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Recovery and characterization of *Proteus mirabilis* persisters

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ABSTRACT

Bacterial persistence is a phenomenon in which a subpopulation of cells survives antibiotic treatment. The occurrence of bacterial persisters is associated with recurrence of chronic infections. In this study, we aimed to isolate, characterize persister subpopulation in *Proteus mirabilis.* Persister cells isolation was done by the dose-dependent killing of ciprofloxacin. Their characterization was achieved by determining their growth rates. Our results revealed that 1.3% of persister cells could be recovered from the *Proteus mirabilis* test isolate. Upon resuscitation, these cell subpopulations exhibited slow growth rate than wild-type cells. As a common phenomenon demonstrated among microbial pathogens, *Proteus mirabilis* persisters could be isolated with ciprofloxacin. The slow growth rate is one of its characters recorded in the study for persister cells of such bacterial species.

Keywords: *Persisters; Multidrug tolerance; Proteus mirabilis; Ciprofloxacin; Survival*

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1. INTRODUCTION

Microbial persistence is the particular capacity of microbes to be able to tolerate immediate contact with lethal ranges of bactericidal antibiotics **[1]**. In 1944, the first scientist discovered bacterial persistence was Joseph Bigger when he was trying to eliminate *Staphylococcus aureus* population with penicillin. A small number of cells was found that could stand high lethal antibiotic concentrations **[2]**. They are dormant, multidrug tolerant, phenotypic variants rather than mutants and have been produced by all microbial pathogens analyzed to date. On removal of the antibiotic stress, they switch back to a growing state, by this means giving rise to a new population that is genetically identical to the wild-type cell **[3,4]**. Their tolerance to antibiotics is mechanistically distinct from resistance **[5,6]**. Additionally, the formation of persisters is growth phase-dependent, and their particular number is low inside exponentially growing cultures (0.0001%) and this number attains its maximum inside stationary phase which accounts up to 10% from the whole population **[7]**.

Antibiotics mainly target actively developing cells whereas the drug-tolerant cells are generally dormant. Therefore, this was the first direct

statement to the mechanism of persistence. The second mechanism, activation of the toxin-antitoxin module that can account for the concurrence of two different subpopulations with varying sensitivity to the antibiotic **[8]**. Transient growth arrest arises in those cells that the toxin concentration rises above the threshold, enabling such cells to persist. Consequently, a transitory growth arrest is the main functional phenomenon associated with persistence **[9]**. Persisters may be a middle stage in the development of drug resistance owing to their ability to undergo cellular division during persistence. In addition, persisters can accelerate mutagenesis and horizontal gene transfer due to their stress-response mechanisms **[3]**. Therefore, persisters may play a role in the development of multidrug-resistant bacteria **[3]**. Eliminating persisters or preventing their emergence should be a substantial target in the treatment of bacterial infections **[10]**. At the same time, associated problems such as the development of transmissible resistance, toxicity, undesirable side effects should be kept in focus. The aim of the present study was to investigate the formation of persister cells in the pathogen *Proteus mirabilis*, the possible phenotypic change associated with its formation.

2. MATERIALS AND METHODS

2.1. Microorganism and growth conditions

A clinical *Proteus mirabilis* isolates from urine specimen was used in this study. The isolate was identified using their growth characteristics on general, some selective and diagnostic culture media, as well as their biochemical reactions in the classical pathway. The identification was confirmed using API 20E kit (bioMérieux, France). This isolate was cultured in Luria Bertani (LB) medium **[11]**. For different experiments, a loopful from grown culture on LB agar slant was used to inoculate 5 mL LB broth contained in a test tube. The tube was incubated overnight at 37 °C. The culture was centrifuged at 12000 rpm for 5 min at room temperature and the pellets produced were washed twice with 0.9% saline and resuspended in saline. To adjust the count of the resultant suspension, the optical density (OD) was adjusted to 600 nm of 0.1 to get 10^8 CFU/mL and in different experiments; the test organism cell suspension was prepared in the same way.

2.2. MIC determination

The MIC of ciprofloxacin (Amriya Pharm IND, Alexandria, Egypt) was determined using the agar dilution technique. Experiments were carried out in at least three independent replicates **[12]**.

