Antibiotic Resistance in *Pseudomonas Aeruginosa* is Associated with Decreased Fitness

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Key Words

*Pseudomonas aeruginosa* • Nosocomial infection • Bacterial resistance • β-lactam antibiotics

Abstract

The number of clinical specimens containing β-lactam-resistant *Pseudomonas aeruginosa* isolates is increasing. However, whether resistance is associated with reduced fitness is still uncertain in clinical *Pseudomonas aeruginosa* isolates. In this study, we aimed to determine whether β-lactam resistance conferred a fitness cost in *Pseudomonas aeruginosa*. Growth rate, extracellular slime production, elastase activity, proteolytic activity, LasA protease activity, biofilm formation, and pairwise *in vitro* competition experiments were investigated in a collection of 11 isogenic, β-lactam-susceptible and -resistant (≥8-fold increase in minimum inhibitory concentration (MIC)) pairs of *P. aeruginosa* clinical isolates; each pair was recovered from a different patient treated with β-lactam antibiotics. All β-lactam-resistant *Pseudomonas aeruginosa* isolates showed a significant reduction in elastase activity. In addition, 90.9% (10/11) of β-lactam-resistant *Pseudomonas aeruginosa* isolates were associated with markedly lower growth rate and proteolytic activity, and 81.8% (9/11) of β-lactam-resistant *Pseudomonas aeruginosa* isolates had less extracellular slime production, compared to susceptible isolates. Meanwhile, LasA protease activity and biofilm formation ability were variable among isolates. Pairwise *in vitro* competition experiments showed that 72.7% (8/11) of β-lactam-susceptible strains could outgrow resistant strains. In conclusion, resistance development with β-lactam exposure confers a fitness cost, resulting in a decreased invasion potential, while the effect on viability varied. Thus, the potential for the dissemination of β-lactam-resistant *Pseudomonas aeruginosa* clinical isolates should not be underestimated.
Introduction

Pseudomonas aeruginosa is an opportunistic pathogen and a leading cause of nosocomial infection [1]. In the United States, the National Nosocomial Infection Surveillance System ranked P. aeruginosa first among all nosocomial pathogens related to pneumonia in intensive care units [2]. In China, Nosocomial Pathogens Resistance Surveillance date showed that P. aeruginosa ranked first among all Gram-negative pathogens [3]. P. aeruginosa infection is life threatening as the clinical strains are often difficult to treat because of their virulence and antimicrobial resistances [4]. β-lactam antibiotics are currently the most effective treatments for P. aeruginosa infection. However, β-lactam resistance is increasing [1, 5-7], and trends toward greater resistance rates have been found in the Asia-Pacific region [1, 6]. β-lactam resistance in combination with widespread infection and the unique virulence of this pathogen makes it a significant public health concern, leaving few therapeutic options against P. aeruginosa [8].

The ability of P. aeruginosa to cause a broad range of infections in humans is due, at least in part, to its adaptability and its capacity to regulate the expression of key virulence genes in response to unique environmental conditions [9]. Fitness cost, characterized by impaired bacterial physiology, often accompanies antibiotic resistance, enabling susceptible bacteria to outcompete resistant strains [10, 11]. Several studies support this concept, but in some cases, fitness cost has been found to be low or overcome in clinical strains [12, 13]. For example, P. aeruginosa strains may accumulate drug resistance genes, while maintaining the ability to cause bacteremia in humans [14]. Correlation between resistance and physiology in laboratory-obtained mutants is not novel [15-17], however, the evolution to compensate for fitness loss is different in clinical isolates. Most previous studies focusing on the fitness cost of clinical isolates have not used isogenic strains. And to the best of our knowledge, the fitness cost of β-lactam resistance in P. aeruginosa has not been measured in clinical isolates.

The objective of the present work was to determine whether β-lactam-resistant P. aeruginosa clinical strains have decreased fitness after exposure to β-lactams. Therefore, we analyzed the fitness of isogenic P. aeruginosa clinical strains isolated from patients before and after treatment with β-lactam antibiotics. Investigations into these mechanisms may lead to the discovery of new therapeutics.

Materials and Methods

Strain collection

Five hundred eight clinical P. aeruginosa strains were isolated before and after treatment with β-lactam antibiotics from 140 patients at the First Affiliated Hospital of Shantou University Medical College from July 2005 to August 2006 and from July 2008 to January 2010. All isolates were identified based on typical morphology by Gram-negative staining, a positive oxidase reaction, and conventional biochemical and susceptibility tests using a VITEK60 Gram-negative identification card (bioMérieux, Marcy l'Etoile, France).

