Effect of subinhibitory concentration of piperacillin/tazobactam on Pseudomonas aeruginosa

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Subinhibitory concentrations (sub-MICs) of antibiotics, although not able to kill bacteria, can modify their physico-chemical characteristics and the architecture of their outermost surface and may interfere with some bacterial functions. This study investigated the ability of sub-MIC piperacillin/tazobactam (P/T) to interfere with the bacterial virulence parameters of adhesiveness, cell-surface hydrophobicity, motility, biofilm formation and sensitivity to oxidative stress. Antimicrobial activity against five Pseudomonas aeruginosa clinical isolates, representative of clonal lineages of 96 strains of nosocomial origin, and six control strains (ATCC 27853, PAO1, AK1, MT1562, PT623, PAO1algC) was evaluated in vitro using the NCCLS microdilution method. The effects of sub-MIC on bacterial adhesion and biofilm formation were studied using a modified microtitre plate assay. The relative cell-surface hydrophobicity of P. aeruginosa strains was determined by measuring their ability to adhere to n-hexadecane. P. aeruginosa that had been exposed overnight to P/T and incubated with P/T in the plate were also screened for their ability to swim using flagella and to twitch and for their sensitivity to oxidative stress. The results obtained showed that the impact of sub-MIC P/T on bacterial characteristics was different for the various strains of P. aeruginosa. There was a change in bacterial morphology and hydrophobicity that could explain a significant decrease in adhesion values in all clinical isolates and controls tested, a decrease in biofilm formation, a significant increase in sensitivity to oxidative stress, a significant decrease in flagellum-mediated swimming and a decrease in type IV fimbriae-mediated twitching. The results obtained indicate that sub-MIC P/T interferes with the pathogenic potential of P. aeruginosa.

INTRODUCTION

Infection is a major problem resulting from the use of intravascular catheters and other prosthetic devices such as artificial heart valves and prosthetic joints. Bacteria adhere to biomaterials and proliferate, causing clinical infection and disease (Hoyle & Costerton, 1991; Rupp & Hamer, 1998). One important agent that causes these infections is Pseudomonas aeruginosa, and its pathological effects are attributed to various characteristics, including the elaboration of many cell-associated virulence/survival factors (Van Delden & Iglewski, 1998), such as fimbriae, interaction with host defences and, most importantly, their adhesive and biofilm-formation abilities (Gristina, 1987; Gristina et al., 1987; Wilson & Schurr, 2002).

Piperacillin is a potent, broad-spectrum ureidopenicillin and, when combined with the triazolymethyl penicillin acid sulphone β-lactamase inhibitor tazobactam, results in a broader spectrum of activity against β-lactamase-producing Gram-negative, Gram-positive and anaerobic organisms. This combination is often used against Pseudomonas species in clinical practice (Kim et al., 2001). Treatment with subinhibitory concentrations (sub-MIC) of some antibiotics may influence bacterial virulence parameters (Wolter & McCormack, 1998), such as adherence (Wilson & Schurr, 2002; Wolter & McCormack, 1998; Trafny et al., 1995), cell-surface hydrophobicity (CSH) (Tateda et al., 1993), biofilm formation (Drago et al., 2001; Kim et al., 2001; Sonstein & Burnham, 1993), sensitivity to oxidative stress (Hassett et al., 1999) and motility (Braga et al., 2000; Drago et al., 2001).

Together with cell-surface structures, the polar flagellum is responsible for one type of motility in aqueous environments (Evans et al., 1991). Another cell-surface structure acting as a virulence factor is the type IV fimbria. These mediate adherence to biotic and abiotic surfaces and are responsible for surface translocation or twitching motility (Horii et al., 2003; Ichimiya et al., 1994; Kawamura-Sato et al., 2000).

Abbreviations: CI, clinical isolate; CSH, cell-surface hydrophobicity; P/T, piperacillin/tazobactam.
Many biocides are good oxidizing agents (e.g. H₂O₂, HOCl), and understanding the mechanisms by which bacteria react to oxidative stress is important as a possible way of predicting reactions to oxidative burst from neutrophils (Hassett et al., 1999).

To date, there is no information in the literature concerning the influence of sub-MIC piperacillin/tazobactam (P/T) on P. aeruginosa virulence parameters. These parameters were therefore tested in this study.

