J Fluoresc (2011) 21:1421–1429
DOI 10.1007/s10895-010-0826-7

ORIGINAL PAPER

Mechanisms of Ceftazidime and Ciprofloxacin Transport through Porins in Multidrug-Resistance Developed by Extended-Spectrum Beta-Lactamase *E.coli* Strains

Beatrice Mihaela Radu · Mihaela Bacalum · Adela Marin · Carmen-Mariana Chifiriuc · Veronica Lazar · Mihai Radu

Received: 27 September 2010 / Accepted: 29 December 2010 / Published online: 14 January 2011

Abstract Resistance towards antibiotics stands out today as a major issue in the clinical act of treatment of bacterial-generated infections. This process was characterized in proteoliposomes reconstituted from an *E.coli* strain isolated from invasive infections (blood culture) occurred in patients with a cardio-vascular device admitted for surgery. Fluorescence spectroscopy and patch-clamp technique have been used. Two types of antibiotics have been targeted: ceftazidime and ciprofloxacin. Antibiotics addition in proteoliposomes suspension undergoes a quenching in tryptophan residues from outer membrane porins structure, probably due to the formation of a transient non-fluorescent porin-antibiotic complex. Patch-clamp recordings revealed strong ion current blockages for both antibiotics, reflecting antibiotic–channel interactions but with varying strength of interaction. The present study puts forward the mechanism of multidrug-resistance in extended-spectrum beta-lactamase *E.coli* strains, as being caused by alterations of the antibiotics transport across the porins of the outer bacterial membrane.

B. M. Radu (✉) · A. Marin
Department of Anatomy, Animal Physiology and Biophysics,
Faculty of Biology, University of Bucharest,
Splaiul Independentei, 91-95,
Bucharest 050095, Romania
e-mail: beatrice_macri@yahoo.com

M. Bacalum · M. Radu
Department of Life and Environmental Physics, 'Horia Hulubei'
National Institute for Physics and Nuclear Engineering,
PO Box MG6, Magurele 077125, Romania

C.-M. Chifiriuc · V. Lazar
Department of Botanics and Microbiology, Faculty of Biology,
University of Bucharest,
Aleea Portocalelor 1-3,
Bucharest, Romania

Keywords Outer membrane porins · Ceftazidime · Ciprofloxacin · Non-fluorescent complex · Extended-spectrum beta-lactamase *E.coli* strain

Introduction

Bacteria may exhibit resistance to antibacterial drugs through a variety of mechanisms. Of greater concern are cases of acquired resistance, where initially susceptible populations of bacteria become resistant to an antibacterial agent and proliferate and spread under the selective pressure of the respective agent. Several mechanisms of antimicrobial resistance are readily spread to a variety of bacterial genera. First, the organism may acquire genes encoding antibiotic-inactivating enzymes, such as β-lactamases. Secondly, bacteria may acquire efflux pumps that extrude the antibacterial agent from the cell before reaching its target site. Third, bacteria may acquire several genes for a certain metabolic pathway that ultimately produces altered bacterial cell walls, no longer containing the binding site of the antimicrobial agent, or bacteria may acquire mutations that can limit access of antimicrobial agents to the intracellular target site via downregulation of porin genes.

Two of the most important resistance mechanisms occurred in *Gram-negative* bacteria following spontaneous mutations are: downregulation or alteration of an outer membrane protein channel required for the drug entry into the cell, or upregulating pumps that expel the drug from the cell (efflux of fluoroquinolones in *S. aureus*) [1].

There are particular classes of integral proteins located in the outer membrane of *Gram-negative* bacteria which are called porines, which allow the passive translocation in and

out of the periplasmic space of small molecules and antibiotics. They belong to a large family of proteins with a highly conserved aminoacid sequence especially for the transmembrane domains [2, 3] which are named as follows: OmpF, OmpC, OmpD, PhoE, LamB, OmpA, OmpK36, and OmpK36 (*Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Salmonella typhimurium*) [4–6]. In *Escherichia coli*, there are cation-selective (OmpF and OmpC) and anion-selective (PhoE) porins, with opposite voltage-dependences for OmpF and PhoE [7].

Patch-clamp studies indicate that OmpF is opened at membrane potentials under 100 mV, and the current increase is voltage-dependent [8]. The critical voltage of OmpF is affected by pH [9] and is lowered by membrane olygosaccharides [10], polycations [11], and by pressure [12]. Polyamines (putresceine, cadaverine, spermidin and spermine) exhibit different concentration- and voltage-dependent inhibitory effects on OmpF and OmpC [7, 13]. Shorter and more charged antimicrobial peptides (i.e. HPA3P) are more accessible to the inner volume of the OmpF [14].

