Introduction

We have recently shown that the inflammatory response to Pseudomonas aeruginosa infection in the lungs of patients with cystic fibrosis (CF) may cause P. aeruginosa to undergo conversion to the mucoid form. Mucoid forms cannot usually be eliminated from the lungs despite aggressive antibiotic treatment. However, aggressive antibacterial treatment has been shown to improve lung function and survival rate in CF patients. As a natural consequence of intensive treatment, the bacteria have become increasingly resistant to the anti-pseudomonal drugs, including an increase in the number of highly β-lactamase-producing P. aeruginosa isolates.

Mucoid and non-mucoid phenotypes of P. aeruginosa, with apparent differences in their antimicrobial susceptibility pattern, are frequently isolated simultaneously from patients with CF and chronic lung infection. The reason for this difference in antibiotic susceptibility is not clear. The aim of the present study was to determine whether mucoid/non-mucoid paired isolates share the same genotype and to characterize their β-lactamase activity (basal and induced levels), as this is one of the most common mechanisms of resistance to β-lactam antibiotics in CF P. aeruginosa strains. Outer membrane protein patterns for the mucoid/non-mucoid pairs were also analysed for possible changes associated with resistance to the anti-pseudomonal antibiotics.

Materials and methods

CF patients and P. aeruginosa isolates

Forty-two Danish CF patients (23 females, 19 males) with a mean age of 25 years (range 15.3–37.6 years) and an average duration of the chronic P. aeruginosa infection of...
16.6 years (range 6–23 years) were included in this study. Thirty-two of these patients represent a cohort of patients infected with a multiresistant, non-mucoid strain, as described by Pedersen et al. All patients had been treated with regular courses of iv antipseudomonal therapy, as described previously. Sputum samples obtained by expectoration or endolaryngeal suction were Gram stained and examined under the microscope to confirm their origin from the lower airways. Material was cultured as reported previously. 
P. aeruginosa was identified by conventional biochemical tests. All these isolates were collected in 1997 and kept at −80°C in broth supplemented with 10% glycerol. One pair of mucoid/non-mucoid isolates was obtained from each patient.

Eight CF P. aeruginosa isolates from the strain collection of the Department of Clinical Microbiology, Rigshospitalet, isolated in 1986 (one isolate from each of two patients), 1988 (two isolates from each of two patients) and 1991 (one isolate from each of two patients) were included in the study for comparison.

All P. aeruginosa isolates were cultured overnight at 37°C in sterile beef broth.

**MIC determination**
The MIC of piperacillin (Lederle, Carolina, USA), ceftazidime (Glaxo-Welcome, Brøndby, Denmark), meropenem (Zeneca Limited, Macclesfield, UK), aztreonam (Bristol-Myers Squibb, Bromma, Italy), tobramycin (Tobramycin-sulfate, Sygehusapotekerne, Denmark), colistin (Lundbeck, Valby, Denmark) and ciprofloxacin (Miles Inc., IL, USA) for each isolate was determined by the agar plate dilution method using IC₅₀ agar (Statens Serum Institut, Copenhagen, Denmark), using an inoculum of c. 10³–10⁴ cfu/spot.
The isolates were classified as susceptible or resistant according to the MICs of at least three antibiotics. The following breakpoints for resistance were used: piperacillin, MIC ≥ 128 mg/L; ceftazidime and aztreonam, MIC ≥ 32 mg/L; meropenem, MIC ≥ 16 mg/L; tobramycin, MIC ≥ 8 mg/L; and ciprofloxacin, MIC ≥ 4 mg/L.

**β-Lactamase assay**
β-Lactamase production in basal conditions and after induction with benzyl-penicillin (500 mg/L) was measured spectrophotometrically using nitrocefin as substrate as described previously.

**Outer membrane proteins**
Outer membrane proteins were prepared by the sarcosyl method, separated on 12.5% SDS–PAGE gels and detected by Coomassie staining. The protein patterns were analysed visually.

**Alginate assay**
Alginate production was measured using a borate-carbazol method. D-Mannuronate lactone (Sigma, St Louis, MO, USA) was used to calibrate a standard curve.