METHODS

Bacterial strains. Six control strains were used, namely, three reference strains, PAO1 (Stephen Lory, Harvard Medical School, USA), AK1 (H. C. Van Der Mei, Groningen University) and ATCC 27853, and three defined mutants from the wild-type strain PAO1 with deviating surface characteristics, MT1562 (fla- mutant) (Tsuda & Iino, 1983), PT623 (pilA mutant) (Kohler et al., 2000) and PAO1ΔgC (non-mucoid; no LPS) (Louws et al., 1994). Four clinical isolates of P. aeruginosa resistant to P/T (CI 56, 67, 77 and 88) and one clinical isolate sensitive to P/T (CI 27) were also used, selected on the basis of different enterobacterial repetitive intergenic consensus PCR and methylation-specific PCR DNA profiles (Louws et al., 1994; Versalovic et al., 1991) of 96 isolates obtained from patients hospitalized at Hospital de S. João, Porto. CI 27 and 88 were from urine, CI 56 was from a bronchial secretion, CI 67 was from a catheter and CI 77 was from pus.

Bacteria were stored at −70 °C in brain heart infusion (BHI) medium (Merck) containing 20 % glycerol.

Antibiotics. Stock solutions of the antibiotics piperacillin and tazobactam (Wyeth) were prepared in accordance with the recommendations of the NCCLS (2000).

Antimicrobial susceptibility. Determination of MICs was carried out using a microbroth dilution method (NCCLS, 2000); an adjusted inoculum of the test organism was inoculated into Mueller–Hinton broth (Merck) containing twofold dilutions of an initial antibiotic solution so that each well contained approximately 1.0 × 10⁷ c.f.u. ml⁻¹. Results were observed after overnight incubation at 37 °C. MIC was defined as the lowest concentration that inhibited visible growth.

Adhesion assay. In the adhesion assay, all strains were grown overnight (37 °C, 150 r.p.m.) in Luria–Bertani (LB) broth (Difco) in the presence or absence of P/T at a subinhibitory concentration (0.5 MIC). Bacteria were washed twice in PBS, pH 7.4 (Sigma), by centrifugation for 20 min at 1000 g and resuspended in PBS in a standard inoculum corresponding to approximately 1.0 × 10⁸ c.f.u. ml⁻¹. Results were observed after overnight incubation at 37 °C and the MIC was defined as the lowest concentration that inhibited visible growth.

Biofilm formation assay. Biofilm formation was examined in microtitre plates (O’Toole et al., 1999). Briefly, strains were grown overnight in LB broth (37 °C, 150 r.p.m.) in the presence or absence of 0.5 MIC P/T and added to wells in a standard inoculum corresponding to approximately 1.0 × 10⁸ c.f.u. ml⁻¹, as described for the adhesion assay. After 16 h incubation at 37 °C, bacteria were quantified as for the adhesion assay.

Sensitivity to oxidative stress. Assays were performed on each of the eight strains grown overnight with 0.5 MIC P/T and inoculated on to plates with medium containing 0.5 MIC P/T. Control plates contained no antibiotics. The non-motile mutant strains MT1562 (fla- mutant) and PT623 (pilA mutant) were used as controls.

Swimming. The medium for swimming assays was composed of 1 % tryptone (Difco), 0.5 % NaCl and 0.3 % agar. Briefly, the plates were inoculated in the centre with a sterile toothpick and incubated for 16 h at 25 °C (Deziel et al., 2001). Motility was assessed by observation of the circular turbid zone formed by bacteria migrating away from the point of inoculation.

Twitching. Cells were stab-inoculated with a toothpick through a thin (approx. 3 mm) 1 % LB agar layer to the bottom of the Petri dish. After incubation for 24–48 h at 30 °C, a hazy zone of growth at the interface between the agar and the polystyrene surface was observed. The ability of bacteria to twitch strongly on the polystyrene surface was examined by removing the agar, washing away unattached cells with a stream of tap water and staining the attached cells with a 1 % (w/v) crystal violet solution (Deziel et al., 2001). Each assay was performed in triplicate and repeated three times.

