Decreased virulence of a strain of *Pseudomonas aeruginosa* O12 overexpressing a chromosomal type 1 β-lactamase could be due to reduced expression of cell-to-cell signaling dependent virulence factors

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Abstract

*Pseudomonas aeruginosa* produces a large variety of virulence factors and is characterized by its capacity to rapidly develop resistance when exposed to antibiotics. In order to evaluate a possible correlation between acquired resistance to antibiotics and virulence, we examined the virulence of four isogenic variants of *P. aeruginosa* O12 that differ in their resistance phenotypes to various β-lactam antibiotics in a mouse model of acute pneumonia. Strains overproducing a chromosomal type 1 β-lactamase were less virulent in both immunocompetent and immunosuppressed animals. Whereas the production of the exopolysaccharide alginate was similar between the four strains, extracellular virulence factors (elastase, rhamnolipid) that are controlled by the cell-to-cell signaling system circuit were detected in reduced amounts in the supernatant of the two isolates overproducing type 1 β-lactamase. These results suggest that strains overexpressing the chromosomal type 1 β-lactamase could be less virulent because of a reduction of cell-to-cell signaling dependent virulence factor production. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

*Pseudomonas aeruginosa* is one of the commonest causes of infection in intensive care units, and is responsible for numerous nosocomial infections [1]. Chronic colonization of the respiratory tract, complicated by frequent acute respiratory exacerbation, is also a major concern in patients with cystic fibrosis [2]. The virulence of this opportunistic pathogen is due to bacterial binding to host target cells, as well as to the secretion of a vast array of toxins and proteases [3]. A complex regulatory circuit, involving at least two cell-to-cell signaling systems [1], controls the production of several extracellular virulence factors. These exotoxins produce essentially local tissue damage, but are also responsible for the penetration of the bacteria into the bloodstream leading to systemic infection [1]. Despite the use of potent antibiotics, invasive *P. aeruginosa* infection is associated with high mortality [4]. In the past decade, acquired multiresistance, related to selective antibiotic pressure, has emerged in several countries and in some cases, infections caused by multiresistant *P. aeruginosa* have been untreatable [5]. Even if multiresistant strains are not directly related to severe infection, antibiotic resistance is often believed, in clinical practice, to be associated with virulence. However, this opinion is not based on experimental observations.

The aim of this work was to evaluate the virulence of four isogenic strains of *P. aeruginosa* O12 that differ by their resistance pattern to β-lactams in a mouse model of pneumonia and to explore, in vitro, any modification observed in the production of virulence factors.
2. Materials and methods

2.1. Bacteria

The initial \textit{P. aeruginosa} O12 (strain 1) was isolated from a patient with a severe burn infection. This strain produced a penicillinase (Bla-PSE1), and was resistant to ciprofloxacin, amikacin, netilmicin, and gentamicin but susceptible to fosfomycin. Three isogenic variants of this strain were obtained independently by serial passages in vitro in the presence of sub-inhibitory concentrations of antibiotics. Bacteria were initially grown for 72 h at 37°C in Mueller-Hinton medium containing 0.25 mg l\(^{-1}\) of antibiotic. The concentration of antibiotic was further increased in each subsequent 72-h culture period. Strain 2 was obtained after 24 subsequent passages in the presence of imipenem in order to obtain a D2 porin impermeability associated with the initial resistance. Strain 3 was obtained from strain 1 after 42 subsequent passages in the presence of ceftazidime. This strain had a spontaneous mutation and overexpressed the chromosomal type 1 \(\beta\)-lactamase [6]. Strain 4 was obtained after 42 subsequent passages in a medium containing both ceftazidime and imipenem. This strain had all three resistance mechanisms described. The phenotypes obtained are shown in Table 1.

### 2.1.1. Antibiogram

Minimal inhibitory concentrations (MICs) were determined by the agar dilution method in Mueller-Hinton medium. In order to evaluate the antibiotic resistance stability, antibiotic susceptibility profiles were determined before and after infection in each animal by disk diffusion.