Minimum Inhibitory Concentration (MIC) tests

MIC values for selected antibiotics were determined using the standard broth dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI) [18]. P. aeruginosa ATCC 27853 was used as a control.

Random amplified polymorphic DNA (RAPD)

Pairs of susceptible and resistant (≥8-fold increase in MICs) P. aeruginosa clinical isolates treated with conventional β-lactams (ceftazidime, cefepime, imipenem, and meropenem) were genotyped by RAPD as described previously [19, 20]. Genomic DNA was extracted using the EZ-10 Spin Column Bacterial Genomic DNA MiniPrep Kit (Bio Basic Inc., Markham, ON, Canada), and DNA concentration was adjusted to 10 ng/µl. RAPD reaction mixtures were set up and incubated with primers 272 (5’-AGC GGG CCA A-3’) and 208 (5’-
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ACG GCC GAC C-3’) [19]. RAPD products were separated by electrophoresis, and strains with identical band patterns were considered to be isogenic.

In vitro competition experiment

P. aeruginosa clinical isolates, isolated from the same patient, were mixed in a 1:1 ratio and diluted in a 0.9% saline solution. Approximately 10^3 cells from each mixture were inoculated into three 10-ml Luria-Bertani (LB) LB broth flasks and grown at 37°C with shaking at 150 rpm for 16-18 h. Serial 10-fold dilutions were plated in duplicates onto LB agar (LBA) alone and LBA with the corresponding antibiotics. The plates were incubated overnight at 37°C to determine the total colony-forming units (CFUs) and the CFUs of the resistant strains that were post-treatment isolates. To obtain the CFUs of the sensitive strains (isolated prior to β-lactam treatment), we subtracted the CFUs of the resistant strains (post-treatment isolates) from the total CFUs. The competition index (CI) was defined as the resistant/susceptible ratio [16].

Growth rates

The growth rate in the liquid medium was measured using the method described by Deptula [21]. Briefly, the strains were cultured at 37°C for 20 h and then centrifuged at 3,000 g for 10 min. The pellets were washed twice with phosphate-buffered saline (PBS) and then resuspended in 3 ml of PBS. Absorbance was measured at 480 nm by a microplate reader (Thermo Labsystems, Finland).

Phenotypic assays

The strains were cultured in LB broth for 20 h at 37°C with intense shaking. The cultures were then centrifuged at 3,000 g for 10 min. The supernatant was aliquoted into 1 ml dilutions and frozen at -20°C for future analyses. Optical density (OD) was used to normalize the results of exoproduct activity of the supernatants, using ATCC 27853 as the reference. The following experiments were repeated eight times for each isolate.

Proteolytic activity (mg/L) was determined in culture supernatants using azocasein as the substrate as previously described, with modifications [22, 23]. The results were expressed in mg/L based on a standard curve obtained with bacterial proteinase (Sigma, St. Louis, MO, USA).

Elastase (LasB) activity was examined using elastin–Congo Red conjugate (Sigma, St. Louis, MO, USA) as the substrate using the method detailed by Sanchez [17]. Elastase activity (mg/L) was established using a standard curve based on standard elastase solutions.

Extracellular slime production was measured using the Congo red binding assay [21].

Biofilm formation was quantified using the method described by Jackson [24] with some modifications [25]. Sterile LB medium was used as a negative control. The experiment was performed eight times, and the results were averaged.

LasA protease activity was evaluated directly as described by Kong [15] using boiled S. aureus cells as the substrate.

Statistical analysis

Values obtained for growth rate, extracellular slime production, biofilm formation, proteolytic activity, elastase activity, lasA protease activity, and in vitro competition experiments were analyzed using a paired-sample t-test (SPSS 17.0). A P value of <0.05 was considered significant.

Results and Discussion

Fitness costs of resistance are an important determinant of the prevalence of resistant strains in a clinical environment. Whether fitness costs are associated with β-lactams resistance after β-lactams exposure is important for the choice and design of antibiotics therapies. In this study, 140 pairs of β-lactams-susceptible and –resistant (≥8-fold increase) strains were genotyped by RAPD. Eleven paired isolates from each patient were considered isogenic, and each was found to belong to a different clone (data not shown). The MICs of 11 paired isolates to selected antibiotics are shown in Table 1.
Growth rate determination and competition experiment are often used to detect the fitness of a bacterium. In this study, growth rates and CI values obtained for each pair are presented in Figure 1. The growth rate in the liquid medium was less for most of the \textit{P. aeruginosa} post-treatment isolates (10/11) than for the strains before treatment (\textit{P}<0.05).