CSH assay. Estimation of CSH was determined by growing the 11 strains in LB broth in the presence or absence of 0.5 MIC P/T and using a standard microbical adhesion to n-hexadecane test, as described previously (Perez et al., 1998; Fonseca et al., 2003). After incubation, bacteria were washed twice in PBS, pH 7.4, by centrifugation for 20 min at 1000 g and resuspended in PBS to a standard inoculum corresponding to approximately 10⁶ c.f.u. ml⁻¹, as described above. n-Hexadecane (250 μl) was added to each triplicate sample and the two phases were mixed by vortexing for 1 min. The two phases were allowed to separate by letting the solution stand at 30 °C for 30 min. The lower aqueous phase was collected and the OD₆₅₀ was measured.

The results were expressed as the percentage decrease in the OD of the lower aqueous phase (OD₉) compared with the OD of the initial cell suspension (OD₀): [1 - (OD₂/OD₀)] × 100. Each assay was performed in triplicate and repeated three times.
**Data analysis.** The inhibition percentage of the virulence parameters tested was calculated as: \[\left(\frac{\text{OD}_{600} \text{ (without P/T)}}{\text{OD}_{600} \text{ (with P/T)}}\right) \times 100.\] The statistical significance of the differences was calculated using a paired t-test. Each test was repeated three times for each variable and means ± SD were determined. The difference was considered statistically significant for \( P \leq 0.05.\)

**RESULTS AND DISCUSSION**

The P/T MICs for the strains of *P. aeruginosa* tested were: 16 µg ml\(^{-1}\) for AK1; 8 µg ml\(^{-1}\) for ATCC 27853, PAO1, MT1562, PT623 and PAO1algC; 28 µg ml\(^{-1}\) for CI 27; 64 µg ml\(^{-1}\) for CI 56 and 32 µg ml\(^{-1}\) for CI 67, 77 and 88.

**Effect of P/T on bacterial adherence**

Previous studies have shown that certain antibiotics can suppress virulence factors, including macrolides, fluoroquinolones, carbapenems and aminoglycosides (Evans et al., 1991; Horii et al., 2003; Ichimiya et al., 1994; Kawamura-Sato et al., 2000; Tanaka et al., 1999, 2000; Wolter & McCormack, 1998; Yasuda et al., 1999; Zhanel et al., 1993).

An important determinant of virulence is the ability to adhere (Kawamura-Sato et al., 2000), and exposure of bacteria to sub-MICs of antibiotics generally weakens this ability (Schifferli & Beachey, 1988; Shibl, 1985).

Although there was some strain-to-strain variability, 0.5 MIC P/T caused a significant decrease in adhesion of all reference strains, one mutant (PAO1algC) and all clinical isolates. Although the adherence of CI 56 was very low, the percentage inhibition was significant (Fig. 1, Table 1). Strains MT1562 and PT623 are fla\(^-\) and pilA mutants, respectively, and thus acted as surface-adhesion-deficient controls. As can be seen in Fig. 1, there was no inhibitory effect of 0.5 MIC P/T on these two strains. The anti-adhesive effects of P/T could be explained by (i) a change in CSH, as suggested by the correlation between adhesion of *P. aeruginosa* strains to polystyrene microtitre plates and CSH (Table 1, see Fig. 3), (ii) an inhibition of motility (swimming) (see below) and (iii) a possible decrease in adhesins in the bacterial cell surface due to the induction of long filaments (Fig. 2), as shown previously (Braga et al., 1999).

The induction of bacterial filamentation by sub-MIC P/T was found for all strains tested, and is shown for CI 77 in Fig. 2. These altered forms of bacteria usually exhibit lower patho-

**Table 1. Inhibition of adhesion and CSH parameters following incubation with 0-5 MIC P/T**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Inhibition (%)</th>
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<tbody>
<tr>
<td></td>
<td>Adhesion</td>
</tr>
<tr>
<td>ATCC 27853</td>
<td>43.4*</td>
</tr>
<tr>
<td>PAO1</td>
<td>69.1*</td>
</tr>
<tr>
<td>AK1</td>
<td>76.8*</td>
</tr>
<tr>
<td>CI 27</td>
<td>93.7*</td>
</tr>
<tr>
<td>CI 56</td>
<td>41.2*</td>
</tr>
<tr>
<td>CI 67</td>
<td>86.0*</td>
</tr>
<tr>
<td>CI 77</td>
<td>90.0*</td>
</tr>
<tr>
<td>CI 88</td>
<td>77.2*</td>
</tr>
<tr>
<td>MT1562</td>
<td>0</td>
</tr>
<tr>
<td>PT623</td>
<td>0</td>
</tr>
<tr>
<td>PAO1algC</td>
<td>37.0*</td>
</tr>
</tbody>
</table>

*\( P \leq 0.05.\)

**Fig. 1.** Effect of 0-5 MIC P/T on adhesion of *P. aeruginosa* strains. Strains were grown in the absence (filled bars) or presence (open bars) of P/T.

