa was isolated from a 54-year-old patient admitted to the burn ward who died of severe burns and infection. [The ethics committee of the Iran University of medical sciences has approved the study protocol (code:IR.IUMS.FMD.REC.1398.161). The patient signed a written informed consent form before died.] The exponential phase of the planktonic cells was prepared by the inoculation of 105 CFU/ml of bacteria in LB broth and incubation for 3 h at 37 °C. Stationary phase of the planktonic cells was obtained by the inoculation of 105 CFU/ml of bacterial cells in LB broth and incubation for 18 h at 37 °C (Spoering and Lewis 2001).

#### Identification of type II TA systems

Type II TA systems in P. aeruginosa were predicted by TADB database, and as reported in Table 1, specific primers were designed using NCBI designing primer tool (Chuang et al. 2012).

| Gene Names | Sequence (5′→3′) | Annealing temperature (°C) | Product length | Reference |
|------------|------------------|---------------------------|---------------|-----------|
| T.relE     | F: GGACGCCATCTACGACCCATT R: ACGATGCCTGAAACGGCTTTT | 58 | 72 | This study |
| T. Xre-COG | F: CAATTGGTCGACACCTGGA R: GTAGAACAGGCTGGCTCGT | 58 | 177 | This study |
| vapBC */Xre-PIN | F: CCCCTGTTGGGAGCAATTACGAG R: AGGGTTTCCAGCTTTGCTCAG | 58 | 76 | This study |
| -/Xre-GNAT | F: CAAGCATGCTTCCGACCACC R: GTCCGGTGATGCTGTAGACAG | 58 | 178 | This study |
| 16S. PSEU  | F: GCCCTACAGTTGCATTCCG R: ATGAAGATCAGTGGCGG | 58 | 99 | This study |

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Polymerase chain reaction (PCR) was performed to screen the studied isolates for the existence of type II TA system genes, including *relBE*, *Xre-COG5654*, *vapBC*, and *Xre-GNAT*. PCR was prepared in a total volume of 25 μl containing 12.5 μl MasterMix PCR (Ampliqon, Denmark), 1 μl of primers, 2 μl of template DNA, and 6.5 μl DDW. Thermal cycling included an initial denaturation step at 95 °C for 5 min; 34 × (95 °C for 45 s, annealing at 58 °C for 45 s, and extension at 72 °C for 30 s), with a final extension step at 72 °C for 5 min. PCR products were analyzed through electrophoresis on 1% (w/v) agarose gel (Merck, Germany) containing DNA safe stain. Agarose gels were visualized via gel documentation (Bio-Rad, USA).

**Minimum inhibitory concentration (MIC) determination**

The microdilution method was employed to determine the MIC of ciprofloxacin (Sigma-Aldrich) and colistin (Sigma-Aldrich) against *P. aeruginosa* isolates according to Clinical and Laboratory Standards Institute (CLSI-2019). To determine the MICs of ciprofloxacin and colistin (Sigma-Aldrich), 100 μL of overnight bacterial culture (dilution of 1:100 in MHB, set according to 0.5 McFarland) and 512 mg/l of the tested antibiotic were diluted serially (512–0.25 mg/l) in 96-well microtiter plates and incubated at 37 °C for 18 h. MIC was defined as the minimum concentration of the antibiotic preventing the visible bacterial growth after 18 h of incubation. MIC values for the examined antibiotics were determined in triplicate (Sobouti et al. 2020).

**Persistence assay in the exponential and stationary phases with antibiotics ciprofloxacin and colistin**

Persister cells of *P. aeruginosa* isolates were investigated through exposing the isolates in the exponential and stationary phases to fivefold MICs of ciprofloxacin and colistin. To measure the number of persister cells, a single colony of the isolates was inoculated into 5 mL LB broth (Merck, Germany) for 24 h. Then, 50 μl of sub-cultured isolates were re-suspended in 50 mL of LB broth and bacterial cultures were incubated at 37 °C in an incubator shaker (Bio-Rad, USA) at 220 rpm until reaching an optical density (at 600 nm) of 0.4 after 3 h of incubation (exponential phase). Also, the optical density of 1.3 after 18 h of incubation was designated as the stationary phase. To induce persister cells, isolates in the exponential and stationary phases were treated with fivefold MICs of ciprofloxacin and colistin and incubated at 37 °C in an incubator shaker at 220 rpm for 3.5 h (Möker et al. 2010).