In our study, we are investigating the antimicrobial resistance mechanism exerted by means of porins, that is altered by the antibiotic transport. By means of fluorescence and patch-clamp techniques, we have studied the properties of porins reconstituted in proteoliposomes that contain outer membrane fragments from the selected *E. coli* strain, in the presence/absence of two antibiotics: ceftazidime (CAZ) and ciprofloxacin (CIP).

Material and Methods

Bacterial Strain

This study was performed on one *Escherichia (E.) coli* strain isolated from invasive infections (blood cultures) occurred in patients with an implanted cardio-vascular device admitted for surgery at the Institute for Cardiovascular Diseases Prof. 'C.C. Iliescu', Bucharest, Romania, selected on the basis of its resistance to the 3rd generation cephalosporins (the strain was encoded *E. coli* no. 2662) and the reference *E. coli* ATTC 25922 strain recommended for the internal quality control of the antibiotic susceptibility testing. The microbial strain was identified using VITEK I automatic system. Microbial suspensions of 0.5 McFarland density (0.125 O.D. at λ=550 nm) obtained from 15 to 18 h microbial cultures developed on solid media were used in our experiments in order to determine the antibiotic susceptibility patterns, using Mueller Hinton medium and the following Oxoid disks: ampicillin, ampicillin plus clavulanic acid, ceftazidime, ceftriaxone, cefpirome, imipenem, amikacin, gentamycin, nalidixic acid, ciprofloxacin, norfloxacin, tetracycline. The plates were incubated aerobically at 37°C for 18 h. Results were expressed as susceptible or resistant according to the criteria adopted by CLSI (Clinical and Laboratory Standards Institute) [15]. The minimal inhibitory concentration (MIC) was assessed by classical serial broth dilutions method. The strain was selected based on its resistance to ceftazidime and ciprofloxacin (Fig. 2). The phenotypic screening of beta-lactamases production was performed by double disk diffusion test (DDDT) and chromogenic nitrocephine hydrolysis.

Preparation of Outer Membrane Fractions

The standardized 0.5 MacFarland microbial strains were plated on 3 agar plates and incubated for 24 h at 37°C. The obtained bacterial cultures were harvested from the plates in order to recover the biomass, followed by 3 washing steps with PBS (pH 7.4). After a first centrifugation step (5,000× g, 5 min), the pellet was resuspended in 3 ml PBS and 0.75 ml were transferred into an Eppendorf tube. After a new centrifugation step (5,000×g, 5 min), the pellet was resuspended in 0.75 ml water. An equivalent volume of 90% phenol (pre-heated at 65°C) was added, and upon 1 min of stirring, the mixture was incubated for 10 min. After cooling, the samples were centrifuged (12,000 x g, 3 min), different layers being visible in the suspension. The residual phenol is separated from the aqueous phase by a 3-step procedure with diethyl-ether. After the separation of the diethyl-ether phase from the aqueous phase (which contains outer membrane fragments), the sample was placed on a water bath for 1 h in order to evaporate the diethyl-ether residues.

Reconstitution of Bacterial Membrane Fragments into Liposomes

The proteoliposomes were prepared from lecithin and fragments from the outer membrane fragments of E. coli. Lecithin and N,N,N –Trimethyl – 4 – (6 – phenyl – 1,3,5 – hexatrien – 1 – yl)phenylammonium p – toluenesulfonate (TMA-DPH) was parched from Sigma and the fragments were obtained following the protocol previously described. All solutions were prepared in 10 mM PBS solution, pH 7.4.

The liposomes were prepared after a protocol recommended in the literature for low concentrated liposome suspensions, as in our case [16, 17]: the lipids (lecithin) were dissolved in chloroform at a given concentration as stock solutions and preserved at −20°C. For each experiment an appropriate volume from stock solution was mixed with a mixture of methanol-chloroform (1/1, v/v) and then solvent was removed by drying under nitrogen flow. The

lipid film formed on the bottom of a tube was hydrated with 3 ml PBS. After hydration, the solution was vigorously vortexed to obtain a suspension of multilamellar vesicles. This suspension was sonicated in an ultrasonic bath (MRC D80H Ultrasonic cleaner 80 W) until clarity (~ 30 min) resulting in a suspension of SUV which was used in the experiments after a proper dilution to a final lipid concentration of ~78 μM.

