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SHORT COMMUNICATION

# Reduced expression of virulence factors in multidrug-resistant *Pseudomonas aeruginosa* strains

**Aleksander Deptuła · Eugenia Gospodarek**

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**Abstract** MDR *Pseudomonas aeruginosa* strains are isolated from clinical specimens with increasing frequency. It seems that acquiring genes which determine antibiotic resistance usually comes at a biological cost of impaired bacterial physiology. There is no information on investigations comparing phenotypic differences in MDR and MDS *P. aeruginosa* strains in literature. The study included 150 clinical *P. aeruginosa* isolates (75 classified as MDS and 75 as MDR). PFGE analysis revealed five pairs of identical isolates in the group of MDR strains and the results obtained for these strains were not included in the statistical analyses. MDR strains adhered to polystyrene to a lesser extent than MDS strains. The growth rate in the liquid medium was significantly lower for MDR strains. Detectable amounts of alginate were present in the culture supernatants of seven MDS and six MDR strains. The MDR *P. aeruginosa* strains which were investigated produced significantly lower amounts of extracellular material binding Congo Red, lower lipolytic, elastase, LasA protease, phospholipase C activity and pyocyanin quantity in culture supernatants when compared with MDS strains. No significant differences were observed between MDR and MDS strains in proteolytic activity. In conclusion, the MDR *P. aeruginosa* strains have impaired virulence when compared to MDS strains.

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A. Deptuła (✉) · E. Gospodarek
Department of Microbiology, Nicolaus Copernicus University, Collegium Medicum in Bydgoszcz, 9 M. Skłodowskiej, Curie St, 85-094 Bydgoszcz, Poland
e-mail: deptul@o2.pl

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**Abbreviations**

| | |
|---|---|
| MDS | Multidrug sensitive |
| MDR | Multidrug resistant |
| OD | Optical density |

## Introduction

Multidrug-resistant (MDR) *Pseudomonas aeruginosa* strains have been isolated all over the world from clinical specimens with increasing frequency (D'Agata 2004; Lambiase et al. 2006; Navon-Venezia et al. 2005; Sánchez et al. 2002). Currently, there are no perspectives for introducing new drugs that could be used against this nosocomial pathogen (Landman et al. 2002), therefore investigations into its physiology are justified as they may lead to the discovery of new therapeutic opportunities.

It seems that acquiring genes which determine antibiotic resistance usually comes at a biological cost of impaired bacterial physiology (Andersson 2006; Andersson and Levin 1999; Spratt 1996), regardless whether the genes had been acquired in vivo (Schrag and Perrot 1996) or in vitro (Sánchez et al. 2002). Correlation between antibiotic resistance and virulence expression is not a new issue; it seems to be a task quite rarely undertaken when compared with those concerning antibiotic resistance, biofilm formation and epidemiology of infections caused by *P. aeruginosa*. Acquisition of multidrug-resistance genes

may also affect bacterial quorum sensing that controls gene expression and the release of some virulence factors (Linares et al. 2005; Sánchez et al. 2002; Köhler et al. 2001).

From the evolutionary standpoint, the selection of MDR has been taking place for quite a short time—since the 1940s. Experimental studies have proved that MDR microorganisms, when in the presence of selective agents preserving the antibiotic resistance genes, are able to recover their previous levels of fitness during several hundreds of passages (Schoustra et al. 2006; Schrag and Perrot 1996). Some authors suggest that *P. aeruginosa* strains may accumulate drug resistance genes and are still able to cause bacteremia in humans (Hocquet et al. 2007).

No information on investigations comparing phenotypic differences in the expression of virulence factors in MDR and multidrug-sensitive (MDS) *P. aeruginosa* clinical strains can be found in literature.

## Materials and methods

The study included 150 clinical *P. aeruginosa* isolates. Seventy-five of them were classified as MDS (sensitive to most of the tested antibiotics), and 75 as MDR, on the basis of contemporary definitions of multidrug resistance (Deplano et al. 2005; Navon-Venezia et al. 2005; Schina et al. 2006; Hill et al. 2005; Tam et al. 2005; Obritsch et al. 2004; Hsu et al. 2005). All strains were isolated in the Department of Microbiology of the Dr. A. Jurasz University Hospital between 2000 and 2005. Strains isolated from the same patient were not included in the study.

The strains were identified by routine methods used in clinical microbiology. The antibiotic sensitivity tests were performed according to the current recommendations of National Committee for Clinical and Laboratory Standards (2004) and National Reference Centre for Anitmicrobial Sensitivity Testing (Hryniewicz et al. 2004) using disk diffusion. The MIC values for selected antibiotics were determined with Etest (AB Biodisk).

