Amisano F.[1], Silvestri M.[1], Mercuri P.S.[1], Gagliardi A.[2], Bini L.[2], Frère J.M.[1] and Galleni M.[1]

[1]Macromolécules Biologiques, Centre for Protein Engineering, University of Liège, Liège, Belgium
[2]Functional Proteomics Laboratory, Department of Life Sciences, University of Siena, Siena, Italy

Study of the outer membrane permeability of *Pseudomonas aeruginosa* to ß-lactam antibiotics

Introduction

β-Lactams are the most potent and widely used antibiotics but their activity depend on the presence in the target bacteria of resistances caused by the interplay between four independent factors:

(i) the sensitivity of the target enzymes, the penicillin-binding proteins
(ii) the properties and concentration of the periplasmic β-lactamases
(iii) the permeability of the outer membrane
(iv) the efficiency of the active efflux system

On this basis, Zimmermann and Rosselet [1] proposed a model which allowed a quantitative prediction of the MICs for Gram-negative bacteria and it was applied with success to *Escherichia coli* and *Enterobacter cloacae.*

This model seems to be not applicable to *Pseudomonas aeruginosa* due to its low outer membrane permeability that is mostly influenced by the combined result between a remarkable reduction of the functional porins expression and an over-expression of the efflux system (e.g. MexA-MexB-OprM, MexC-MexD-OprJ, MexE-MexF-OprN and MexX-MexY-OprM) [2, 3]; this decrease in permeability causes difficulties in the directs measures of the permeability coefficient resulting in few and highly variable coefficients published for *P. aeruginosa.*

For this purpose BlaR-CTD, the C-terminal domain of a highly sensitive penicillin binding protein derived from *Bacillus licheniformis*, expressed in the periplasmic space has been used for the direct determination of the concentrations of different β-lactam antibiotics in this cell compartment and consequently to have reliable measures of the permeability coefficients [4].

This method can avoid the problem of the low outer membrane permeability and can give information about the permeability coefficients of different β-lactams thanks to the following model:

$$I_e \underset{k_d}{\rightleftharpoons} I_p + \begin{matrix} E_1 \xrightarrow{k_{f1}} E_1I^* \\ E_2 \xrightarrow[k_{f2}]{} E_2I^* \xrightarrow{k_3} E_2 + X \end{matrix}$$

The equations describing the variations of I_p, E_1I^* and E_2I^* are the following:

$$d[I_p]/dt = k_d \cdot ([I_e] - [I_p]) - k_{f1} \cdot [E_1] \cdot [I_p] - k_{f2} \cdot [E_2] \cdot [I_p]$$
$$d[E_1I^*]/dt = - d[E_1]/dt = k_{f1} \cdot [E_1] \cdot [I_p]$$
$$d[E_2I^*]/dt = - d[E_2]/dt = k_{f2} \cdot [E_2] \cdot [I_p] - k_3 \cdot [E_2I^*]$$

Legend:
I_e : external concentration of antibiotic
I_p : periplasmic concentration of antibiotic
E_1 : periplasmic concentration of BlaR-CTD
E_2 : periplasmic concentration of β-lactamase
E_1I^* and E_2I^* are the corresponding acyl -enzymes
X :degradation product of β-lactam
k_d : first-order rate constant for antibiotic diffusion through the outer membrane
k_{f1} *and* k_{f2} are the second-order rate constants for the formation of E_1I^* and E_2I^*, respectively
k_3 : first-order rate constant for deacylation of the β-lactamase
P: permeability coefficient
A: outer membrane area, equal to 132 $cm^2 \cdot mg\ (dry\ weight)^{-1}$
V_p: periplasmic volume equal to 1 µl · mg (dry weight)$^{-1}$
nE_1I^*: labeled BlaR-CTD (pmol)

It is possible to define the permeability coefficient (P) considering:

$$k_d = P \cdot A/V_p$$
$$[E_1I^*] = nE_1I^*/V_p$$
$$d(nE_1I^*)/dt = P \cdot A \cdot [I_e]$$

Aims

- **Periplasmic production of BlaR-CTD as a probe**
- **Quantization of different β-lactams in the periplasm and measure of their permeability coefficients (*P*)**
- **Analysis of a PAO1 porin mutants collection to study the role of each single channel in the β-lactams permeability**
- **Characterization of the influences of the low outer membrane permeability of *P. aeruginosa* PAO1 and TNP065 (*ΔoprC, ΔoprD*) in the periplasmic proteome composition.**