**Fig. 2.** Photographs of Gram-stained cells of CI 77 in the absence (a) and presence (b) of 0-5 MIC P/T. Magnification ×250.
genicity than their respective normal counterparts, such as decreased adherence to epithelial cells, altered susceptibility to phagocytosis and decreased output of bacterial enzymes (Labro et al., 1988).

**Effect of P/T on CSH**

The findings presented in Table 1 and Fig. 3 show that 0.5 MIC P/T caused a statistically significant decrease in CSH of two reference strains (ATCC 27853 and AK1), three clinical isolates (CI 56, 87 and 88) and one mutant (PAO1algC), which could explain the decrease in adhesion values. There was no inhibitory effect of P/T in strains PAO1, MT1562 and PT623 and CI 27 and 67, which started off with relatively low hydrophobicity without P/T. Strain PAO1algC is non-mucoid and does not have LPS, which could explain why this was the only mutant affected by 0.5 MIC P/T in the adhesion and CSH assays (Table 1).

The bacterial structures that affect CSH include outer-membrane proteins, lipoproteins, phospholipids, LPS and fimbriae (Mozes et al., 1988, 1989). Among these components, the contribution of fimbriae to CSH in P. aeruginosa has been investigated extensively. The fimbriae of P. aeruginosa are known to be proteinaceous, polar structures with highly hydrophobic domains and are classified as N-methylphenylalanine fimbriae (Paranchych et al., 1979; Watts et al., 1983).

It was previously reported that sub-MICs of azithromycin inhibit the expression of fimbriae in Neisseria gonorrhoeae, which is known to possess the same N-methylphenylalanine fimbriae as P. aeruginosa (Gorby & McGee, 1990; Paranchych et al., 1979).

Thus, for the P. aeruginosa strains tested, adherence could be prevented by sub-MIC P/T, which thus acts as an antipathogenic drug, affecting the initial step of colonization and most probably the development of infection.

**Effect of P/T on bacterial motility**

**Swimming.** The different circular turbid zones formed by bacterial cells under different conditions are a measure of one type of motility of P. aeruginosa strains.
When the strains were subjected to 0.5 MIC P/T (overnight and during the incubation of the microtitre plates), the turbid zones decreased significantly (Fig. 4a). The only exception was CI 56.

The inhibitory effect of 0.5 MIC P/T was more marked for the three reference strains and for CI 27, 67, 77 and 88 when P/T was present both overnight and in the plate (total inhibition of swimming was found in strain AK1 and CI 27, 67 and 77) compared with the effect of P/T present only overnight (Table 2). This could be explained by a change in bacterial morphology (filamentation; Fig. 2). In strain ATCC 27853, the effect of P/T when present only overnight did not fit with the behaviour of other strains. CI 56 was the only strain where 0.5 MIC P/T had no effect on swimming motility values, which is in agreement with its low adhesion values. This observation shows the interaction between adhesion and swimming motility (Figs 1 and 4a).

**Twitching.** With the exception of reference strain AK1, all other strains demonstrated significantly reduced twitching after treatment with 0.5 MIC P/T (Fig. 4b). Interestingly, strain AK1 and CI 56, 77 and 88 had a relatively high value of twitching motility without P/T, which was in agreement with the CSH assay (compare Figs 3 and 4b).

The reduced twitching could also be a result of morphological changes in *P. aeruginosa* (such as different levels of filamentation; Fig. 2) induced by 0.5 MIC.

The inhibitory effect of 0.5 MIC P/T was much more pronounced for the two reference strains ATCC 27853 and PAO1 and for the clinical isolates when P/T was present overnight and in the plate, compared with the effect of P/T present only overnight (Fig. 4b, Table 2).

Inhibition of motility could also explain the reduction in adhesion and, consequently, the decrease in formation of new colonies and spread of infection from the first point of contact (Braga *et al.*, 1999; Paranchych *et al.*, 1979).

