



**Master Biologie structurale, génomique**  
**Parcours : Biochimie structurale (BS)**  
**Année 2024-2025**

## MASTER 2 INTERNSHIP STRUCTURAL BIOLOGY 2024-2025

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**Title:** Measurement of antibiotic translocation through porins by electron paramagnetic resonance (EPR)

### State-of-the-art :

Bacterial resistance represents a growing threat to human and animal health worldwide. In Europe, it is responsible for around 25,000 deaths per year; and the medical costs associated with the resulting social costs are estimated at some 1.5 billion euros per year. New forms of resistance are emerging and spreading, leaving clinicians with few options for infection control. At the same time, despite the recognized need to develop new antimicrobial compounds, the reality is that only two new classes of antibiotics have been introduced to the market in the last three decades. In scientific terms, there is an urgent need to better understand how antibiotics work, how resistance develops, and what molecular mechanisms could be exploited to hijack bacterial resistance. Between 2013 and 2018, the European research program IMI-TRANSLOCATION aimed to better understand antibiotic transport and accumulation, as well as the emergence of multidrug resistance in problematic Gram-negative bacteria belonging to the ESKAPE pathogen group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter spp.*). Significant results were obtained concerning (i) the translocation of antibiotics through outer membrane porins, and (ii) the impact of efflux pumps on the accumulation and activity of antibiotics in enterobacteria.

### Rational :

The effectiveness of antibiotics depends on their ability to accumulate inside bacteria until they reach a threshold concentration to interact with their target(s) and inhibit their activity. However, the outer membrane of Gram-negative bacteria is highly impermeable, and antibiotics diffuse through the narrow channel of porins. At the same time, they are substrates for transmembrane efflux pumps that expel them<sup>1</sup>. As a result, many clinical isolates with multiple antibiotic resistance show functional changes or loss of porins, associated with an overproduction of efflux pumps.

Porins account for a substantial fraction of the total number of OM proteins and promote the diffusion of solutes with molecular weights of up to 600 Da. *E. coli* produces two main general porins: OmpC and OmpF<sup>1</sup>. OmpF/C channels are hourglass-shaped, with the narrowest part called the constriction region (CR) due to the folding of the L3 loop. The L3 loop also generates a strong transverse electric field across the CR, resulting from a row of positively charged residues on the barrel wall facing negatively charged residues on the L3 loop. This electric field has direct consequences for molecular permeation<sup>2</sup>. The structures of several OmpF/C orthologues have been solved by X-ray crystallography. Despite structural similarities, OmpC porins have a smaller pore radius, lower conductance, higher

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cation selectivity, and lower transverse electric field intensity. Experiments predicting liposome permeability and swelling have shown that the atomic charge and size of solutes are major limitations to their diffusion through porins<sup>3</sup>. We have recently shown that ceftazidime (CAZ), which has a net negative charge, is active on bacteria expressing OmpF porins. In contrast, cefepime zwitterionic (FEP) kills bacteria irrespective of the porin expressed. Quantification of accumulated molecules by liquid chromatography with tandem mass spectrometry (LC-MS/MS) and experiments on isolated systems confirmed that CAZ shows a preference for OmpF to enter bacteria<sup>4</sup>.

The specificity of porins towards antibiotics is an important issue for antibiotic efficacy and the development of new antibacterial molecules. Here, we aim to develop an approach using protein biochemistry and biophysics (Site-directed spin labeling (SDSL) and Electron Paramagnetic Resonance (EPR) spectroscopy) to measure differences in affinity between antibacterial molecules and porins. Purified *K. aerogenes* Omp35 and Omp36 porins will be reconstituted in liposomes using a protocol favoring "outside-out" orientation. First, the orientation of these porins will be tested. Cysteine substitutions will be made in extra- and intracellular loops of both porins. The functionality and overall structure of all mutants will be analyzed by phenotypic assays *in vivo* and by circular dichroism *in vitro*. Proteoliposomes containing these reconstituted variants will then be incubated with a cysteine-specific fluorophore and a membrane-impermeable fluorescence quencher in the absence or presence of detergent<sup>5</sup>. Similarly, EPR signal quenching of protein tagged with a nitroxide spin label will be studied as a function of protein orientation. A complete biophysical analysis (size, heterogeneity) will be carried out on proteoliposomes before and after incorporation of the tagged protein (fluorescent probe or nitroxide probe) by Dynamic Light Scattering (DLS).

The second step will analyze the potential conformational changes of Omp35 and Omp36 porins in the presence of antibiotics. To this end, we have selected amino acids in Omp35 and Omp36 located above and below the CR that will be individually cysteine-substituted for labeling with a nitroxide probe. All mutants were generated by site-directed mutagenesis using pColdIV-*omp35/36* plasmids as templates and expressed in BL21Δ*omp8*. Mutant expression levels and correct localization to the outer membrane will be tested before purification by ion exchange chromatography (in-house MCT protocol). The same mutations will be generated in pBAD24-*omp35/36* plasmids to confirm that they do not affect antibiotic sensitivity in *E. coli* K12. Cys mutant proteins will be labeled with a nitroxide probe and EPR spectra will be analyzed in the absence and presence of antimicrobials (FEP and CAZ). Spectral changes are expected if the antibiotic comes into contact with the nitroxide probe and establishes favorable interactions allowing it to cross the constriction region and reach the channel exit<sup>6-8</sup>. The positive outcome of these experiments will provide a rapid and powerful *in vitro* test to screen a whole chemical library of antimicrobials and determine the ability of certain molecules to penetrate the porins of enterobacteria.

**Methods :**

- Microbiology: antibiotic susceptibility assays
- Molecular biology: site-directed mutagenesis
- Protein biochemistry: purification and reconstitution of membrane proteins ; estimation of membrane protein orientation in liposomes
- Biophysics: SDSL-EPR, CD, DLS

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