2.3. Persistence assay

Persistence was measured by determining survival upon exposure to ciprofloxacin (Cip) by using dose-dependent killing procedure **[13]**. In this method, the test isolate was challenged with various concentrations of Cip beginning with 10 fold MIC for 24 h. *Proteus mirabilis* cell suspension was prepared as described above to be treated with different concentrations of Cip for 24 h at 37 °C and 200 rpm. After treatment, the cells were washed twice with and resuspended in 0.9% w/v saline. The number of viable persister cells was then quantified by spotting 10 µL aliquots of the cell suspension on MacConkey's agar plates. The log CFU/mL values of survivors were plotted against Cip concentrations. A distinct biphasic killing pattern with a plateau of surviving persisters could be obtained. One test concentration of ciprofloxacin within the plateau part of the dose-dependent ciprofloxacin killing curve was selected to be used for recovery of persister cells of the test organism in further experiments **[13]**.

2.4. Persister revival assay

Persister cells were recovered by exposing the cells suspension of the test organism, prepared as mentioned before to 30 μg/mL ciprofloxacin for 24 h. The cells were collected by centrifugation at 12000 rpm for 5 min then washed twice with 0.9% w/v saline followed by resuspension in 5 mL LB broth. The persister cells were allowed to resuscitate for 6 h at 37 °C during which the growth was monitored every hour by measuring the optical density of the cell suspension at 600 nm. As a control, the wild-type cells without ciprofloxacin exposure were similarly treated. The experiment was carried in triplicates and the growth as a function of time was presented graphically **[14-16]**.

2.5. Statistical analysis

All values are expressed as means ± standard deviations (SD). The results of persister cells revival were statistically analyzed using student t-test at 95% confidence interval using Graph Pad Prism® Version 5.0 (GraphPad Software, La Jolla, CA, USA).

3. RESULTS

3.1. Persister cell isolation

Ciprofloxacin dose-dependent killing (MIC= 0.031μg/mL; sensitive) of *Proteus mirabilis* test isolate experiment showed a biphasic killing pattern **(Fig. 1)** The bulk of the population was effectively killed with 0.31 μg/mL ciprofloxacin (10 fold MIC) followed nearly by a plateau with minimal decrease in survival up to 40 μg/mL ciprofloxacin concentration. The percentage of survival at 30 μg/mL ciprofloxacin was recorded to be 1.3% of the initial cell count and that at 40 μg/mL ciprofloxacin (1000 fold MIC) was about 1.5%. The survivors along the plateau of ciprofloxacin dose-dependent killing curve could be considered as persisters as they survived the antibiotic treatment. To ensure the persistence, the MIC of these survivors against ciprofloxacin showed no change in its value as compared to the wild-type cell population of the test organism. Additionally, no colonies appeared on the 24 h at 37 °C incubated LB agar plates containing 30 μg/mL ciprofloxacin that were pre-inoculated with survival cells as compared to their growth on LB agar plates alone without added ciprofloxacin.

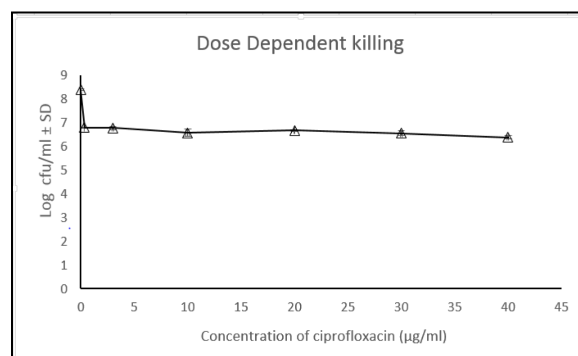

Fig. 1. Ciprofloxacin dose-dependent killing of clinical *Proteus mirabilis* isolate. The survivors after exposure of the test organism cell suspension to various concentrations of ciprofloxacin was determined after 24 h contact period and the error bars represent the standard deviation of log-values of the means of three independent experiments were then calculated.

3.2. Characterization of persister cells

The persister cells obtained after prolonged treatment with ciprofloxacin (24 h) were characterized by determining their growth profile after regeneration in the nutrient medium. Regeneration in LB show growth profile similar to the normal wild type cells but with a lower rate **(Fig. 2)**. After 5 h incubation in LB broth under the growth conditions, the persister cells attained an OD approximately equal to 30% that of the wild-type cells. The results were analyzed using student t-test at 95% confidence interval giving

p<0.01 that indicates the significance of the results.