*PFGE analysis of genomic profiles* was adapted from the procedure published by Jung et al. (2002). The optimal time of incubation with 1 ml of 0.5 M EDTA solution, pH = 9.5 was established at 15 min at room temperature. Isolated chromosomal DNA was digested with *SpeI* enzyme in Tango Buffer (Fermentas) at final concentration of 40 U $ml^{-1}$ for 20 h at 37°C. The results were analysed using the Molecular Analyst Fingerprint software (Dice coefficient, position tolerance 1.5%). Strains indicating over 96% similarity were considered as identical and were not included in further analyses.

### Evaluation of selected virulence factors

The strains were cultured in 2% Proteose Peptone (PP, Oxoid) for 20 h at 37°C with intensive shaking. The cultures were then centrifuged (3,000*g*/10 min). The supernatants were divided into 1 ml portions, frozen at −70°C and used for further analyses. The pellets were washed twice with 3 ml of PBS (pH 7.4), then resuspended in 3 ml of PBS in order to measure the optical density (OD) at microplate reader (Synergy HT, BioTek) $\lambda = 480$ nm. The OD was used to normalize the results of exoproducts activity of supernatants and as a measure of the growth rate. *P. aeruginosa* PAO1 strain had been used in all experiments as a control. The strain was kindly provided by prof. Fiona Brinkman and prof. Bob Hancock.

*Proteolytic activity* was examined using the method described by Sieprawska-Lupa et al. (2004) with asocaseine (Sigma) as a substrate. The experiment was carried out in triplicate. The results were expressed in mg $l^{-1}$ on the basis of standard curve obtained with bacterial proteinase (Sigma).

*Elastase activity* was estimated using the method described by Grimwood et al. (1989) using elastin-Congo red conjugate (Sigma) as a substrate. Elastase activity (mg $ml^{-1}$) was established using the standard curve based on elastase solution (Sigma).

*LasA protease activity* was evaluated directly as described by Ołdak and Trafny (2005) with boiled *S. aureus* cells as a substrate.

*Lipolytic activity* was evaluated using the method described by Lonon et al. (1988). The results were expressed in U defined as an enzymatic activity that increases the OD of the sample of 0.001 during 2 h incubation.

*Phospholipase C activity* was evaluated using the method described by Kurioka and Matsuda (1976) with p-nitrophenylphosphorylocholine (Sigma) as a substrate. The results were established on the basis of the standard curve obtained from the reaction with *Clostridium perfringens* phospholipase C (Sigma), and expressed in U $ml^{-1}$.

*Alginate assay* was performed using the borate-carbazole method developed by Knutson and Jeanes (1968) with further modifications (Bagge et al. 2004). The concentration of alginate was established on the basis of the standard curve obtained from the reaction with alginic acid sodium salt (Sigma) solution.

*Pyocyanin production* was quantified using the spectrophotometric method described by Dietrich et al. (2006).

*Polystyrene adhesion assay* was performed using the method described by Chirstensen et al. (1985) with some modifications (Jackson et al. 2004). Sterile PP medium was used as a negative control. The experiment was carried out in triplicate. The results were averaged.

*Congo red binding assay* was applied to measure the extracellular slime production using the method described by Ishiguro et al. (1985) with some modifications. The pellet obtained from the cultures grown during 20 h at 37°C in PP was resuspended in PBS, pH 7.4, 500 µl of obtained suspensions was placed in Eppendorf tube, and 500 µl of Congo red (Sigma) solution (150 µg $ml^{-1}$) was added. The mixtures were shaken intensively at 1400 RPM for 5 min, and subsequently centrifuged (13500 RPM/10 min). The supernatants were placed in the microplate wells and the OD was measured at Synergy HT microplate reader (wavelength $\lambda = 570$ nm). The negative control used in the experiment was a mixture of 500 µl PBS and 500 µl of Congo red solution. The results were divided by the suspension OD.

*Statistical analyses* were performed with Statistica 6.0 PL (Statsoft). Non-parametric *U*-Mann–Witney's test was used, with the significance level $p < 0.05$. Results for both groups of strains are presented as averages with standard deviation (SD) range.

## Results and discussion

The strains included in the study were isolated from urine (42.7% MDS and 34.7% MDR); wound swabs (17.3% MDS and 22.7% MDR); tracheal secretion (14.7% MDS and 17.3% MDR) BAL (10.7% MDR and 10.7% MDS); blood cultures (1.3% MDS and 2.7% MDR) and other clinical specimens (13.3% MDS and 12.0% MDR). Their antibiotic susceptibility rates are presented in Table 1.

