ß-Lactams Translocation through *Pseudomonas aeruginosa* Outer Membrane

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Abstract (edited)

Background

Permeation of small molecules through *P. aeruginosa* outer membrane is an important issue, still poorly understood. Its importance stems from the fact that the low permeability of *P. aeruginosa* is one of the causes of its intrinsic resistance to different antibiotics such as β-lactams. For example, the deletion of the porin OprD can lead to strains highly resistant towards carbapenems. Many studies have characterized different single channels and their roles in determining antibiotic permeation, but we still lack a global view of the effects of single/multiple porin(s) deletion(s) for the translocation of antibiotics in *P. aeruginosa*.

Methods

Strains: *P. aeruginosa* PAO1 and different *P. aeruginosa* PAO1 deletion mutants studied are reported in Tab. 1; the strains TNP004, TNP065, TNP067 and YY200 produce low amount of OprD porin.

MIC determination: MICs were determined by broth microdilution following CLSI recommendations; results are reported in Tab. 1.

Growth curves: the growth rates of *P. aeruginosa* PAO1 and different porin(s) mutants were determined by following the absorbance at 600 nm for 12 hours of cultures grown in LB medium.

Permeability determination: BlaR-CTD, the C-terminal domain of a highly sensitive penicillin binding protein (Tab. 2) from *Bacillus licheniformis*, was expressed in the periplasmic space of *P. aeruginosa* thanks to the pKT240blaR plasmid Fig.1 [1]. We performed a direct measure of the β-lactam accumulation in the periplasmic space of the bacteria by fluorescence analysis (Fig. 2, Fig. 3 and Fig. 4).
The permeability coefficients of the external membrane to different antibiotics were measured for *P. aeruginosa* PAO1 and for different mutant strains, lacking in one or multiple porins (Tab. 3).

qRT-PCR: the porins OprD and OpdP mRNA were quantified at 4 different moments of growth to determine any change as a function of growth phase; the relative expression of these 2 genes was determined on the basis of 3 independent reference genes (*PA3340*, *gyrA* and *cysG*).

Results

Growth curves show a similar progress between *P. aeruginosa* PAO1 and its porin mutants, thus confirming the ability of the bacteria to adapt its growth at porin loss (Fig. 5).

The permeability coefficients obtained in this study are reported in Tab. 3.
We noticed a 150-fold reduction of imipenem permeability in strains were OprD was deleted. These results are in good agreement with MICs values that increase from 1 to 8 µg/mL when OprD is poorly or not expressed. We also pointed out that the absence of OprD did not affect the permeability coefficient of *P. aeruginosa* PAO1 for meropenem and biapenem, differently from the MICs that rise from 0,5 to 4 µg/mL for both antibiotics. However, for these antibiotics, in ARC5782 (*P. aeruginosa* PAO1 ΔoprD, ΔopdP) the *P* values decreased to 6.5 · 10⁻³ and 0.16 nm/s respectively. We could not appreciate differences in permeability coefficients for the strains lacking the major efflux pumps systems, probably due to a slow recognition between the antibiotic and the efflux pumps.

qRT-PCR allowed us to observe that, in absence of OprD, *P. aeruginosa* ARC5990 increases the expression of the related porin OpdP (Fig. 6B).
We also showed a different ratio between OprD and OpdP as a function of growth phase in *P. aeruginosa* PAO1 (Fig. 6A and 6B). Thus, in accordance with the experimental results obtained by permeability coefficient determination.

