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Short Communication

Carbapenem resistance in cystic fibrosis strains of *Pseudomonas aeruginosa* as a result of amino acid substitutions in porin OprDCharlotte Richardot^a, Patrick Plésiat^a, Damien Fournier^a, Laura Monlezun^b, Isabelle Broutin^b, Catherine Llanes^{a,*}^a Laboratoire de Bactériologie EA4266, Faculté de Médecine-Pharmacie, Université de Franche-Comté, Besançon, France^b UMR8015, Laboratoire de Cristallographie et RMN biologiques, Paris, France

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ABSTRACT

The aim of this work was to investigate the impact of single amino acid substitutions occurring in specific porin OprD on carbapenem resistance of cystic fibrosis (CF) strains of *Pseudomonas aeruginosa*. A PAO1 Δ oprD mutant was complemented with the oprD genes from five carbapenem-resistant CF strains exhibiting very low amounts of mutated OprD porins in their outer membrane despite wild-type levels of oprD transcripts. Compared with wild-type porin from strain PAO1, single amino acid substitutions S₄₀₃P (in periplasmic loop 8), Y₂₄₂H, S₂₇₈P and L₃₄₅P (in β -sheets 10, 12 and 14, respectively) were found to result in reduced amounts of OprD in the outer membrane, increased carbapenem resistance, and slower growth in minimal medium containing gluconate, an OprD substrate, as the sole source of carbon and energy. This indicates that in CF strains of *P. aeruginosa*, loss of porin OprD may not only result from mutations downregulating the expression of or disrupting the oprD gene, but also from mutations generating deleterious amino acid substitutions in the porin structure.

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1. Introduction

Carbapenems are key antibiotics in the treatment of *Pseudomonas aeruginosa* infections. However, resistance rates to these β -lactams have been reported to be gradually increasing in many countries because of the emergence and diffusion of strains producing carbapenem-hydrolysing β -lactamases (carbapenemases) [1] and/or that are deficient in porin OprD, the major route of entry of these molecules into the micro-organism [2]. Unless carbapenemases are produced, loss of OprD is usually associated with a 4- to 16-fold increase in the minimum inhibitory concentrations (MICs) of all carbapenems [3]. As shown by several recent studies [4,5], deficient OprD production mainly results from mutations or insertion sequences disrupting the oprD gene or its promoter region. In some strains, carbapenem resistance is associated with mutations occurring in genes (*parRS*, *mexS* and *czcS*) that are involved in the inverse co-regulation of expression of oprD and various efflux operons (*mexXY*, *mexEF-oprN* and *czcCBA*, respectively) [4]. Here we demonstrate for the first time that some single amino acid substitutions drastically decrease the stability and/or insertion in the outer membrane of the otherwise highly polymorphic porin and

account for the resistance to carbapenems of cystic fibrosis (CF) *P. aeruginosa* strains.

2. Materials and methods

2.1. Bacterial strains and plasmids

Recently, 5 (12%) of 42 imipenem-resistant (MICs > 8 mg/L) CF strains of *P. aeruginosa* (named 1–10, 1–13, 1–17, 5–11 and 6–20) were found to have poor or undetectable amounts of porin OprD in their outer membrane despite expression of their oprD genes similar to that of wild-type susceptible strains [5]. Sequencing of these genes revealed the presence of single amino acid substitutions (L₃₄₅P, Y₂₄₂H, S₂₇₈P, V₄₀₁A and S₄₀₃P, respectively) in the corresponding OprD proteins compared with homologues from reference strains PAO1, LESB58 and PA14 [5]. An extensive search among the already edited OprD porins of *P. aeruginosa* (<http://www.pseudomonas.com>) led to the conclusion that these mutations were unique to these strains.

2.2. Mutant frequency

Phenotypic mutation frequencies of clinical isolates were determined by resistance to rifampicin based on the method described by Oliver et al. [6]. Strains giving rise to mutants able to resist

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10-fold the baseline resistance levels to rifampicin at rates ≥ 20 -fold that of reference strain PAO1 were considered as hypermutators.