### 2.1.2. Growth curves

Each strain was grown for 24 h at 37°C in liquid, antibiotic-free, Mueller-Hinton medium. Bacteria were counted by plating serial dilutions every 2 h.

### 2.2. In vitro measurement of virulence factors

The production of proteases, LasB elastase and rhamnolipid was determined semi-quantitatively using specific agar plates as previously described [7]. Proteolytic activity was detected on casein containing agar plates. LasB elastolytic activity was measured using elastin fiber containing agar plates. Rhamnolipid production was determined using SW plates.

Alginic production was determined by the carbazole method [8]. Bacteria were grown overnight on trypticase soy agar (Biomerieux, Lyon) for direct confirmation of mucoidy. These plates were washed with 10 ml of 0.9% NaCl, and the cell suspension was thoroughly mixed and centrifuged for 30 min at 13 700 \(\times\) g, as well as numbered by serial dilutions. The cell pellet was washed three times with saline solution for quantitative recovery of alginate. These supernatants were combined and centrifuged to remove the remaining cell material. Three volumes of ice-cold 95% ethanol were added to the culture supernatant to precipitate alginate. After centrifugation at 13 700 \(\times\) g, alginate was recovered and suspended in 1 ml of phosphate buffered saline (PBS). Alginate determination was carried out as described by Knutson and Jeanes [8], with \textit{Macro-cystis pyrifera} (Sigma, Saint-Quentin Fallavier, France) as internal standard.

### 2.3. Mice

Five-week-old female BALB/c mice (Charles River, Saint-Aubin-les-Elbeuf, France) were kept in a containment facility in groups of five, in filter-topped cages with sterile litter, water and food. Half of the animals were immunosuppressed, 4 days before challenge, by intravenous injection of 200 mg kg\(^{-1}\) cyclophosphamide (Sigma, Saint-Quentin Fallavier, France) [9].

### 2.4. Infections

Mice were slightly anesthetized with ether and were infected by nasal instillation. Immunocompetent animals were infected with 50 \(\mu\)l of bacterial suspensions containing \(10^6\), \(10^7\) or \(5 \times 10^4\) colony forming units per mouse (cfu/a). Immunosuppressed mice were infected with \(10^6\), \(10^5\) or \(10^4\) cfu/a.

### 2.5. Bacterial counts

Mice were killed at 3, 6, 24 or 48 h after challenge by an intraperitoneal dose of pentobarbital (Sanoﬁ Santé Animale, Libourne, France). Lungs were dissected from the main bronchi, and suspended in sterile PBS (Sigma, Saint-Quentin Fallavier, France). Serial dilutions (10-fold) were

| Strain | MIC (mg ml\(^{-1}\)) |
|--------|---------------------|
| Ticarcillin | \(\geq 2048\) | \(\geq 2048\) | \(\geq 2048\) | \(\geq 2048\) |
| Ceftazidime | 4 | 4 | 64 | 64 |
| Imipenem | 2 | 32 | 2 | 32 |

Table 1

Presentation of the four different isogenic variants of \textit{P. aeruginosa} O12
plated on trypticase soy agar medium. Bacterial counts from lung homogenates were expressed in log10 cfu ml⁻¹ as means ± S.E.M. The number of mice was five per point.

2.6. Histological examinations

Histological examinations were performed 24 h after infection. Lungs were filled with 500 μl of 10% formaldehyde by intratracheal injection, and then dissected from the main bronchi. Tissues were embedded in paraffin blocks. Sections (4 μm) were cut and stained with hematoxylin-eosin-safron, periodic acid Schiff reaction, Gram stain and Masson’s trichrome stain.

2.7. Statistical analyses

Means were compared with Student’s t-test. Survival rates were compared by the use of the Yates or the Mantel–Hetzel χ² test.

3. Results

3.1. Growth rates

Growth rates, measured in vitro, were identical for all strains. Strain 4, however, produced relatively smaller colonies than the other strains (0.7 mm and 1.3 mm respectively). Hence acquired resistance did not influence bacterial multiplication. The antibiotic resistance of the four strains remained stable after infection.