**Colony counting**

The number of formed persister cells was specified by plating the antibiotic-treated cultures on LB agar plates followed by measuring the colony forming unit (CFU), representing the number of cells that survived the antibiotic exposure. For this purpose, samples were washed twice with ice-cold PBS and were added to LB broth, and incubated at 37 °C in incubator shaker at 220 rpm. Samples were subsequently diluted in the same buffer (PBS). Samples were plated on LB agar plates and cultured on LB agar for four times every 20 min. Bacterial culture without antibiotics was used as the control (Möker et al. 2010).

**Quantitative real-time polymerase chain reaction**

For assessing the expression of type II TA system genes, RNA of isolates in the exponential and stationary phases was extracted in the presence of fivefold MIC of ciprofloxacin and colistin. Bacteria were pelleted through centrifugation at 2500×g for 15 min. Total RNA was isolated using the RNA extraction kit (Gene all, Korea). NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) and gel electrophoresis analysis were utilized to assess the concentration, quality, and integrity of the extracted RNA. Next, the extracted RNA was treated with DNase1 (Roche, Germany) according to the manufacturer’s protocols with total RNA reverse transcribed to cDNA via the cDNA synthesis Kit (Betabairn, Germany).

Finally, quantitative real-time PCR was carried out in a Rotor-Gene thermal cycler (Corbett 6000; Australia) using SYBR Green method (AccuPower Green Star qPCR Master Mix; Bioneer; Korea). Specifically, a 20 μl reaction containing 2 μl of cDNA, 12.5 μl SYBR Green Master-Mix, 4.5 μl nuclease-free water, and 1 μl of each primer (5 pmol) was subjected to PCR as follows: an initial activation step at 95 °C for 12 min, followed by 40 cycles of denaturation at 95 °C for 20 s, and annealing at 58 °C for 35 s. The 16 s rRNA gene was applied as an internal control for normalizing the mRNA levels and fold changes. mRNA expression was calculated via the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

**Statistical analyses**

All the statistical analyses were carried out by GraphPad Prism (GraphPad Software, Inc). Student’s *t* test (for two groups) and analysis of variance (two-way ANOVA) were employed. *P* value of less than 0.05 was considered statistically significant.
Result

Detection of type II TA system genes by PCR

The results indicated the existence of all the studied genes (relBE, Xre-COG5654, vapBC, Xre-GNAT) coding for the TA systems in the examined isolates.

Minimum inhibitory concentration of \textit{P. aeruginosa} isolates

In this research, we first specified the MIC ranges of ciprofloxacin and colistin against \textit{P. aeruginosa} strains using the broth microdilution assay. MIC range of the strains with the selected antibiotics was 1 μg/mL, while the value for the clinical isolate with colistin was 0.5 μg/mL.

Persister formation assay in the exponential and stationary phases and viable bacterial counts

To assess the effects of ciprofloxacin and colistin on bacterial survival in the exponential and stationary phases, isolates were exposed to fivefold MICs of ciprofloxacin and colistin antibiotics for 3.5 h. Most isolates were killed by ciprofloxacin and colistin, and only a small portion (w1%) survived the challenge of these antibiotics. Viable counts were measured for 1 h at 20 min intervals.

As shown in Figs. \ref{fig:1} and \ref{fig:2}, the growth of \textit{P. aeruginosa} isolates was strongly prevented in the presence of fivefold MIC of ciprofloxacin and colistin. In the exponential and stationary phases, bacterial count reduced for 1 CFU/ml compared to the control, after 3.5 h exposure to the tested antibiotics. Accordingly, fivefold MIC of ciprofloxacin and colistin reduced the bacterial growth. These results revealed the variations in bacterial survival after antibiotic treatment owing to persister cells. According to the statistical analysis, at times 20, 40, 60, and 80 min, the counts of PAO1 and clinical isolates in the exponential and stationary phases

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Time-dependent killing of PAO1 and clinical isolates exposed to ciprofloxacin. The bacterial culture without any antibiotic treatment served as the control. The values are indicative of the means of three biological replicates and error bars indicate the standard deviation (independent samples \textit{T} test, repeated measure, LSD)}
\end{figure}
treated with ciprofloxacin and colistin were significantly different from the control group (Figs. 1 and 2).