2.3. Gene inactivation and complementation

A $\Delta oprD$ mutant from reference strain PAO1 (<http://v2.pseudomonas.com>) was constructed and was complemented by genomic insertion of the five *oprD* genes and their respective promoter regions. Mutant PAO1 $\Delta oprD$ was obtained by using overlapping PCRs with specific primers (PCRioprD1, 5'-CAATTTGTGCACGGAGTTTG-3'; PCRioprD2, 5'-CAGGTCGTGCTTCGATGAACCCCTTC-3'; PCRioprD3, 5'-GAAGACACGACCTGAACCTAGCCTCTCT-3'; and PCRioprD4, 5'-GGGGTGTTCGACAGAGTAA-3') and recombination events as described previously [7]. Allelic exchange and deletion of 910 bp in the *oprD* gene were confirmed by PCR and DNA sequencing. Then, the *oprD* genes of clinical strains were amplified from whole DNA extracts using the oligonucleotide pair IntoprDCh1 (5'-CGGAAGCTTAGCCATTTTCCAATTTGTGC-3') and IntoprDCh2 (5'-GCCGGATCCGGGGTGTTCGACAGAGTAA-3'). The PCR conditions used were as follows: 5 min of denaturation at 98 °C; 30 amplification cycles each consisting of 30 s at 98 °C, 30 s at 61 °C and 2 min at 72 °C; with a final extension step at 72 °C for 7 min. The resulting 1828 bp DNA fragments were cloned into the *Bam*HI/*Hind*III restricted plasmid mini-CTX1 and were then transferred from *Escherichia coli* CC118 to *P. aeruginosa* PAO1 $\Delta oprD$ by conjugation [8]. A control recombinant strain, named CRPAO1, complemented with the wild-type *oprD* allele of PAO1 was engineered in parallel.

2.4. Immunodetection of OprD

Immunoblotting experiments were carried out on purified outer membrane extracts or whole-cell lysates with an OprD-specific polyclonal antiserum (diluted 1:10,000) as reported previously [9].

2.5. Susceptibility testing and growth conditions

All of the bacterial strains were cultured at 37 °C in Mueller–Hinton broth (MHB) or on Mueller-Hinton agar (MHA), both of them containing adjusted concentrations of divalent cations (Bio-Rad, Marnes-la-Coquette, France). MICs were determined by the serial two-fold macrodilution method in MHA according to protocols recommended by the Clinical and Laboratory Standards Institute (CLSI) (<http://www.clsi.org>). Bacterial growth experiments were carried out in MHB and minimal medium supplemented with 0.5 mM gluconate as previously described [10].

2.6. OprD structural bioinformatics analysis

The amino acid substitutions were mapped on the most recently solved structure of OprD (Protein Data Bank code: 3SY7) using the Coot program [11]. The geometry was refined locally using planar peptide restraints. All of the graphical representations were performed using PyMOL software (<http://pymol.sourceforge.net/>).

3. Results and discussion

Complementation of PAO1 $\Delta oprD$ with the various *oprD* alleles failed to fully restore carbapenem susceptibility in four of the five transconjugants. Indeed, CR1-10, CR1-13, CR1-17 and CR6-20 appeared to be two- to four-fold less susceptible than CRPAO1 to imipenem, meropenem and doripenem (Fig. 1A). These recombinant strains were also as resistant as PAO1 $\Delta oprD$ to carbapenems.