Acknowledgments

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Tab. 1

Strains and MIC determination

Antibiotic (µg/mL) (CLSI standard)	PAO1	TNP004 [2]	TNP064 [2]	YY100 [2]	TNP065 [2]	TNP066 [2]	YY200 [2]	TNP067 [2]	ARC5990 [3]	ARC5170 [3]	ARC5782 [3]	ARC5998 [3]	PAO200 [4]	PAO509 [4]
Relevant characteristics		↓oprD	ΔoprC	ΔoprE	ΔoprC, ↓oprD	ΔoprC, ΔoprE	↓oprD, ΔoprE	ΔoprC, ↓oprD, ΔoprE	ΔoprD	ΔopdP	ΔoprD, ΔopdP	ΔoprD, ΔopdP, ΔopdB, ΔopdC, ΔopdT	ΔmexAB-oprM	ΔmexAB-oprM, ΔmexCD-oprJ, ΔmexJK, ΔmexXY, ΔmexEF-oprN
Ampicillin	2000	1000	1000	2000	1000	2000	2000	1000	1000	1000	1000	1000	500	125
Piperacillin (1-8)	2	2	2	2	2	2	2	2	8	8	8	8	0.25	0.25
Cephaloridin	>2000	>2000	>2000	>2000	>2000	>2000	>2000	>2000	>2000	>2000	>2000	>2000	>2000	>2000
Cefoxitin	1000	500	500	500	500	500	500	500	1000	1000	1000	1000	1000	1000
Cefuroxime	250	250	250	250	250	250	250	250	500	500	500	500	250	250
Cefotaxime (8-32)	16	8	16	16	16	16	16	16	16	16	16	16	0.5	0.5
Cefepime (1-8)	1	1	1	1	1	1	1	1	8	8	8	8	1	0.12
Imipenem (1-4)	1	8	1	1	8	1	8	8	8	1	8	8	1	1
Meropenem (0.25-1)	0.5	2	0.5	0.5	2	0.5	2	2	4	0.5	4	4	0.12	0.12
Ertapenem (2-8)	8	32	8	8	16	8	16	16	32	8	32	32	2	2
Doripenem (0.12-0.5)	0.25	1	0.25	0.25	1	0.25	1	0.5	1	0.25	1	1	0.5	0.12
Biapenem (0.5-2)	0.5	4	0.5	0.5	4	0.5	4	4	4	0.5	4	4	0.25	0.25

Table 1: Table 1 reports the properties of different strains used in this study; MIC values for a selection of antibiotics used in this study are also reported. CLSI reference values for *P. aeruginosa* are reported in brackets.

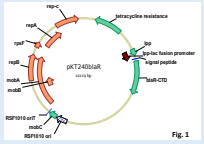

Fig. 1

Figure 1: pKT240blaR shuttle plasmid *E. coli*/*P. aeruginosa* used in this study to produce BlaR-CTD in the periplasm of *P. aeruginosa*.

Tab. 2

Antibiotic	k_2/K ($\mu M^{-1}s^{-1}$)
Ampicillin	1.3 ± 0.1
Cephaloridin	5.9 ± 0.2
Imipenem	0.77 ± 0.23
Meropenem	0.83 ± 0.16
Ertapenem	1.13 ± 0.15

Table 2: Table 2 reports the k_2/K values for different antibiotics used in this study.

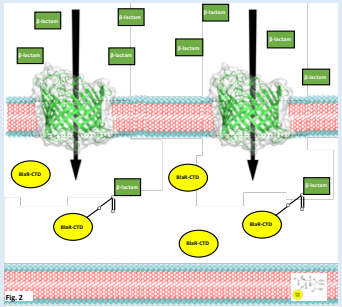

Fig. 2

Figure 2: Schematic representation of the periplasm of *P. aeruginosa*; BlaR-CTD is produced in the periplasm and it will act as a probe for different β-lactams that will pass the outer membrane through specific porins. The complex BlaR-CTD- β-lactam is stable and will be quantified after bacterial lysis.

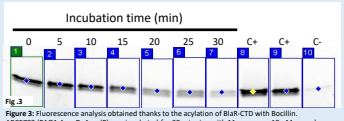

Fig. 3

Figure 3: Fluorescence analysis obtained thanks to the acylation of BlaR-CTD with Bocillin. ARC5782 (PAO1 ΔoprD, ΔopdP) was incubated for 30 minutes with Meropenem 10 µM; samples were harvested every 5 minutes; two samples (C+) were obtained to quantify the total BlaR-CTD produced by 1 mL of culture. One sample (C-) was obtained to assess the presence of BlaR-CTD in the medium.

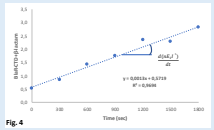

Fig. 4

Figure 4: Increase of BlaR-CTD-Meropenem complex (E_1I^*) as a function of time. The slope of the line represents the value of $d(nE_1I^*)/dt$ and it is used to determine the permeability coefficient.

$$P = \frac{d(nE_1I^*)/dt}{A \cdot [I_e]}$$

where, E_1I^* is the periplasmic concentration of BlaR-CTD acylated with the β-lactam, ***P*** is the permeability coefficient, ***A*** is the outer membrane area and it is assumed to be 132 $cm^2 \cdot$ mg (dry weight)$^{-1}$ and $[I_e]$ is the external concentration of antibiotic.