**Typing methods**
All strains were genotyped by automated ribotyping in the RiboPrinter system, as recommended by the manufacturer (Qualicon, Wilmington, DE, USA). In brief, all strains were subcultured three times on 5% blood agar and a single colony from a 24 h culture was suspended in sample buffer and heated at 80°C for 15 min. Mucoid isolates were grown in BHI broth (SSI-Diagnostika, Hillerød, Denmark) for 6 h, and harvested from 1 mL of broth before suspension in sample buffer. After addition of lytic enzymes, samples were transferred to the RiboPrinter. Further analysis, which included EcoRI restriction of DNA, was carried out automatically. The RiboPrint profiles were aligned according to the position of the molecular size standard and compared with patterns obtained previously, then stored in a database consisting of 750 validated RiboPrints supplied from the manufacturer. The RiboPrint profiles were analysed in BioNumerics (Applied Maths, Kortrijk, Belgium).

**Statistical analysis**
The description and analysis of the data were carried out using StatView 4.5 software for Apple Mac. Logarithmic transformation of the data was performed to stabilize the variance.

Paired t-test was used for comparison of the data of the paired mucoid/non-mucoid isolates. The data were presented as non-mucoid/mucoid ratios with 99% confidence intervals. The level of significance was 1%.

One-way analysis of variance (ANOVA) was used to determine the effect of the four most common ribogroups on the antibiotic susceptibility data. For the parameters that looked as though they could be statistically significant, Scheffé’s F-test was used as a comparison procedure. The level of significance was 5%.

**Results**

**Susceptibility to antibiotics, β-lactamase production and ribotypes**
The analysis of the genotypes determined by RiboPrinting showed that 13 different types existed among the 84 isolates. The same ribotype was observed in paired mucoid/non-mucoid isolates in 18 of 42 patients.

The majority of the isolates were assigned to four types: 73-S2 (n = 39), 73-S1 (n = 16), 207-S3 (n = 8) and 227-S8 (n = 6). The isolates with the ribotype 73-S2 produced
Paired mucoid and non-mucoid P. aeruginosa

Table 1. MIC<sub>50</sub>/MIC<sub>90</sub> and ranges (mg/L) for various antimicrobial agents

<table>
<thead>
<tr>
<th>Ribotype</th>
<th>CAZ</th>
<th>PIP</th>
<th>ATM</th>
<th>MEM</th>
<th>TOB</th>
<th>COL</th>
<th>CIP</th>
<th>(\beta_{\text{basal}})</th>
<th>(\beta_{\text{induced}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>73-S2 ((n = 39))&lt;sup&gt;a&lt;/sup&gt;</td>
<td>400/400</td>
<td>400/400</td>
<td>200/400</td>
<td>25/100</td>
<td>12.5/25</td>
<td>3.1/25</td>
<td>6.2/12.5</td>
<td>74</td>
<td>2059</td>
</tr>
<tr>
<td>73-S1 ((n = 16))&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.2/25</td>
<td>50/400*</td>
<td>12.5/50</td>
<td>12.5/50</td>
<td>3.2/12.5*</td>
<td>6.2/50</td>
<td>1.6/12.5</td>
<td>43.5</td>
<td>710*</td>
</tr>
<tr>
<td>207-S3 ((n = 8))&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.2/400</td>
<td>25/400</td>
<td>6.2/400</td>
<td>1.6/100*</td>
<td>12.5/25</td>
<td>6.2/400</td>
<td>3.1/25</td>
<td>63.3</td>
<td>1842</td>
</tr>
<tr>
<td>227-S8 ((n = 6))&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.2/400</td>
<td>12.5/400*</td>
<td>6.2/400</td>
<td>0.8/50*</td>
<td>3.1/50</td>
<td>1.6/6.2</td>
<td>40.5</td>
<td>1738</td>
<td></td>
</tr>
<tr>
<td>73-S2 ((n = 3))&lt;sup&gt;c&lt;/sup&gt;</td>
<td>400/400</td>
<td>400/400</td>
<td>200/200</td>
<td>3.2/6.2</td>
<td>3.2/200</td>
<td>1.6/3.2</td>
<td>0.2/0.4</td>
<td>146</td>
<td>194</td>
</tr>
<tr>
<td>227-S8 ((n = 5))&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.8/400</td>
<td>6.2/400</td>
<td>0.8/200</td>
<td>0.1/0.8</td>
<td>6.2/12.5</td>
<td>6.2/6.2</td>
<td>0.8/0.8</td>
<td>11</td>
<td>2196</td>
</tr>
</tbody>
</table>