Results

The reference strain *P. aeruginosa* PAO1 was transformed with pKT240blaR plasmid (fig. 1) in order to produce BlaR-CTD in the periplasm.
We also receive a *P. aeruginosa* PAO1 collection of porin mutant strains (tab.1) [6], used for the outer membrane permeability determination (TNP004, *ΔoprD*) or for the proteomic analysis (TNP065, *ΔoprC, ΔoprD*).
For the permeability assay *P. aeruginosa* cultures, at their stationary phase, were incubated with β-lactam at room temperature; 1mL samples were collected at different incubation times and the excess of antibiotic was hydrolysed by the addition of 2 µg of VIM-4 metallo-β-lactamase for 2 minutes; successively EDTA was added, prior the lysis of the bacteria, to have 1 mM final concentration. The soluble crude extract, obtained after sonication, was isolated and incubated in presence of 2.5 µM Bocillin. The samples were then analysed by SDS-PAGE and quantified with a densitometric method.
The quantification result refers to the BlaR-CTD-Bocillin complex, while the quantification of the BlaR-CTD-β-lactam complex was obtained subtracting the different time values from the quantification of the total BlaR-CTD.
We report here the permeability tests made for Imipenem in PAO1 at 0.01 µM (fig. 2 and 3) and at 2 µM for TNP004 (fig. 4 and 5).

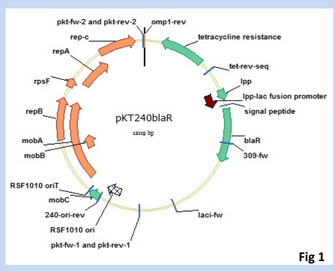

Fig 1

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

Table 1: Table 1 shows the collection of *P. aeruginosa* PAO1 porin mutant strains made up by Yoneyama *et al.* [6].

Tab. 1	Sample	Relevant properties
1	PAO1	wild type
2	TNP064	*ΔoprC*
3	TNP004	*ΔoprD*
4	YY100	*ΔoprE*
5	TNP065	*ΔoprC, ΔoprD*
6	TNP066	ΔoprC, *ΔoprE*
7	YY200	*ΔoprD, ΔoprE*
8	TNP067	*ΔoprC, ΔoprD, ΔoprE*

PAO1

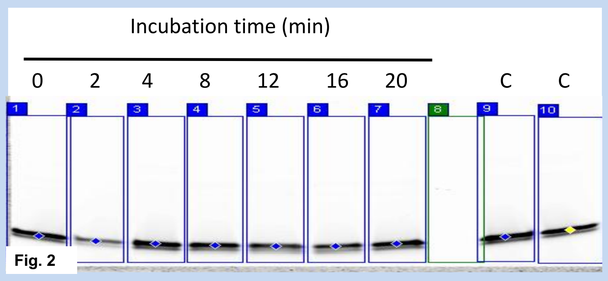

Fig. 2

Figure 2: Figure 2 shows the fluorescence analysis for the determination of the outer membrane permeability to Imipenem in *P. aeruginosa* PAO1; the samples were taken at increasing times; two samples (C) were obtained, after outer membrane lysis, in order to quantify the total BlaR-CTD produced by 1 mL of culture of PAO1.

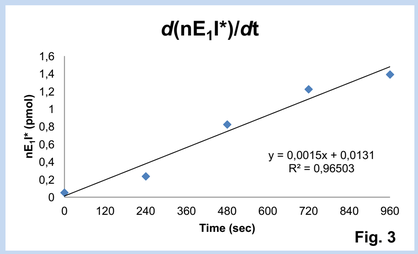

Fig. 3

Figure 3: Figure 3 shows the increase of BlaR-CTD-Imipenem 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.

$$\boldsymbol{P} = d(nE_1I^*)/dt \,/\, (A \cdot [I_e]) = \mathbf{1.6 \cdot 10^{-6}\ cm \cdot sec^{-1}}$$

TNP004 (*ΔoprD*)

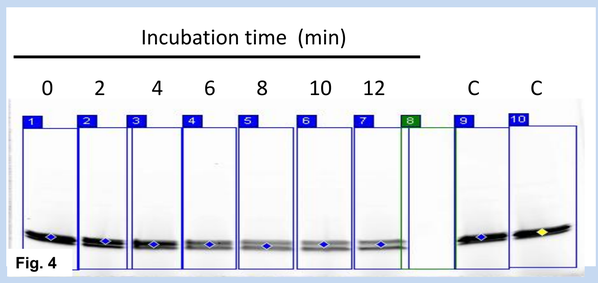

Fig. 4

Figure 4: Figure 4 shows the fluorescence analysis for the determination of the outer membrane permeability to Imipenem in *P. aeruginosa* TNP004; the samples were taken at increasing times; two samples (C) were obtained, after outer membrane lysis, in order to quantify the total BlaR-CTD produced by 1 mL of culture of TNP004.

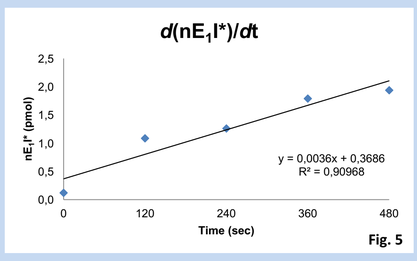

Fig. 5

Figure 5: Figure 5 shows the increase of BlaR-CTD-Imipenem 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.

$$\boldsymbol{P} = d(nE_1I^*)/dt \,/\, (A \cdot [I_e]) = \mathbf{1.4 \cdot 10^{-8}\ cm \cdot sec^{-1}}$$

Each antibiotic was tested at different concentrations and we report (tab. 2) the permeability coefficients measured for PAO1 and TNP004.
We used the acylation rate constants (k_2/K) of BlaR-CTD for different β-lactams published [7] and we determined with a spectrophotometric competition assay the others [8] (tab. 2).
MICs for the used antibiotics were determined with the broth microdiluition method as described by CLSI (tab. 2).
Some published permeability coefficients for *Escherichia coli* for the antibiotics tested are here reported for comparison (tab. 2).