3.2. Virulence in immunocompetent mice

When immunocompetent mice were infected with 10⁶ cfu/a, strains 1 and 2 killed almost all animals within 24 h. In both cases, mouse lungs presented pulmonary hemorrhagic lesions. Strains 3 and 4 were significantly less virulent. The number of survivors was 15/30 (50%) with strain 3 and 16/30 (53%) with strain 4 (Table 2). Neither hemorrhage nor bacteria were seen in lungs of animals infected with strains 3 and 4 (data not shown). Comparison of survival rates after challenge with strain 1 and either 3 or 4 reached statistical significance (P < 0.001).

When mice were infected by 10⁷ cfu/a, all the animals survived regardless of the isogenic variant inoculated. Pulmonary bacterial counts, obtained 24 h after infection, showed a statistically significant reduction between the four variants (Table 3). We observed 4.5 log10 cfu/a with strain 1, 3.9 log10 cfu/a with strain 2, 3.7 log10 cfu/a with strain 3 and 3.2 log10 cfu/a with strain 4.

When the animals were infected with only 10⁴ cfu/a, regardless of the variant inoculated, all the animals survived and all the bacteria were cleared 24 h after infection as determined by both plating of lung homogenates and histological examination.

3.3. Virulence in immunosuppressed mice

When mice were rendered neutropenic by an injection of cyclophosphamide, an inoculum as low as 10⁶ cfu/a killed all the animals within 24 h regardless of the strain. All animals presented clinically a septic shock syndrome, associated with extensive lung necrosis.

At a lower inoculum, 10⁵ cfu/a, strains 1 and 2 caused a fatal pneumonia within 48 h in all animals (n = 8) (Table 4). During the first 3 h post infection, bacterial lung counts decreased slightly in these animals compared to the initial inoculum, but increased again by 3 log units within 48 h. In contrast, at this inoculum strains 3 and 4 were not lethal for the mice, and bacteria were cleared from the lungs within 48 h.

| Strain | 1       | 2       | 3       | 4       |
|--------|---------|---------|---------|---------|
| log10 cfu | 4.5 ± 0.06 | 3.9 ± 0.09 | 3.7 ± 0.08 | 3.25 ± 0.06 |
| P value | 1–2: P < 0.02 | 1–3: P < 0.01 | 2–3: NS | 2–4: P < 0.02 |

NS: not significantly different.
3.4. In vitro production of virulence factors

When compared for in vitro virulence factor production, strain 1 was not distinguishable from strain 2, and strain 3 was similar to strain 4 (Table 5). Strains 1 and 2 both produced amounts of proteases, LasB elastase and rhamnolipids comparable to the P. aeruginosa laboratory strain PAO1. However, the production of the same virulence factors was almost abolished for strains 3 and 4, as assayed by plate assays.

All the strains produced low levels of alginate. Strain 1 produced 53.0 mg l\(^{-1}\) of exopolysaccharide, strain 2 40.2 mg l\(^{-1}\), strain 3 40.6 mg l\(^{-1}\) and strain 4 32.5 mg l\(^{-1}\).

4. Discussion

P. aeruginosa is one of the most important opportunistic bacteria isolated in intensive care units and is often responsible for pulmonary infections complicating mechanical ventilation. It is also responsible for the highest mortality in this clinical setting [10,11]. Colonization of the respiratory tract by P. aeruginosa is very frequent in intubated patients, and is almost impossible to eradicate. Antibiotic therapy leads only to rapid emergence of acquired antibiotic resistance. Isolated clinical observations in our intensive care unit have, however, given the impression of a balance between colonizing multiresistant strains and some intubated patients and suggested a decrease of the virulence of multiresistant strains. The aim of this work was to evaluate the correlation between the virulence and the resistance patterns to β-lactams of four isogenic variants of a strain of P. aeruginosa O12 isolated from a burn patient.