**Table 1.** MICs for 11 paired \textit{P. aeruginosa} isolates before and after treatment with \textit{\beta}-lactam antibiotics. *MIC, minimum inhibitory concentration; CAZ, ceftazidime; FEP, cefepime; IMP, imipenem; MEM, meropenem; TZ, piperacillin/tazobactam; SB, ceferozapen/sulbactam; ATM, aztreonam; CTX, cefotaxime; LEV, levofloxacin; G, gentamicin; A, amikacin.

| Pairs | CAZ | FEP | IMP | MEM | TZ | SB | ATM | CTX | LEV | G | A |
|-------|-----|-----|-----|-----|----|----|-----|-----|-----|----|----|
| I     | <=1 | 8   | <=1 | <=1 | 16 | 8  | <=1 | 16  | <=1 | 32 | 128 |
| II    | <=1 | 8   | <=1 | <=1 | 8  | 8  | <=1 | 32  | <=1 | 4  | <=1 |
| III   | <=1 | 4   | <=1 | <=1 | 4  | 8  | <=1 | 16  | <=1 | 8  | <=1 |
| IV    | >128 | 128 | <=1 | 32 | 128 | 8  | >128 | <=1 | >128 | >128 | >128 |
| V     | 8  | 8   | <=1 | 32 | 32 | 32 | >128 | 4   | >128 | >128 | >128 |
| VI    | <=1 | 4   | 16  | 4   | 64 | 32 | 4   | 64  | 4   | >128 | >4  |
| VII   | 8   | <=1 | 32 | <=1 | 64 | 32 | 32  | 128 | 4   | 64  | <=1 |
| VIII  | 8   | <=1 | 32 | <=1 | 64 | 32 | 32  | >128 | 8   | >128 | >128 |
| IX    | 32  | 8   | 64  | 4   | 64 | 64 | 32  | >128 | 4   | >128 | >128 |
| X     | <=1 | 16  | <=1 | <=1 | 16 | 32 | 8   | <=1 | 16  | <=1 | <=1 |
| XI    | 16  | 8   | 64  | 4   | 32 | 64 | 128 | >128 | 4   | >128 | >128 |

**Fig. 1.** Pairs are indicated by I, II, III, IV, V, VI, VII, VIII, IX, X and XI in the figure. A. Results of the \textit{in vitro} competition experiment. \textit{In vitro} competitions were performed in LB broth flasks in which bacteria were grown at 37°C and 150rpm for 16 to 18 h, corresponding to approximately 20 generations, as described in Material and Methods. The competition index (CI) was defined as the \textit{\beta}-lactam-resistant \textit{P. aeruginosa} (post-treatment isolate) /\textit{\beta}-lactam-susceptible \textit{P. aeruginosa} (isolated before treatment) ratio. The CI values obtained for each of the eight independent experiments are plotted. The median CI values are shown in parentheses. The CI ratio was compared to 1.0. \textit{p}<0.05 (I, II, IV, VI, VII, VIII, IX, X, XI); \textit{p}>0.05 (III, V). B. The growth rate in the liquid medium was estimated by measuring the changes in OD \textit{A} at \textit{t} = 0. The strains isolated post treatment versus the strains isolated before treatment, \textit{p}<0.05 (All pairs).

Growth rate determination and competition experiment are often used to detect the fitness of a bacterium. In this study, growth rates and CI values obtained for each pair are presented in Figure 1. The growth rate in the liquid medium was less for most of the \textit{P. aeruginosa} post-treatment isolates (10/11) than for the strains before treatment (\textit{P}<0.05).
A previous study found that multidrug-resistant *P. aeruginosa* showed significantly reduced growth rates compared with the multidrug-sensitive *P. aeruginosa* [21]. Similar results were also observed in other bacteria species, for example, quinolone-resistant Salmonella strains had decreased growth compared to quinolone-susceptible strains [26], indicating that the development of resistance can damage the multiplication capacity of bacteria. Competition experiments showed that after treatment, 63.6% (7/11) isolates showed a marked decrease...
in fitness (median CI<0.5; P<0.05), one isolate (pair IX) showed only a modest reduction (median CI 0.5243; P<0.05), and one isolate (pair IV) showed little reduction (median CI 0.8531; P=0.015), while two isolates (pairs III and V) did not display a significant fitness cost after treatment (P>0.05). There appeared to be no correlation between the growth rate and the competition ability without antibiotics, and fitness costs could not be measured only by growth rate determination.