Table 2: Table 2 shows the Permeability coefficients (P) for different β-lactams in *P. aeruginosa* PAO1 and TNP004.
The relative MICs for the antibiotic tested are reported as well as the affinity of BlaR-CTD for the antibiotics.
Permeability coefficients published for *E. coli* are also reported.

Tab. 2 Antibiotic	k_2/K BlaR-CTD ($\mu M^{-1} \cdot sec^{-1}$)	PAO1 MIC (µg/mL)	PAO1 Permeability coefficient ($cm \cdot sec^{-1}$)	TNP004 MIC (µg/mL)	TNP004 Permeability coefficient ($cm \cdot sec^{-1}$)	*E. Coli* Permeability coefficient ($cm \cdot sec^{-1}$)
Ampicillin	1.3 ± 0.1 [7]	2 mg/mL	$(8.2 \pm 4.3) \cdot 10^{-10}$	1 mg/mL	$6.4 \cdot 10^{-10}$	$2.8 \cdot 10^{-6}$ [9]
Cefotaxime	0.043 ± 0.003 [7]	16	$(1.1 \pm 0.4) \cdot 10^{-11}$	16	-	$1.8 \cdot 10^{-5}$ [10]
Imipenem	0.77 ± 0.23	1	$(1.7 \pm 0.7) \cdot 10^{-6}$	8	$(1.3 \pm 0.6) \cdot 10^{-8}$	$1.8 \cdot 10^{-4}$ [11]
Meropenem	0.83 ± 0.16	1	$(5.9 \pm 0.9) \cdot 10^{-9}$	4	$(3.6 \pm 1.6) \cdot 10^{-9}$	$3.0 \cdot 10^{-5}$ [11]
Ertapenem	-	8	$(6.0 \pm 2.1) \cdot 10^{-9}$	-	-	-
Doripenem	-	0.25	$(5.6 \pm 3.8) \cdot 10^{-8}$	-	-	-

Proteomics

Proteomic analysis of the periplasmic fraction following the spheroplasting by lysozyme and sucrose method [9] on:
i) PAO1 wt; *ii*) PAO1+pKT240neg; *iii*) PAO1+pKT240blaR; *iv*) TNP065 (PAO1 *ΔoprC, ΔoprD*)

Spots were selected on the basis of a statistically difference between the different conditions and 76 proteins were identified by mass spectrometry; we report here the comparison between PAO1 and TNP065 (fig. 6).

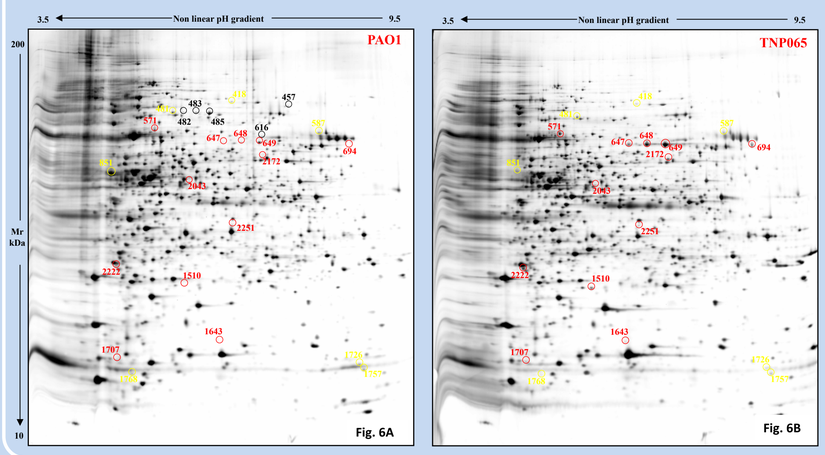

Fig. 6: Figure 6 shows the 2D gel result of periplasmic proteins of PAO1 (6A) and TNP065 (6B).
Differences in protein expression are highlighted:
• 5 spots present only in PAO1
• 7 spots with quantitative differences more expressed in PAO1
• 12 spots with quantitative differences more expressed in TNP065

Conclusion

We validated the use of BlaR-CTD for the determination of permeability coefficient (P) in *P. aeruginosa*.
We determine the permeability coefficients of different β-lactams in *P. aeruginosa* PAO1.
We confirmed the specificity of OprD for Imipenem permeability resulting in a 100 fold decrease between PAO1 and TNP004 (*ΔoprD*).
Finally, the proteomic analysis of the periplsmic proteome is in progress.

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