CAZ, ceftazidime; PIP, piperacillin; ATM, aztreonam; MEM, meropenem; TOB, tobramycin; COL, colistin; CIP, ciprofloxacin.

*a* The basal levels of \(\beta\)-lactamase \(\beta_{\text{basal}}\) and after induction with 500 mg/L benzyl-penicillin \(\beta_{\text{induced}}\) are expressed in mU. The values of \(\beta\)-lactamase are expressed as median (ranges) values of the groups.

*b* The most frequent ribotypes of 1997 isolates.

*c* The two ribotypes of eight CF isolates collected before 1991.

**P** represents the statistical significance between the most common ribogroup (73-S2) and each of the other ribogroups.

Table 2. Ratios (99% confidence intervals) of the MICs of various antibiotics and the ratios of the basal and induced levels of \(\beta\)-lactamase between paired mucoid and non-mucoid CF P. aeruginosa isolates

<table>
<thead>
<tr>
<th>Paired P. aeruginosa isolate with:</th>
<th></th>
<th></th>
<th></th>
<th></th>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CAZ</td>
<td>PIP</td>
<td>ATM</td>
<td>MEM</td>
<td>TOB</td>
<td>COL</td>
<td>CIP</td>
<td>(\beta_{\text{basal}})</td>
<td>(\beta_{\text{induced}})</td>
<td></td>
</tr>
<tr>
<td>Same ribotype ((n = 18))</td>
<td>2.62</td>
<td>1.77</td>
<td>2.82</td>
<td>2.71*</td>
<td>1.30</td>
<td>3.55*</td>
<td>1.30</td>
<td>1.46</td>
<td>1.92</td>
</tr>
<tr>
<td>((0.16, 7.97))</td>
<td>((0.58, 5.42))</td>
<td>((0.78, 10.24))</td>
<td>((1.03–7.12))</td>
<td>((0.73, 2.34))</td>
<td>((0.98, 12.77))</td>
<td>((1.45, 2.48))</td>
<td>((0.421–5.09))</td>
<td>((0.72–5.08))</td>
<td></td>
</tr>
<tr>
<td>Different ribotype ((n = 24))</td>
<td>27.66*</td>
<td>11*</td>
<td>13.83*</td>
<td>7.94*</td>
<td>3.79*</td>
<td>2.66</td>
<td>2.42*</td>
<td>2.58*</td>
<td>0.92</td>
</tr>
<tr>
<td>((8.97, 85.11))</td>
<td>((4.24, 28.37))</td>
<td>((4.42, 43.15))</td>
<td>((2.81, 22.38))</td>
<td>((2.24–6.39))</td>
<td>((0.90–7.8))</td>
<td>((0.435)</td>
<td>((1.02, 6.54))</td>
<td>((0.11, 8.45))</td>
<td></td>
</tr>
</tbody>
</table>

CAZ, ceftazidime; PIP, piperacillin; ATM, aztreonam; MEM, meropenem; TOB, tobramycin; COL, colistin; CIP, ciprofloxacin.