The preparation of proteoliposomes containing the membrane fragments was done using a protocol described in the literature [18], briefly presented in the following: SUV suspension and membrane fragments were mixed (3 ml of liposomes with 50 μl fragments) and then the mixture was subjected to three freeze/thaw cycles.

FRET Spectra Recording and Simulation

The porins structures include tryptophan residues, particularly six tryptophans in the case of OmpF trimer. This allows using the fluorescence of this endogen fluorophore as a tool to follow the insertion of fragments in the SUV lipid bilayer and the interaction of antibiotics with the porins channels [19, 20]. The insertion of membrane fragments in lipid bilayer was analyzed by fluorescence resonance energy transfer (FRET) between porins' tryptophan residues (as donor) and TMA-DPH (as acceptor). TMA-DPH is a fluorescent molecule that has a hydrophobic chain (DPH) and a hydrophilic part that allows this molecule to be anchored at the surface of membrane. The technique was used in previous experiments for proving the insertion of gramicidin A and OmpF molecules in lipid bilayers of SUV [19]. Records were done for TMA-DPH concentration in the range of 0–2 μM. Fluorescence measurements were performed on a Fluoromax 3 (Horiba Jobin-Yvon) spectrofluorometer. The excitation was set at 280 nm and the spectra recorded between 300 and 500 nm. All the recorded spectra were corrected for the spectral sensitivity of the emission channel of the fluorometer and by subtracting the liposomes contribution as background. In Fig. 1 is shown the fluorescence spectrum (normalized data) of the outer membrane bacterial fragments with a maximum at ~350 nm where it is known the tryptophan residues emits. The absorption DPH spectrum and the emission spectra of TMA-DPH in PBS and in proteoliposomes are also shown in Fig. 1 as normalized spectra. TMA-DPH has a weak fluorescence when is found in a hydrophilic medium (PBS) and becomes fluorescent only when reaches in a hydrophobic medium (lipid bilayer in proteoliposomes). The prerequisite condition of overlapping of membrane fragments emission and DPH absorbtion spectra is well fulfilled according to Fig. 1.

Using the fluorescence intensity of Trp from proteolipisomes (I), the FRET efficiency (Eq. 1) was calculated by:

$$E = 1 - I/I_0 \tag{1}$$

where I and I_0 mean the intensity when the acceptor is present and absent, respectively.

For FRET efficiency simulation a 2D theoretical model was used [21]. The model considers uniform distributions of donor and acceptor in the same plan. In this case the membrane thickness is neglected and a minimal distance between the donor and acceptor molecules (exclusion radius, R_e) can be calculated. According to [21], the FRET efficiency (Eq. 2) is given by:

$$E = 1 - \int_0^\infty e^{-\lambda} e^{\left[-\pi CT(2/3\lambda/\alpha)\lambda^{1/3}\right]} e^{\left[\pi C\alpha^{1/3}\left(1-e^{-\lambda/\alpha}\right)\right]} d\lambda \tag{2}$$

where:

$$\alpha = (R_e/R_0)^6 \tag{3}$$

R_e and R_0 being the exclusion radius between donor and acceptor, and Forster radius respectively;

$$\lambda = t/\tau \tag{4}$$

τ and t being the life time of donor in excited state in absence of the acceptor, and time respectively;

$$C = {R_0}^2 c \tag{5}$$

c being the two-dimensional concentration of acceptors and $\Gamma(x,y)$ is the Gamma function.

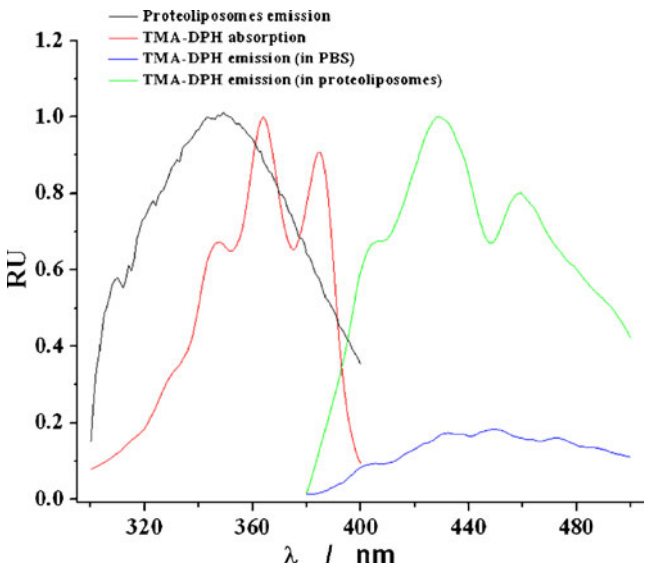

Fig. 1 Emission spectra of tryptophan (from proteoliposomes containing outer membrane fractions of *E. coli* no. 2662 strain) and TMA-DPH (in PBS and in liposomes) and absorption spectrum of TMA-DPH