**Effects of ciprofloxacin and colistin on the expression of type II TA system genes in *P. aeruginosa* in the exponential and stationary phases**

Our results indicated the different expression levels of TA systems (relBE, Xre-COG5654, vapBC, Xre-GNAT) in *P. aeruginosa* strains in the exponential and stationary phases. Furthermore, the results of the binary comparison revealed a significant difference between the expression of TA system genes (relBE, Xre-COG5654, vapBC, Xre-GNAT) in the standard strain (PAO1) and clinical isolate of *P. aeruginosa*. Persister cells the PAO1 strain formed after ciprofloxacin exposure showed the significant increased expression of relBE/RHH-RelE, /Xre-COG5654, /Xre-GNAT, vapBC */Xre-PIN TA systems in the exponential phase. A similar trend was observed in the clinical isolate, except for the vapBC */Xre-PIN system. In the stationary phase, the expression of relBE/RHH-RelE was decreased in the clinical isolate, and in the PAO1 strain, the expression of the relBE/ RHH-RelE and vapBC */Xre-PIN was decreased (Fig. 3).

During the exponential phase of persister cells caused by colistin exposure, the expression of vapBC */Xre-PIN and /Xre-COG5654 systems decreased in both clinical and PAO1 isolates. In the stationary phase, the expression of /Xre-GNAT and /Xre-COG5654 systems decreased for both clinical and PAO1 isolates (Fig. 3). Our results can provide information about the potential role of the TA systems in the persister cell formation.

**Discussion**

Persistent infections are generally arising from difficult-to-remove pathogens that have not developed classical resistance. *P. aeruginosa* has the ability to adapt to the host environment, causing a chronic infection that cannot totally be removed by antibiotic treatment. Failure of infection treatment has been linked with the evolution of antibiotic resistance as well as the presence of antibiotic-tolerant persister cells (Li et al. 2018). Different mechanisms have been
introduced as the cause of persister cell formation, among which is the TA systems. TA systems can be categorized into five groups based on their gene products (type I, II, III, IV, or V). TA toxins can interact with RNA, ribosomes, or DNA gyrase, leading to a bacteriostatic or bactericidal reaction within cells and causing the formation of persister cells (Wang et al. 2012; Yamaguchi et al. 2011). In this study, we investigated the role of type II TA systems in the induction of persister cells in clinical and standard (PAO1) *P. aeruginosa* isolates by evaluating the expression of type II TA system genes (*relBE/RHH-RelE, vapBC*/Xre-PIN, /Xre-COG5654, and /Xre-GNAT*) in the exponential and stationary phases after exposure to ciprofloxacin and colistin. RelE is a sequence-specific, ribosome-dependent mRNA endoribonuclease that inhibits translation during amino acid starvation (the stringent response).

In this study, we showed that type II TA systems were involved in the formation of persister cells in *P. aeruginosa* strains. Different expression levels of these systems were observed in the exponential and stationary phases, indicating different functions of TA systems in persister cell formation in different phases of bacterial growth (Fig. 3). Similar alterations in the expressions of type II TA system genes were observed in both bacterial isolates in the exponential and stationary phases. Also, there was no difference between the ability of the tested antibiotics in changing the expression of the systems except for the vapBC system. In fact, expression of the vapBC system was different in both clinical and PAO1 strains in the exponential and stationary phases after exposure to ciprofloxacin and colistin antibiotics (Fig. 4). In 2014, Oksana I. Demidenok et al. reported that the vapBC system in *Mycobacterium smegmatis* causes a dormant mode (Demidenok et al. 2014). In a follow-up study by Cooper et al. in 2009, the global transcriptional analysis of *S. solfatarius* was carried out following heat shock (temperature shift from 80 to 90 °C). According to their results, several
vapBC genes were triggered by the thermal shift, suggesting implication of this system in heat-shock response (Cooper et al. 2009). VapC is a tRNA-(fMet) endonuclease, and it cleaves both charged and uncharged tRNA-(fMet) between positions 38 and 39 at the anticodon stem–loop boundary. Overexpression in *E. coli* inhibits translation, and leads to loss of cell growth and degradation of tRNA(fMet); these effects are neutralized by expression of cognate antitoxin VapB. In this research, we also showed that the expression of the vapBC system was different in *P. aeruginosa* compared to other type II TA systems here. These results are consistent with the studies mentioned above and indicate the role of this system in the persister cell formation. What this study adds to the previous data is the different behavior of this system in the strains. Amraei F et al. showed that the increased expression of type II TA systems in *Brucella* may lead to antibiotic resistance and persister cell formation (Amraei and Narimisa 2020). The contribution of TA systems in bacterial persister cell formation was initially discovered in 1983 by Harris Moyed. He found that two mutations in the hipA gene enhanced bacterial tolerance to antibiotics (Moyed and Bertrand 1983).