PFGE analysis revealed five pairs of closely related isolates in the group of MDR strains, and the results obtained for these strains were removed from further analyses. All isolates in the group of MDS strains were unrelated.

MDR strains adhered to polystyrene to a lesser extent than MDS strains (0.111 vs. 0.130; $p = 0.005$; PAO1 = 0.277) expressed in OD units (Fig. 1). Some authors have indicated that MDR *A. baumannii* isolates strongly adhered to polystyrene and epithelial cells (Lee et al. 2008). Investigations over MDR *Klebsiella pneumoniae* strains show strong adhesion to intestinal epithelial cells (Di Martino et al. 1997). The adhesive properties depended on the antibiotic resistance profile of these strains.

The growth rate in the liquid medium was significantly lower for MDR strains (0.924 OD) than for the MDS strains (1.234 OD); $p < 0.001$ (Fig. 2), whereas for PAO1 strain, it was 1.421. Kugelberg et al. (2004) obtained similar results investigating *P. aeruginosa* strains resistant to fluoroquinolones. Also MDR *Mycobacterium tuberculosis* strains grow slower than MDS strains (Toro et al. 2006).

**Table 1** Susceptibility rates of 150 *P. aeruginosa* strains

| Antibiotic | MIC range (µg/ml) | MDS strains ($n = 75$) | | | | | MDR strains ($n = 75$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Susceptibility rate (%) | | | $MIC_{50}$ | $MIC_{90}$ | Susceptibility rate (%) | | | $MIC_{50}$ | $MIC_{90}$ |
| | | S | I | R | | | S | I | R | | |
| Piperacillin | ND | 100 | 0 | 0 | ND | ND | 0 | 0 | 100 | ND | ND |
| Piperacillin/tazobactam | 0.125–256 | 100 | 0 | 0 | 3.0 | 4.0 | 1.3 | 6.7 | 92.0 | ≥256 | ≥256 |
| Carbenicillin | ND | 98.7 | 0 | 1.3 | ND | ND | 0 | 0 | 100 | ND | ND |
| Ticarcillin/clavulanic acid | ND | 98.7 | 1.3 | 0 | ND | ND | 0 | 1.3 | 98.7 | ND | ND |
| Aztreonam | ND | 100 | 0 | 0 | ND | ND | 10.7 | 38.7 | 50.7 | ND | ND |
| Imipenem | 0.016–32 | 98.7 | 0 | 1.3 | 2.0 | 4.0 | 17.3 | 16.0 | 66.7 | ≥ 32 | ≥32 |
| Meropenem | 0,016–32 | 100 | 0 | 0 | 0.38 | 1.0 | 4.0 | 5.3 | 90.7 | 24 | ≥32 |
| Cefotaxime | ND | 10.7 | 85.3 | 4.0 | ND | ND | 0 | 9.3 | 90.7 | ND | ND |
| Ceftazidime | 0.125–256 | 100 | 0 | 0 | 1.5 | 3.0 | 18.7 | 13.3 | 68.0 | 96 | ≥256 |
| Cefoperazone | ND | 100 | 0 | 0 | ND | ND | 0 | 0 | 100 | ND | ND |
| Cefoperazone/sulbactam | ND | 100 | 0 | 0 | ND | ND | 1.3 | 33.3 | 65.3 | ND | ND |
| Cefepime | ND | 98.7 | 0 | 1.3 | ND | ND | 4.0 | 28.0 | 68.0 | ND | ND |
| Gentamicin | ND | 100 | 0 | 0 | ND | ND | 0 | 0 | 100 | ND | ND |
| Tobramycin | 0.125–256 | 100 | 0 | 0 | 0.75 | 1.5 | 0 | 0 | 100 | 48 | ≥256 |
| Amikacin | ND | 100 | 0 | 0 | ND | ND | 4.0 | 5.3 | 90.7 | ND | ND |
| Netilmicin | ND | 100 | 0 | 0 | ND | ND | 4.0 | 2.7 | 93.3 | ND | ND |
| Ciprofloxacin | 0.016–32 | 100 | 0 | 0 | 0.125 | 0.38 | 2.7 | 1.3 | 96.0 | 24 | ≥32 |
| Colisitin | ND | 100 | 0 | 0 | ND | ND | 100 | 0 | 0 | ND | ND |