Extracellular slime production, proteolytic activity, lasA protease activity, elastase activity, and biofilm formation are presented in Figure 2, respectively. Slime produced by P. aeruginosa markedly impairs human polymorphonuclear neutrophil functions and it is also a potent activator of both nuclear factor kappa B and activator protein-1, which are involved not only in tumor necrosis factor-alpha induction but also in many of the inflammatory responses triggered in the course of infection with P. aeruginosa [27]. Proteases secreted by P. aeruginosa play a major role in pathogenesis, especially during acute infections [28]. In this study, we used azocascin as a substrate in order to determine the activity of a wide range of proteases, including LasA, LasB, alkaline protease, elastase A and B, and type IV protease. Among these proteases, LasA and LasB play an important role in the invasion of P. aeruginosa [29]. LasB has a strong elastolytic activity capable of inactivating a wide range of biological tissues and immunological agents, while LasA possesses a low level of elastolytic activity but can enhances the activity of LasB [30]. As previously noted, lower extracellular slime production and LasA, and LasB activities were observed in multidrug-resistant (MDR) P. aeruginosa clinical strains, while no significant difference was observed in the production of proteases between MDR and multidrug-susceptible P. aeruginosa [21]. In this study, most of the β-lactam-resistant strains (9/11) produced decreased amounts of extracellular material as evidenced by Congo Red binding, and lower LasB activity was also observed in all of the β-lactam-resistant strains, compared to the susceptible strains, which is in agreement with a previous study [21]. However, the proteolytic activity decreased in 90.9% (10/11) of post-treatment isolates, and production of LasA increased in 6 β-lactam-resistant isolates when compared with their isogenic β-lactam-susceptible isolates. Obviously, the invasion ability decreased with the development of resistance to β-lactam antibiotics.

It has become clear that cells in biofilms express properties distinct from planktonic cells; for example, cells in biofilms are more resistant to antimicrobial agents. However, few studies have determined whether the ability of biofilm formation is associated with the increased resistance. The ability of biofilm formation has been shown to be increased in quinolone-resistant P. aeruginosa [13], while no data are available for β-lactam-resistant P. aeruginosa. As an important unique survival strategy exhibited by P. aeruginosa [31], biofilm formation should be included in the determination of fitness. In this study, biofilm formation was reduced in 54.5% of β-lactam-resistant strains, while 36.4% of β-lactam-resistant strains produced more biofilm than their isogenic susceptible stains. These results indicate that although most of the susceptible strains outcompete the resistant strains, increased biofilm formation may help some resistant strains survive even without antibiotics.

As precious study on laboratory-obtained P. aeruginosa mutant strains has shown that ampR mutants that upregulate expression of AmpC have an increased production of LasA protease but lower levels of LasB protease than the wild-type strain [15]. Overexpression of the β-lactam-resistant determinants MexABOprM (nalB mutant) decreased the production of proteases and increased the capability for forming biofilms, compared with their isogenic strains [17]. These data suggest that over-expression of AmpC and efflux pumps may contribute to the increased resistance and lower production of virulence factors, and various resistance mechanisms may lead to different fitness costs in the clinical isolates researched in this study.

The spread of bacterial antibiotic resistance is thought to be constrained by their fitness cost. Whether resistance confers fitness costs is controversial. Different conclusions may be made with different resistance genotypes in different strains. In this study, β-lactam exposure conferred a fitness cost that was associated with resistance. The growth rate decreased in resistant strain, and most of the susceptible stains outcompeted resistant
strains without antibiotic. Expression of invasive factors, such as slime, proteases and LasB elastase, decreased, but virulence factors important for long-term persistence such as the ability to form biofilm were variable. Of particular interest, the bacteria survived despite the decreased invasion potential, which explained why the resistant bacteria were so widespread. Thus, the importance of implementing efficient strategies to treat and prevent the spread of resistant strains before they have become stably established in a bacterial population is paramount.

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