In 2016, Cheverton and colleagues discovered that GNAT of a type II TA system by acetylation of the primary amine group of charged tRNA caused blocking translation and was associated with the formation of intracellular Salmonella persisters (Cheverton et al. 2016). In this study, according to statistical analysis, the expression of type II TA systems varied depending on the stage of bacterial growth. In conclusion, at each phase, different systems play a role given their mechanism of action. In 2021, Golmoradi Zadeh et al. showed the formation of persister cell within the biofilm of *P. aeruginosa* strains following exposure to fivefold MIC of ciprofloxacin and colistin antibiotics (Zadeh et al. 2021). To

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**Fig. 4** Expression levels of type II TA system genes in PAO1 and clinical isolates in the presence of fivefold MIC of ciprofloxacin (A exponential phase B stationary phase) and colistin (C exponential phase D stationary phase). Graph data are indicated as the means ± SD of three independent replicates. *P < 0.05. **P < 0.01. ***P < 0.001. ****P < 0.0001 (by independent samples T test)
evaluate the persister cell formation in the presence of ciprofloxacin and colistin in each strain, colonies were counted in stationary and exponential phases. The results indicated that the amount of persister cells was different in exponential and stationary phases, such that the value of persister cells in stationary phase was higher than exponential phase in both strains (Fig. 5). However, there was no significant difference between the two antibiotics in terms of inducing persister cell formation in the two strains. There was no significant difference either between PAO1 and clinical strains considering persister cell formation. In the exponential phase, similar numbers of persister cells were formed in both strains, and in the stationary phase, there was no difference in the persister cell formation in both strains after exposure to ciprofloxacin and colistin (Figs. 5, 6). In 2018, Ying Wang et al. found a difference between the rate of persister cell formation in *Staphylococcus aureus* in the stationary and exponential phases after exposure to ciprofloxacin. They showed that the number of persister cells formed in the stationary phase was higher. They also observed that in the exponential phase, the α-ketoglutarate dehydrogenase genes of the tricarboxylic acid cycle (TCA) were expressed in small amounts during persister cell formation, while in the stationary phase, TCA genes were suppressed during the formation of persister cells, and therefore, more persister cells were formed (Wang et al. 2018). Also, in 2004, Keren et al. concluded that when *S. aureus* enters the stationary phase, persister cell formation increases dramatically (Keren et al. 2004). In 2009, Ilana Kolodkin-Gal et al. found that in *Escherichia coli*, the sigma factor during the stationary phase is responsible for the resistance of dormant bacteria to mazEF-mediated cell death (Kolodkin-Gal and Engelberg-Kulka 2009). According to previous studies, suppressed expression of TCA genes or increased expression of sigma factor during the stationary phase may increase the formation of persister cells. Also, according to our study, difference in the expression of type II TA systems involved in the formation of persister cells in each phase may increase the persister cell formation in the stationary phase as compared to the exponential phase, which requires further studies.

**Conclusion**

The results of this study indicated that the expression of type II TA system genes in persister cells of *P. aeruginosa* increased compared to the control in exponential and stationary phases. More studies are required to identify the exact contribution of these genes to the formation of *P. aeruginosa* persister cells.
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Author contributions  This article was part of a study that contributed to an MSC thesis by RGZ. BSK and FMJ designed the study. RGZ and MM performed the experiments of the research and drafted the manuscript, and JP and MB performed the statistical analysis. All authors read and approved the final manuscript.

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Availability of data and materials  All the datasets supporting the conclusions of this article are available. Additional data of this paper can be obtained upon request. The corresponding author (Faramarz Masjedian Jazi: Masjedian.f@iums.ac.ir) should be contacted if someone wants to request the data from this study.

Declarations

Conflict of interest  Rezvan Golmorad Zadeh, Maryam Mirshekar, Behrooz Sadeghi Kalani, Johar Pourghader, Mahmood Barati, and Faramarz Masjedian Jazi declare that they have no conflict of interest.

Ethical approval and consent to participate  The ethics committee of Iran University of Medical Sciences has approved the study protocol (code: IR.IUMS.FMD.REC.1398.161). All methods were performed in accordance with the relevant guidelines and regulations/Declaration of Helsinki. The patient signed a written informed consent form.

Consent for publication  Not applicable.

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