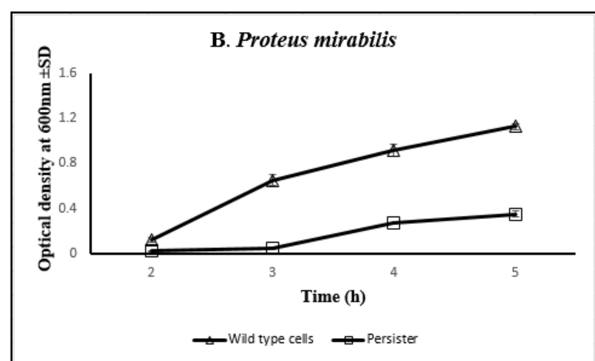

Fig. 2. Growth profile of resuscitated *Proteus mirabilis* persisters recovered at 30 µg/mL ciprofloxacin in normal saline, 37 °C and 200 rpm versus untreated control. The OD values represent the means of triplicates ± SD.

4. DISCUSSION

Bacterial persistence is abundant in many bacterial species. Most previous studies have been investigated on *Escherichia coli*. In this study, the persistence phenomenon was studied in *Proteus mirabilis*. This organism is mostly known to cause recurrent urinary tract infections that are usually occurred in patients under long-term catheterization. As well as increasing drug resistance to ampicillin, trimethoprim, and ciprofloxacin was reported for *Proteus mirabilis* **[17]**.

Persistence is the intermediate stage between sensitive wild strain and resistant mutant before resistance being established **[18]**. Accordingly, if we are capable to inhibit persistence, resistance to antimicrobial agents would decrease and help in ameliorating chronic infections. Persisters represent a small subpopulation of a culture that survives killing with lethal doses of antimicrobial agents **[19-21]**.

Persistence is detected when the bulk of the population is rapidly killed leaving a minor subpopulation persists for a much longer time. In some studies, the authors used 24 h or 48 h to isolate it **[22,23]** while other studies used 3 h or 4 h instead **[20,24]**. The dose-dependent killing curve is characterized by being biphasic, due to the heterogeneous response of the population. When survivor cells are isolated, they allowed to regrow in rich medium and then re-exposed to the same antibiotic treatment. The same heterogeneous response to the drug was observed similar to that in the original population. In accordance with the previous literature, the level of persisters within culture reached approximately 1% to maximum of 10% in stationary phase **[25, 26]**.

In this study, persisters from *Proteus mirabilis* upon exposure to ciprofloxacin (1000 fold) MIC for 24 h represent 1.3% of the total cell population in the stationary phase. The fluoroquinolone ciprofloxacin is the antibiotic of choice for the treatment of this kind of bacterial infections. In the present study, it represents a worthy example of synthetic drugs against which bacteria have quickly advanced resistance. Additionally, it kills both normal growing cells and slowly growing or non-growing stationary-phase cells, leaving dormant persisters intact. In addition, the dose-dependent killing curve shows biphasic killing.

We hypothesize that tolerance to antibiotics may be due to the shutdown of a target function leaving metabolically inactive dormant cells in which antimicrobial agents could not kill. It looks that in most cases inhibition of the target function is the instantaneous cause of antibiotic tolerance, while the decrease in growth rate is a result of this inhibition. Additional experimentations are needed to know the mechanism of *Proteus mirabilis* persistence against ciprofloxacin. According to Dörr *et al.* **[27]**, fluoroquinolones induce persistence in *Escherichia coli* by preventing ligation reactions of gyrase and topoisomerase resulting in double-strand breaks

(DSB). Processing of DSBs leads to the induction of the SOS response, the result of which is the production of various repair proteins **[27]**. This leads to the formation of tolerant persister cells. That is considered an effective bacterial survival strategy for *E.coli* against ciprofloxacin.

Remarkably, the antibiotic tolerance of persisters is not genetically expressed as progenies of persisters are as susceptible as the parent strains. Consistent with a number of studies, persisters among an isogenic bacterial culture temporarily reside in a slow or non-growing state and arise both stochastically **[7,28-31]**. Researchers proved that upon resuscitation of persister exhibit slow growth rate in comparison with the control **[32]**. After resuscitation, regrown persisters remain sensitive to ciprofloxacin similar to the wild-type cell. The result noticeably showed that surviving persisters regenerate the original population and are therefore phenotypic variants of the wild type rather than resistant mutants.

5. CONCLUSION

Overall, this study was the first to report the persistence phenomenon in *Proteus mirabilis.* Persisters can be isolated with antibiotic treatment (ciprofloxacin). The slowdown of metabolic functions of subpopulation persister cells was evidenced by the slow growth rate upon resuscitation.

Conflicts of Interest

The authors declare no conflict of interest.

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