Tab. 3

Permeability coefficients (nm/sec)

Antibiotic	PAO1	TNP004	ARC5990	ARC5170	ARC5782	ARC5998	PAO509
Relevant characteristics		↓oprD	ΔoprD	ΔopdP	ΔoprD, ΔopdP	ΔoprD, ΔopdP, ΔopdB, ΔopdC, ΔopdT	ΔmexAB-oprM, ΔmexCD-oprJ, ΔmexJK, ΔmexXY, ΔmexEF-oprN
Ampicillin	0.008 ± 0.004	0.008 ± 0.003	0.02 ± 0.006	0.01 ± 0.002	0.01 ± 0.002	0.02 ± 0.005	0.03 ± 0.007
Cephaloridin	0.03 ± 0.01	0.02 ± 0.004	-	0.03 ± 0.009	0.03 ± 0.008	0.04 ± 0.01	0.06 ± 0.02
Imipenem	20 ± 9	0.13 ± 0.06	0.14 ± 0.08	15 ± 6	0.13 ± 0.05	0.12 ± 0.07	18 ± 9
Meropenem	0.06 ± 0.01	0.03 ± 0.01	0.03 ± 0.006	0.1 ± 0.04	0.007 ± 0.002	0.01 ± 0.005	0.07 ± 0.02
Ertapenem	0.06 ± 0.02	0.03 ± 0.01	0.04 ± 0.01	0.02 ± 0.008	0.03 ± 0.003	0.02 ± 0.008	0.03 ± 0.01
Doripenem	0.56 ± 0.38	0.20 ± 0.05	0.08 ± 0.03	0.11 ± 0.02	0.14 ± 0.09	0.11 ± 0.05	0.13 ± 0.03
Biapenem	4.7 ± 1.4	3.4 ± 2.0	4.2 ± 2.2	7.2 ± 3.5	0.16 ± 0.09	0.12 ± 0.04	4.0 ± 1.4

Table 3: Permeability coefficient values determined in this study; each value represents the mean of measures performed in duplicate at 3 different antibiotic concentrations.

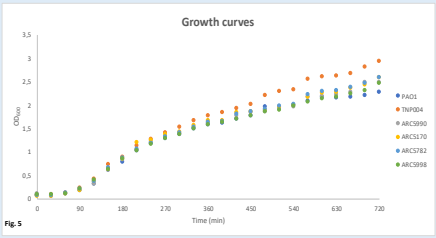

Fig. 5

Figure 5: Growth curves of *P. aeruginosa* PAO1 and 5 different porin(s) mutants grown in LB medium; the increase of absorbance was measured at 600 nm for 12 hours.

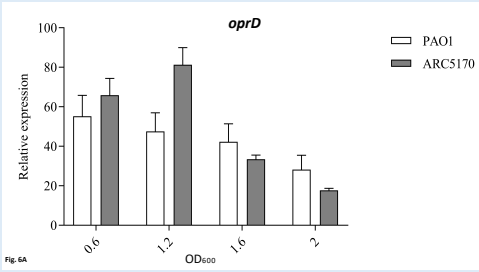

Fig. 6A

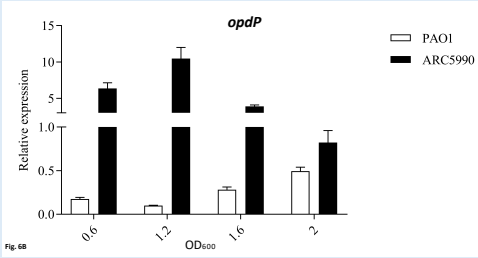

Fig. 6B

Figure 6: Relative expression of OprD **6A** and OpdP **6B** mRNA in *P. aeruginosa* PAO1, ARC5170 (PAO1 ΔopdP) and ARC5990 (PAO1 ΔoprD) respectively; each value was obtained by qRT-PCR in 4 independent biological replicates and 3 different technical replicates. The total RNA extractions were performed at 4 different points of the bacterial growth.

Conclusions

This work allowed us to quantify the permeability of the outer membrane in *P. aeruginosa* and contributes to the modelisation of the intrinsic resistance of *P. aeruginosa* to β-lactams.
Interactions of OpdP and meropenem were already described but we identified the involvement of OpdP in biapenem uptake.
We pointed out the synergic role of OprD and OpdP for carbapenems uptake; we also demonstrated a compensative expression of OpdP when OprD is deleted.
We verified that permeation of carbapenems, except for Imipenem, is not only dependent to OprD contrary to MIC results.
In order to complete our study, we will quantify the role of efflux pumps overexpression in antibiotic resistant strains.

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