R_0 was computed according to Forster theory:

$$R_0 = 9790\left(J\,\kappa\,q\,n^{-4}\right)^{1/6} \quad (6)$$

where κ is the orientation factor for the dipole-dipole interaction, q is the quantum yield, n is the medium refractive index and J (Eq. 7) is the normalized overlap integral:

$$J = I(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda / I(\lambda)d\lambda \quad (7)$$

$I(\lambda)$ being the normalized emission spectrum of the donor (Trp from porins), $\varepsilon(\lambda)$ being the extinction coefficient at the acceptor (DPH) at the wavelength λ. Since it was not possible to estimate the Trp concentration in our samples we use as an approximation of the quantum yield of the Trp from porins the Trp quantum yield in deionized water (q_0= 0.13, [22]).

In our calculus the orientation factor value was considered κ=2/3 for a random orientation and refractive index values was n=1.5 which is the refractive index in the hydrocarbon region of a bilayer [23].

Fluorescence Quenching

Trp emission spectra for excitation at 280 nm were recorded in the range of 300–500 nm for proteoliposomes in the presence of various concentrations of antibiotics (CAZ or CIP). The spectra were corrected for the spectral sensitivity of the emission channel of the fluorometer and by subtracting the liposomes contribution as background. The data were analyzed using the Stern-Volmer formalism [22]:

$$I_0/I = 1 + K_{SV}[Q] \quad (8)$$

where I and I_0 mean the intensity in the absence or presence of the antibiotic, respectively, $[Q]$ is the molar concentration of the antibiotic and K_{SV} is the quenching constant.

Patch-Clamp Electrophysiology

Reconstitution of outer membrane fraction was performed into soybean phospholipids as described [24, 25]. A borosilicate glass capillary (GC150F-10, Harvard Apparatus Ltd., Edenbridge, UK) was pulled into a pipette whose tip diameter gave a resistance of 10–11 MΩ. Patch clamp experiments were performed with the same buffer in the pipette and the bath: 150 mM KCl, 10 μM $CaCl_2$, 0.1 mM K-EDTA, 5 mM HEPES, pH 7.2. Seals of 0.5–1.0 GΩ were formed by bringing the pipette tip into contact with the blister membrane. After seal formation, the patch was momentarily exposed to air and brought back to the buffer (excised patch). This ensured that there was only a single bilayer of membrane at the tip of the pipette. The bath was then perfused with buffer.

The voltage across the membrane was clamped at different values using a WPC-100 amplifier (ESF electronic, Göttingen, DE). The signal was filtered using a 4-pole low-pass Bessel filter at a frequency of 10 kHz and sampled at 50 kHz, acquired using a Digidata 1322 acquisition board (Axon Instruments, Foster City, CA) under pClamp 8.1 in gap-free mode and stored on hard-disk.

After 10 min from the seal formation, we have added the antibiotics: ceftazidime (5 mM) and ciprofloxacin (2 mM).

Data Analysis

Data were analysed with Clampfit 9.0 (Axon Instruments) and Origin 8.0 (OriginLab Corporation, USA).

Results

Minimal Inhibitory Concentration Tests

The phenotypic double disk diffusion screening test evidenced the presence of extended-spectrum beta-lactamase (ESBL) (Fig. 2) in *E. coli* no. 2662 strain. One should notice the high MIC value observed for CAZ (32± 2 μg/mL) compared to CIP (20±3 μg/mL) for the *E. coli* no. 2662 strain (Fig. 3). A possible explanation for this high MIC value could be the presence of a non-enzymatic resistance mechanism, probably mediated by efflux pumps or porines loss [26].