Very low amounts of the OprD variants could be detected in CR1-10, CR1-13 and CR1-17 (Fig. 1B), suggesting that the

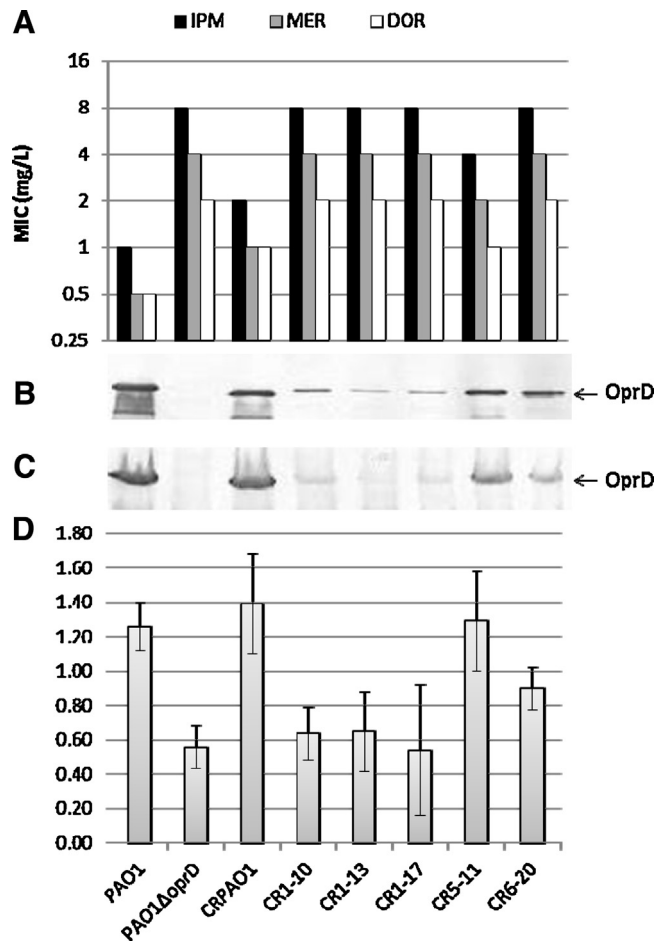


Fig. 1. (A) Resistance levels, (B, C) OprD immunoblots and (D) growth rates of PAO1 $\Delta oprD$ trans-complemented mutants. (A) Minimum inhibitory concentrations (MICs) of imipenem (IPM), meropenem (MER) and doripenem (DOR) were determined at least twice in two independent experiments. (B, C) Detection of porin OprD by western blotting in outer membrane extracts (B) and whole-cell lysates (C) of mutants CRPAO1, CR1-10, CR1-13, CR1-17, CR5-11 and CR6-20. (D) Growth rates of PAO1 $\Delta oprD$ trans-complemented mutants were measured in minimal medium with 0.5 mM gluconate as the sole carbon source.

corresponding amino acid substitutions (L₃₄₅P, Y₂₄₂H and S₂₇₈P, respectively) might either prevent insertion of the porin into the outer membrane or make the protein more susceptible to enzymatic degradation in the cytoplasm or in the periplasmic space. Indeed, some misfolded proteins are known to form aggregates that are rapidly degraded by periplasmic proteases [12]. Consistent with this, only low levels of these OprD variants could be detected in whole-cell lysates from CR1-10, CR1-13 and CR1-17 by western blotting (Fig. 1C). Since OprD allows the uptake of gluconate [10], we measured the growth rates of complemented strains in minimal medium with gluconate as the sole carbon source and in MHB for comparison. CR1-10, CR1-13 and CR1-17 exhibited a growth rate similar to that of PAO1 $\Delta oprD$ (growth rate 0.56), compatible with the slight amounts of porin present in these strains (Fig. 1D). Conversely, in MHB medium all of the transconjugants grew at the same rates as CRPAO1 (data not shown), confirming that in minimal medium with gluconate bacterial growth solely relies upon OprD-dependent penetration of the substrate into the cells. To evaluate the impact of the different mutations on porin architecture, we mapped the corresponding amino acid variations on the structure of OprD. Possibly accounting for defective insertion of the OprD variants into the outer membrane, the model predicted that: (i) the L₃₄₅P substitution (CR1-10), located at the extremity of strand