When immunocompetent mice were infected with 10\(^5\) cfu/a of strains presenting a Bla-PSE1 penicillinase alone, or a Bla-PSE1 associated with the D2 porin impermeability, almost all animals died. In contrast, 50% of the animals infected with the same inoculum of strains overexpressing a type 1 β-lactamase survived. These results suggested a decrease of virulence in strains 3 and 4. This observation was confirmed when the animals were infected with 10\(^7\) cfu/a. This inoculum did not kill the mice but bacterial counts recovered from the lungs 24 h after infection were significantly lower in animals infected with strains producing the chromosomal type 1 β-lactamase.

In immunosuppressed animals, infection with 10\(^6\) cfu/a induced an acute pneumonia with sepsis that killed all mice in 48 h, regardless of the resistance profile of the strain. Infection with 10\(^5\) cfu/a was still 100% lethal for strains producing a Bla-PSE1 penicillinase, regardless of the presence of a D2 porin impermeability. Pulmonary bacterial counts increased drastically at 48 h in these animals. In contrast, infection with strains overexpressing a type 1 β-lactamase did not kill the mice, and pulmonary bacterial counts were decreased by more than 3 logs 48 h after the challenge.

These in vivo experiments in immunosuppressed animals confirmed the in vivo reduced virulence of the strains overexpressing a type 1 chromosomal β-lactamase observed in the immunocompetent mouse model.

The in vitro measurement of the three virulence factors proteases, LasB elastase and rhamnolipids showed that these factors were produced in relatively high amounts by strains 1 and 2, bearing the Bla-PSE1 penicillinase alone, or together with a D2 porin impermeability. In contrast, the production of these virulence factors was significantly reduced for strains 3 and 4, both overexpressing the type 1 chromosomal β-lactamase. This production of extracellular virulence factors, controlled by the cell-to-cell signaling circuitry, including proteases, LasB elastase, and rhamnolipid, has been recently suggested to play an
important role in acute \textit{P. aeruginosa} infections [1]. The coordinated production of proteases and LasB elastase is supposed to lead to local tissue destruction, as well as to invasion of blood vessels [1]. Rhamnolipid, a glycolipid biosurfactant, is believed to solubilize the phospholipids of lung surfactant. Associated with phospholipase C, it might be responsible for the atelectasis associated with \textit{P. aeruginosa} lung infection [1].

Alginate was produced at a low level by all four variants regardless of the resistance profile. This exopolysaccharide, composed of mannuronic and guluronic acids, is mostly synthesized by mucoid strains recovered from cystic fibrosis patients. Strains of \textit{P. aeruginosa} growing in such an alginate 'slime matrix' are protected from phagocytes and complement activity, and have been shown to be more resistant to disinfectants and antibiotics [12]. This increased resistance to antibiotics has been attributed to several factors mainly including slow growth and penetration barriers. The low production of exopolysaccharide observed in the four strains suggests that alginate is not involved in both virulence and resistance to β-lactams.

The correlation between in vivo observations and results obtained in vitro suggests that resistance to β-lactam antibiotics, due to the overexpression of a type 1 chromosomal β-lactamase, might be correlated with a reduction of the production of cell-to-cell signaling dependent virulence factors. In contrast, acquiring a D2 porin impermeability, conferring resistance to imipenem, does not seem to influence the production of these virulence factors. As the overexpression of the type 1 chromosomal β-lactamase was achieved by several subsequent passages, it is possible that multiple mutations occurred, and that the reduced virulence factor production is not directly due to the mutation responsible for the overexpression of the β-lactamase. However, it is remarkable that the same reduced virulence phenotype characterizes only the two strains overexpressing the type 1 β-lactamase (strains 3 and 4), and not strain 2, obtained in similar conditions. These results are striking as cell-to-cell signaling dependent virulence factor production by \textit{P. aeruginosa} has recently been related to resistance due to overexpression of multidrug efflux pumps [13,14].

5. Conclusion

This is the first observation suggesting a link between resistance due to β-lactamase overexpression and virulence. Further experiments using defined mutants overexpressing the chromosomal type 1 β-lactamase are under way to confirm these results.

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