*P* < 0.01. *P* represents the statistical significance between paired mucoid/non-mucoid isolates within each group.
higher basal levels of \( \beta \)-lactamase and presented a \( \beta \)-lactamase hyperinducible phenotype compared with the isolates with the ribotype 73-S1 \( (P = 0.0075) \) (Table 1). This correlated with significantly higher MICs \( (P < 0.05) \) of piperacillin and meropenem compared with the other three ribotypes. The MICs of tobramycin were significantly lower for isolates with 73-S1 ribotype compared with 73-S2 ribotype (Table 1). The median amount of alginate produced by \( P. \) aeruginosa isolates with ribotype 227-S8 was significantly higher \( (P = 0.03) \) than that produced by the isolates with ribotype 73-S2 \([316 \text{ mg/L (range 0–840 mg/L)} \) versus \( 6.89 \text{ mg/L (range 0–688 mg/L)} \), respectively].

Five of the eight archived isolates (ribotype 227-S8) produced lower basal levels of \( \beta \)-lactamase and were more susceptible to antibiotics, as compared with the three isolates of ribotype 73-S2 (Table 1). A similar difference in the susceptibility pattern of these two ribotypes was found among the 1997 isolates (Table 1), showing that this ribotype (227-S8) maintained greater susceptibility to antibiotics in the CF lung.

**Susceptibility to antibiotics, \( \beta \)-lactamase production and phenotype**

The mucoid isolates showed lower \( \beta \)-lactamase activity and had lower MICs of all the antibiotics compared with non-mucoid isolates as indicated by the ratio values (Table 2). As shown in Table 2, significant differences \( (P = 0.01) \) between the mucoid and non-mucoid isolates with the same ribotype were found for the MICs of meropenem and colistin. A similar trend was observed for the other antibiotics with ratios higher than 2.5 for ceftazidime and aztreonam [99% confidence interval \( (1.16, 7.97) \) and \( (0.78, 10.24) \), respectively], although these did not reach statistical significance. For paired isolates belonging to different ribotypes, significant differences \( (P \leq 0.01) \) between the two phenotypes were similarly found for MICs of ceftazidime, piperacillin, aztreonam, meropenem, tobramycin and ciprofloxacin, and in the basal levels of \( \beta \)-lactamase.

**Outer membrane proteins**

Outer membrane protein analysis showed the presence of a characteristic 54 kDa protein in all mucoid isolates, which was not present in the non-mucoid isolates (Figure). We have previously identified this protein as AlgE protein, which has a role in the alginate secretion.\(^1\)\(^{18}\)

The major outer membrane porin OprF was observed in all the isolates with one exception (Figure, lane 4). This mucoid isolate had a higher MIC of ciprofloxacin \( (\text{MIC} = 6.2 \text{ mg/L}) \) compared with its paired strain \( (\text{MIC} = 1.6 \text{ mg/L}) \). Both the mucoid and non-mucoid isolates of this pair were resistant to the other anti-pseudomonal antibiotics.

**Discussion**

The antibiotic susceptibility of paired mucoid and non-mucoid \( P. \) aeruginosa isolates collected in 1997 showed that the mucoid isolates were generally more susceptible to antibiotics, and had lower \( \beta \)-lactamase activity than the corresponding non-mucoid isolates. These results are in accordance with previously published data\(^6\)\(^–\)\(^8\) and raise the question of whether resistance to antibiotics is inversely correlated to alginate production.

As 32 of the 42 patients included in our study belonged to a cohort of patients infected with a non-mucoid, multiresistant strain, our finding of a dominant ribogroup 73-S2 (39 isolates) was to be expected. This strain was resistant to all tested antibiotics, including tobramycin and ciprofloxacin. This ribogroup was also present in three resistant isolates collected before 1991. This is not surprising as it has been shown previously in longitudinal studies that once the chronic lung infection is established, the initially colonizing \( P. \) aeruginosa strain remains at all times.\(^19\) We suspect that intensive antibiotic treatment may have selected for this multiresistant strain. Although the induced levels of \( \beta \)-lactamase of the isolates belonging to the ribogroup 73-
S2 were significantly higher than those produced in the other isolates, this phenotype does not explain the additional resistance to tobramycin and ciprofloxacin. Therefore, other resistance mechanisms, such as enhanced efflux pumps, may be present in these isolates. Jalal et al.\(^20\) found changes in both gyrA and rfxB, and expression of OprN and OprI in two ciprofloxacin-resistant isolates belonging to ribotype 73-S2.