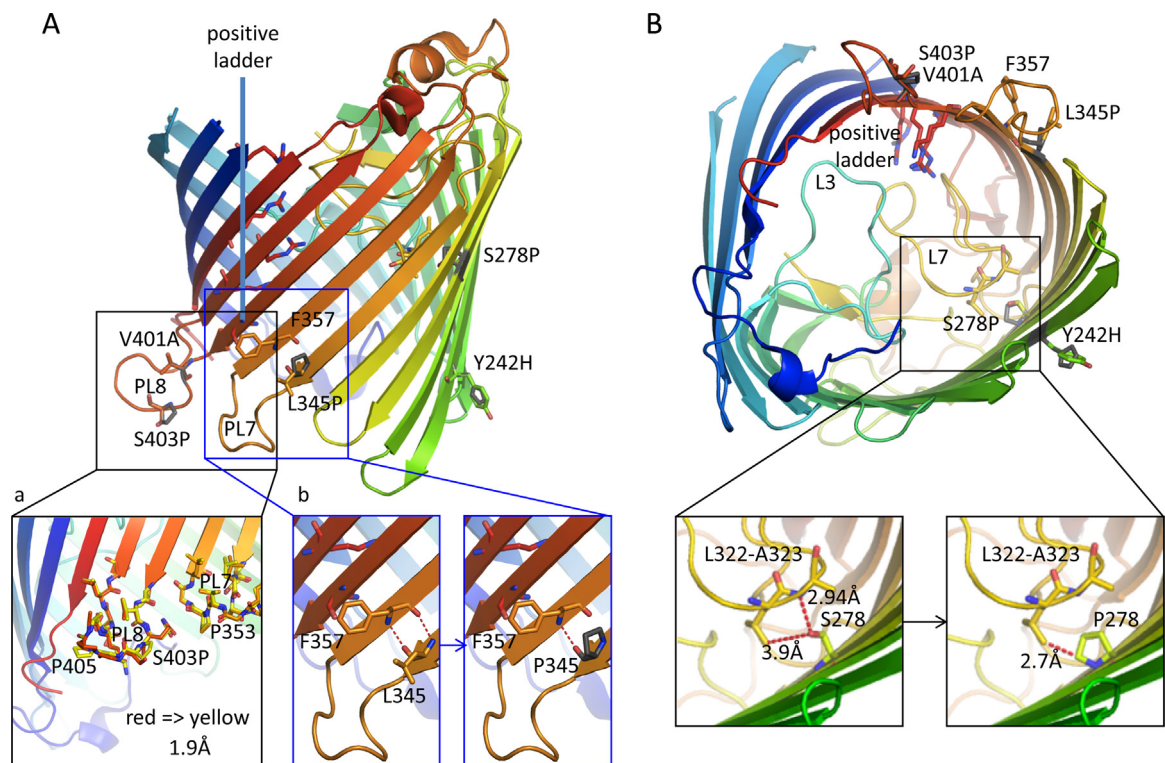


Fig. 2. Cartoon representation of the structure of OprD with the amino acid substitutions L₃₄₅P, Y₂₄₂H, S₂₇₈P, V₄₀₁A and S₄₀₃P in ball and stick. Some additional residues of the positively charged ladder (constituted by amino acids R₅₃, K₃₉₈, R₄₁₂, R₄₁₄ and R₄₃₃) as well as residues L₃₂₂, A₃₂₃ and F₃₅₇ are indicated for clarity. (A) View of the porin through the outer membrane. Inset (a) presents in ball and stick all the residues constituting periplasmic loops PL7 and PL8 in order to highlight the possible displacement of loop PL8 owing to the S₄₀₃P mutation. Amino acid residues in red show the wild-type position of the loop; those in yellow show the modifications that could be induced by the S₄₀₃P substitution. Inset (b) represents the wild-type interaction between L₃₄₅ and F₃₅₇, and the breaking of the hydrogen bond between the carbonyl of P₃₄₅ and the nitrogen of F₃₅₇. (B) Perpendicular view. The insets represent a scaled-up view of the interactions between residue 278 and loop L7 before and after mutation. It highlights the possible steric hindrance between S₂₇₈P and residue L₃₂₂. In addition, the β -sheet could be twisted by the introduction of a proline that could lead to destabilisation of the whole pore.