*S* sensitive, *I* intermediate, *R* resistant, *ND* not determined

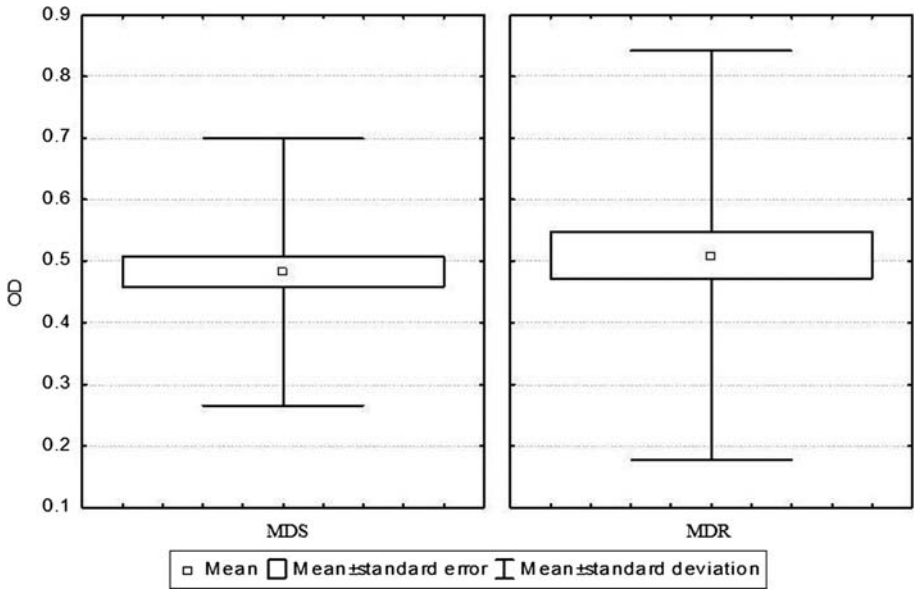


**Fig. 1** Polystyrene adhesion assay for MDS and MDR *P. aeruginosa* strains ($n = 150$)

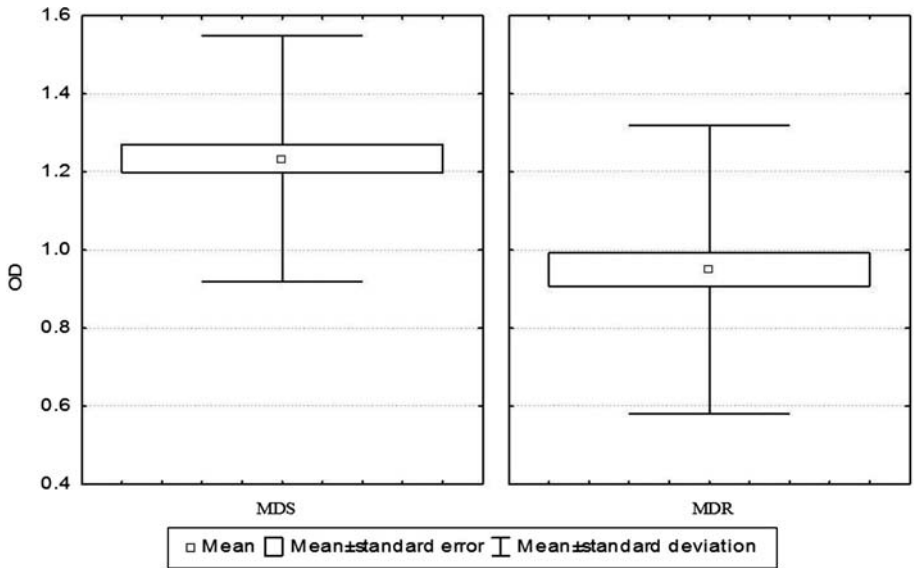


**Fig. 2** Growth rate of MDS and MDR *P. aeruginosa* strains ($n = 150$)

Detectable amounts of alginate were present in the culture supernatants of 7 (9.3%) MDS and 6 (8.0%) MDR strains and the differences were statistically irrelevant. Testing the alginate synthesis using other methods or in different conditions should be considered. However, some authors suggest that alginate is not a significant component of *P. aeruginosa* biofilms (Wozniak et al. 2003) and its overproduction may affect both the biofilm's structure as well as its function (Hentzer et al. 2001).

The MDR *P. aeruginosa* strains which were investigated produced lower amounts of extracellular material binding Congo Red. Congo red binding index equalled 0.224 OD for MDR strains, while for MDS strains it equalled 0.284 OD and for PAO1 −0.154. The differences were statistically relevant ($p = 0.02$).