RiboPrinting profiles of the two largest ribogroups, the resistant 73-S2 (39 isolates) and the susceptible 73-S1 (16 isolates) differed by only one band, indicating a degree of similarity between the two ribogroups. It is possible that the 73-S2 ribogroup might have evolved and spread in the CF population from the susceptible 73-S1 ribogroup under the selective antibiotic pressure. However, no association of these two ribotypes was observed among the 24 paired isolates with different ribotypes.

Isolates of ribotype 227-S8 appear to have maintained greater susceptibility to antibiotics. Interestingly, the isolates with 227-S8 ribotype produced significantly higher amounts of alginate compared with isolates belonging to the 73-S2 ribotype.

The ratios of the MIC of \(\beta\)-lactam antibiotics for the non-mucoid/mucoid isolates showed that, generally, the isolates with mucoid phenotype were more susceptible than the non-mucoid paired isolates, and that this was associated with the levels of \(\beta\)-lactamase production. This difference in susceptibility to anti-pseudomonal antibiotics between pairs of non-mucoid and mucoid *P. aeruginosa*, as well as the maintenance for a long period of time of the antibiotic susceptibility of isolates with ribotype 227-S8 overproducing alginate, indicates that the non-mucoid isolates are exposed to a relatively higher antibiotic selective pressure than the mucoid isolates. This might be due to the biofilm mode of growth.

As proposed previously, biofilm-embedded cells may have different degrees of susceptibility to antibiotics, depending on the site where each individual cell is located within the multiple layers of cells forming the biofilm.\(^21\) In the case of \(\beta\)-lactam antibiotics, the \(\beta\)-lactamase produced by the superficial layer in the biofilm will be able to inactivate the \(\beta\)-lactam antibiotic before it reaches the deep layers.\(^22,23\) Bacterial microcolonies have recently been considered as organized communities with functional heterogeneity.\(^24\) In the current view of the biofilms as microbial societies with their own defence and communication systems,\(^25\) it is possible that the mucoid and non-mucoid phenotypes live in symbiosis within the biofilm. While the mucoid, alginate hyperproducing cells ensure the survival of the biofilm, the non-mucoid cells might play a protective role against antibiotics. Taking into account the fact that the biofilm mode of growth leads to a 1000-fold higher MIC of different antibiotics compared with the MIC that we have observed with planktonic cells, we can understand the therapeutic problems encountered in treating the *P. aeruginosa* chronic infection in CF patients.\(^26\)

Interestingly, a significant difference in the MIC of colistin between the mucoid and non-mucoid isolates sharing the same ribotype was also found. In the Danish CF centre colistin is frequently administered for prophylaxis and for treatment of CF lung infection.\(^27\) Recently, the mechanism of colistin resistance in these strains has been shown to be related to structural modifications of lipid A.\(^28\)

The presence of a 54 kDa outer membrane protein, AlgE, with a role in alginate secretion\(^18\) was found in isolates with high alginate production. The major porin OprF used by most \(\beta\)-lactam antibiotics to penetrate the outer membrane was present in all the isolates, irrespective of their resistance pattern or alginate production, with the exception of one clinical *P. aeruginosa* isolate where OprF was lacking. This OprF\(^+\) isolate was mucoid and resistant to all \(\beta\)-lactam antibiotics, as was its non-mucoid OprF\(^+\) paired strain, but the OprF\(^-\) isolate had an MIC of ciprofloxacin four times higher than its paired OprF\(^+\) strain. The lack of OprF has previously been reported to be associated with resistance to quinolones in Gram-negative bacteria.\(^29\)

In conclusion, our study has shown that mucoid isolates were generally more susceptible to antibiotics than their paired non-mucoid *P. aeruginosa* isolates, and this was associated with lower levels of \(\beta\)-lactamase activity. We propose that the maintenance of the antibiotic susceptibility of alginate overproducing isolates might be explained by the co-existence in the biofilm of non-mucoid resistant isolates that might play a protective role.

**Acknowledgements**

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**References**


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