FRET Experiments Between TMA-DPH and the Tryptophan Residues from the Outer Membrane Fragments of *E. coli* no. 2662 Strain

The insertion of bacterial fragments into the proteoliposomes was proved by FRET technique. In this experiment, the donors were considered the tryptophan residues of the porins from bacterial membrane fragments (particularly OmpF) and the acceptors were TMA-DPH molecules inserted in the lipid bilayer of the proteoliposomes. To reveal FRET, we recorded four types of spectra: the spectrum of liposomes, the spectrum of proteoliposomes (that contains the bacterial fragments), the spectrum of liposomes (without any bacterial fragment incorporated) doped with TMA-DPH and the spectrum of proteoliposomes doped with TMA-DPH. The first two spectra were subtracted to obtain the emission spectrum of the donor and the third and fourth spectra were subtracted to obtain the emission spectra for the probes that contain also the donor and the acceptor.

In Fig. 4 are presented the spectra of proteoliposomes with different concentration of TMA-DPH (increasing concentrations from 0.42 to 1.25 μM); the spectra are

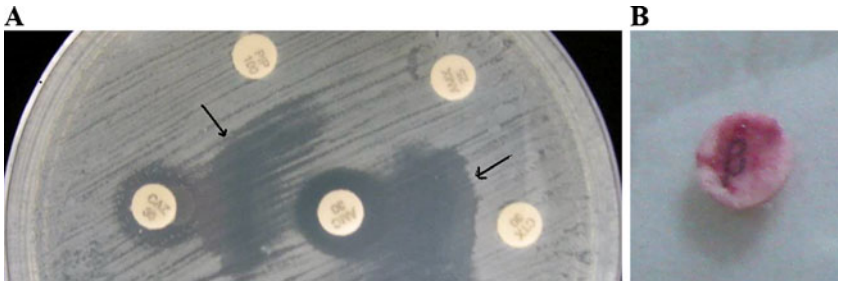

Fig. 2 **a** Aspects of synergism between clavulanic acid and the third generation cephalosporins (ceftazidime and cefotaxime) in *E. coli* no. 2662 strain demonstrating the production of an extended spectrum beta-lactamase. **b** Positive nitrocephine test

corrected as described above. Energy transfer was proved by the decrease of tryptophan emission (the peak seen at approximately 350 nm) simultaneously with the increase of TMA-DPH emission (400–500 nm) for increasing values of acceptor concentration.

A small energy transfer was recorded between the porines' tryptophan residues and TMA-DPH for *E. coli* no. 2662 bacterial strain. This transfer proves that the outer membrane bacterial fragments are incorporated into the lecithin liposomes.

The FRET efficiency was computed using spectra from Fig. 4A and the data are represented in Fig. 4B as FRET efficiency against TMA-DPH concentration. Very low efficiency was obtained, less that 20% suggesting a higher distance among the donor and acceptor molecules. In order to evaluate this distance a simulation of FRET efficiency was done using the 2D FRET model presented in the Experimental section. For computing the Forster radius the spectra from Fig. 1 have been used resulting in a value of 3.1 nm. The exclusion radius was inferred comparing the simulated FRET efficiency (curve in Fig. 4A) and the experimental data resulting in a value of 4.1 nm, a high value explaining the low efficiency of FRET. An explanation for this high exclusion radius is given by the fact that the tryptophan residues are located in the porins from bacterial outer membrane fragments and the acceptor is located in the lipid bilayer formed by lecithin, a restrictive factor being in this case the size of the fragments.

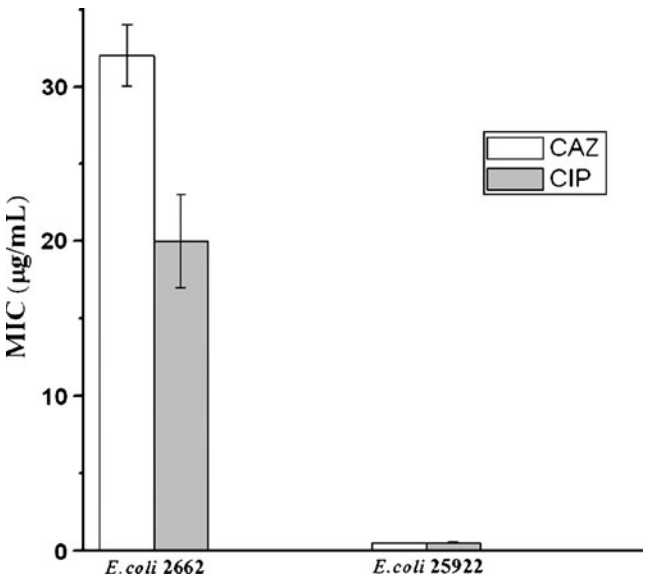

Fig. 3 Graphic representation of MIC for the two tested strains in the presence of CAZ and CIP. Data represent the means ± SD of three independent experiments (* significant $P<0.05$)