No statistically significant differences were observed between MDR and MDS strains tested in proteolytic activity of culture supernatants (1.983 mg $l^{-1}$; SD = 2.573 vs. 2.562 mg $l^{-1}$; SD = 4.418; PAO1 = 3.754). Sánchez et al. (2002) indicated that laboratory mutants overexpressing *nalB* and *nfxB* efflux pumps produce significantly lower proteolytic activity when compared with wild-type strain. Authors stress that the influence of drug resistance mediating genes acquisition in vivo may lead to different biological activity than in mutants obtained in laboratory.

Lower activity of elastase was observed in the supernatants of MDR *P. aeruginosa* strains when compared with the MDS strains (3.451 mg $l^{-1}$; SD = 6.711 vs. 7.511 mg $l^{-1}$; SD = 11.001; PAO1 −15.2 mg $l^{-1}$); $p < 0.01$. Lower activity of LasA protease was also observed in MDR strains

supernatants (1.15 U $ml^{-1}$, SD = 3.97 vs. 0.9 $ml^{-1}$, SD = 1.22; PAO1 −3.41 U $ml^{-1}$); $p < 0.001$. Sánchez et al. (2002) indicated that laboratory obtained mutants of *P. aeruginosa* strains overexpressing efflux pumps, produced lower amounts of elastase. They indicate that in vivo selection may lead to other genetic changes and other changes of virulence factors profile. Cowell et al. (2003) investigated PAO1 knock-out mutants, without *lasA* and/or *lasB* genes. Their results showed that the presence of *lasB* gene correlates stronger with *P. aeruginosa* virulence rabbit corneal epithelium model than the presence of *lasA* gene. These authors claim that the elastolytic activity is the main cause of *P. aeruginosa* virulence. Both, LasB (elastase) and LasA proteases are known to be controlled by two quorum-sensing systems (*las* and *rhl*) virulence factors. Further investigations may elucidate if lower expression of these proteases is caused by altered *las* and *rhl* signalling systems due to multidrug resistance. Bratu et al. (2006) indicated that the enhanced expression of *las* and *rhl* quorum-sensing systems leads to increased production of elastase and pyocyanin. It did not, however, influence the increase in antimicrobial resistance. The MDR strains included in our study usually presented different mechanisms of antimicrobial resistance.

Lack of differences in proteolytic activity of supernatants with differences in LasA and elastase activity may be a result of specificity of substrates used in the study. Azocasein is a substrate used to evaluate total proteolytic activity which may be a sum of activities of many different proteases found in *P. aeruginosa* strains (i.e. LasA, LasB, alkaline protease, elastase A and B and type IV protease). The MDR strains might have enhanced activity of other proteases, which were not tested in the study, so that lower LasA and elastase activity did not affect the total proteolytic activity in the protease assay.

In the culture supernatants of MDR *P. aeruginosa* strains, lower lipolytic activity was observed: (38.191 U $ml^{-1}$; SD = 24.484) versus (47.254 U $ml^{-1}$; SD = 21.95). These differences were statistically significant, $p < 0.001$. PAO1 supernatants reached the activity of 28 U $ml^{-1}$. There is no information on differences between MDR and MDS bacterial strains in lipase production in relevant literature.

MDR strains expressed lower phospholipase C activity in culture supernatants than MDS strains (0.580 U $ml^{-1}$; SD = 0.556 vs. 2.139 U $ml^{-1}$; SD = 6.691; PAO1 −0.436 U $ml^{-1}$). The differences observed were statistically significant ($p < 0.001$). Such differences had not been reported elsewhere.

Pyocyanin synthesis was present in 33 (44.4%) MDS and 31 (41.3%) MDR strains. MDR strains produced less pyocyanin than MDS strains (24.98 μmol $l^{-1}$, SD = 85.85 vs. 47.53 μmol $l^{-1}$, SD = 63.2; PAO1 −53.2 μmol $l^{-1}$) $p < 0.001$. Pyocyanin is a redox-active pigment that plays an important role in *P. aeruginosa* infections (Ran et al. 2003). Its synthesis in bacterial cell is under influence of quorum-sensing system (Bratu et al. 2006; Dietrich et al. 2006). Some epidemic strains had been described as pyocyanin and LasA protease overproducers, which is quite an unusual phenotype (Fothergill et al. 2007).

In conclusion, we found that when compared with MDS strains, the MDR *P. aeruginosa* strains have impaired virulence in vitro study mainly as a result of slow growth and reduced expression of some exoenzymes and pyocyanin. Further studies using molecular biology methods and possible correlation to quorum-sensing alterations in MDR strains are necessary to confirm these observations.

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