**Effect of P/T on biofilm formation**

The effect of 0.5 MIC P/T on biofilm formation is shown in Fig. 5(a). In general, a more significant decrease in biofilm formation was observed when 0.5 MIC P/T was present in both the overnight bacterial culture and in the plate (Table 2). Total inhibition of biofilm formation was found in all clinical isolates except CI 88. Comparison of Figs 1 and 5(a) shows that there was no correlation between bacterial adherence and biofilm formation without P/T. Indeed, some of the strains showed low adherence but high biofilm formation, while others showed the opposite. Together with adhesiveness, CSH and motility, biofilm formation correlates with bacterial pathogenicity, since inhibition of adhesion reduces the possibility of cellular aggregation by twitching and, consequently, of biofilm formation (Fig. 5a, Table 2).

Our results, although different for different strains, indicate that 0.5 MIC P/T (present overnight or in the plate) can lower adhesion, CSH, motility and biofilm formation of *P. aeru-

Table 2. Inhibition of motility, biofilm formation and sensitivity to oxidative stress parameters by 0.5 MIC P/T

<table>
<thead>
<tr>
<th>Strain</th>
<th>Motility (swimming)</th>
<th>Motility (twitching)</th>
<th>Biofilm formation</th>
<th>Sensitivity to oxidative stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 27853</td>
<td>0</td>
<td>0</td>
<td>56.7*</td>
<td>39.5*</td>
</tr>
<tr>
<td>PAO1</td>
<td>23.1*</td>
<td>0</td>
<td>58.8*</td>
<td>18*</td>
</tr>
<tr>
<td>AK1</td>
<td>73.7*</td>
<td>184*</td>
<td>18*</td>
<td>86.7*</td>
</tr>
<tr>
<td>CI 27</td>
<td>62.5*</td>
<td>22.7*</td>
<td>68.2*</td>
<td>29.3*</td>
</tr>
<tr>
<td>CI 56</td>
<td>200*</td>
<td>0</td>
<td>99.0*</td>
<td>100.0*</td>
</tr>
<tr>
<td>CI 67</td>
<td>40*</td>
<td>0</td>
<td>100.0*</td>
<td>100.0*</td>
</tr>
<tr>
<td>CI 77</td>
<td>45*</td>
<td>0</td>
<td>100.0*</td>
<td>100.0*</td>
</tr>
<tr>
<td>CI 88</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*P < 0.05.
ginosa (Tables 1 and 2). Although the experimental conditions were restricted, the ability of P/T to reduce biofilm formation by bacteria could be of clinical significance.

**Effect of P/T on sensitivity to oxidative stress**

In this assay, the zone of inhibition formed by each strain under different conditions was measured. It was found that the zone of inhibition formed by all strains tested was significantly higher in the presence of 0.5 MIC P/T (Fig. 5b). This effect was stronger when the strains were grown with P/T present in the overnight culture and the plate. In fact, there was no bacterial growth in strains PAO1 and AK1 and CI 27, 67, 77 and 88 (Table 2), which represented maximum of inhibition (growth inhibition for the entire plate was 90 mm). In strain ATCC 27853 and CI 56, there was some bacterial growth but inhibition was significant (Fig. 5b, Table 2).

It may be hypothesized that sub-MIC P/T affects either superoxide dismutase, catalase or peroxidase production by *P. aeruginosa* clinical isolates. Reduced levels of these enzymes could explain the limited bacterial ability to survive and proliferate under oxidative stress.

The possible interference of sub-MIC P/T with bacterial virulence and its interaction with phagocytes should be addressed as in previous findings, as it can function as a predictor of sensitivity to oxidative burst from neutrophils (Braga et al., 2000). There are no data in the literature concerning the effect of sub-MIC β-lactamase inhibitor combinations on *P. aeruginosa* sensitivity to oxidative stress. Alksne & Projan (2000) have suggested that targeting those processes that occur early in infection represents the most viable area for chemotherapy that does not directly kill or inhibit growth of bacteria. In this work, we have shown that 0.5 MIC P/T can influence the expression of bacterial virulence parameters of *P. aeruginosa*, namely adhesion, CSH, motility, biofilm formation and sensitivity to oxidative stress. This may prevent the final aggregative stages of *P. aeruginosa* adherence and thus may have clinical significance.

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