S14, could destabilise the end of this strand by breaking the hydrogen bond between the carbonyl of L₃₄₅ and the nitrogen of F₃₅₇ and impact the orientation of the PL7 periplasmic loop; (ii) Y₂₄₂H (CR1-13) introduces a positive charge in the acidic environment of the periplasmic space, as it is localised outside of the pore structure, close to the inner side of the outer membrane; and (iii) S₂₇₈P (CR1-17), located in S12, could both destabilise this strand and change amino acid interactions into the pore by creating a steric hindrance with L₃₂₂ from one of the loops responsible for the inner constriction of the pore, the loop L7 (Fig. 2).

The complemented strain CR6-20 exhibited carbapenem resistance despite the presence of significant amounts of OprD in the outer membrane (Fig. 1B). Based on our model of OprD, the S₄₀₃P mutation in this variant might modify the orientation of the periplasmic loop PL8, thereby disturbing the nearby positively charged molecular ladder (Fig. 2), which promotes the passage of carbapenems through the pore [13]. Consistent with this, CR6-20 exhibited an intermediate growth rate of 0.9 compared with that of the positive and negative controls (CRPAO1 and PAO1 Δ oprD, respectively) (Fig. 1D). Carbapenem resistance in CR6-20 would thus result from both the presence of reduced amounts of OprD in the outer membrane and lower penetration rates of carbapenems through the porin channel, a hypothesis that would require electrophysiology techniques for confirmation.

In the last complemented strain, CR5-11, which produced notable amounts of OprD (Fig. 1B), carbapenem susceptibility was partially restored in comparison with CRPAO1, suggesting that the V₄₀₁A substitution, located in periplasmic loop PL8, does not play a major role in resistance. A trivial explanation would be that a component of the Bam transport machinery, which delivers porins

to the outer membrane, is impaired in the parental strain 5-11, thus leading to carbapenem resistance. Supporting this notion, decreased expression of one Bam component in an in vitro mutant has been reported to significantly affect the ability of porin OprF to localise into the outer membrane [14].

OprD polymorphism could be considered as a consequence of the well-known hypermutable phenotype of *P. aeruginosa* CF strains. To confirm this, we determined mutant frequencies leading to rifampicin resistance for strains 1-10, 1-13, 1-17, 5-11 and 6-20. As expected, all of them exhibited higher (≥ 20 -fold) mutation rates (4.1×10^{-7} , 2.6×10^{-6} , 2.0×10^{-6} , 4.8×10^{-7} and 2.9×10^{-6} , respectively) compared with PAO1 (7.6×10^{-9}), indicating that CF isolates are hypermutators.

4. Conclusion

In conclusion, this study shows that single amino acid substitutions located in periplasmic loops or in β -sheets may result in very low levels of porin OprD in the outer membrane and thus impermeability to carbapenems. Contrary to OprD-deficient mutants, those harbouring permeability-impairing amino acid substitutions in the porin structure require extensive analyses for their identification. This is probably why such mutants have rarely been reported among clinical strains of *P. aeruginosa* and remain under-recognised. Sanbongi et al. suggested that the G₃₁₄D substitution in L7 could impact carbapenem resistance levels [15]. This substitution, initially identified in an in vitro mutant selected for its resistance to carbapenems, was further observed in a clinical strain. Because of its carboxylic group, D₃₁₄ might cause a significant change to the porin conformation [15]. Anyway

compared with frameshifting mutations in the *oprD* gene [4,5], amino acid changes appear to be less frequent but still significant (12%) among imipenem-non-susceptible isolates in the context of CF where the strains are exposed to repeated antibiotic treatments and evolve rapidly because of hypermutator phenotypes.

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