Effect of Antibiotics on Proteoliposomes Reconstituted from *E. coli* no. 2662 Outer Membrane Fragments

The interaction between proteoliposomes and antibiotics (CAZ and CIP) was investigated for the *E.coli* no. 2662 strain, which is characterized by the presence of extended-spectrum beta-lactamase. CAZ concentration ranges between 30.5 and 152.5 μM. Tryptophan residues fluorescence spectra were recorded at 37°C, in the range of 300–500 nm for emission, excitation being set at 280 nm.

In Fig. 5a are presented the spectra of proteoliposomes in the presence of CAZ. The addition of the antibiotic causes a gradually decrease of the OmpF emission intensity. These data prove a quenching of tryptophan fluorescence by CAZ molecules. To analyze in more details this process a Stern-Volmer formalism was used. In Fig. 5c the ratio of fluorescence in presence of CAZ and without CAZ is plotted against the CAZ concentration. The experimental data are well fitted by the linear Stern-Volmer equation (presented in Material and Methods section). The quenching constant for CAZ was obtained as $(27.0\pm2.7)\times10^3$ M^{-1}.

Similar experiments have been done for CIP. CIP concentration ranges between 0.5 and 2.5 μM. Unlike CAZ, CIP is a fluorescence molecule with a maximum of emission at ~410 nm. Spectra were recorded at 37°C, in the range of 300–500 nm for emission, excitation being set at 280 nm. In Fig. 5b are presented the spectra of proteoliposomes in the presence of CIP. The spectrum of liposomes with only CIP was subtracted from spectrum of proteoliposomes in the presence of CIP in order to remove the effect of CIP fluorescence on the tryptophan emission. The addition of the antibiotic causes a gradually decrease of the OmpF emission intensity, but also of the fluorescence

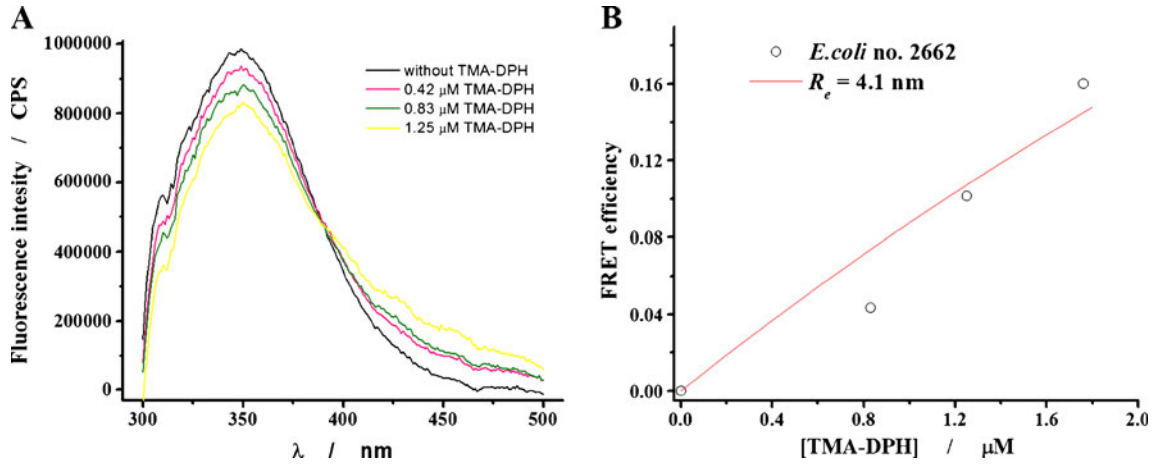

Fig. 4 FRET spectra for various acceptor (TMA-DPH) concentrations recorded on proteoliposomes reconstituted from **a** fragments from *E. coli* no. 2662 strain. **b** FRET efficiency calculated from recorded spectra (open circles) and simulated by the 2D model (line) resulting in an exclusion radius of 4.1 nm

intensity of the CIP (the negative values of corrected spectra prove that the emission at the maximum of CIP decreases). In the literature, it was suggested the possibility of energy transfer between tryptophan and CIP since the absorption spectrum of CIP partially overlaps with emission of tryptophan [20]. In our experiments such a transfer was not observed since the CIP fluorescence decreases. In the case of a FRET the CIP molecules would be acceptors and

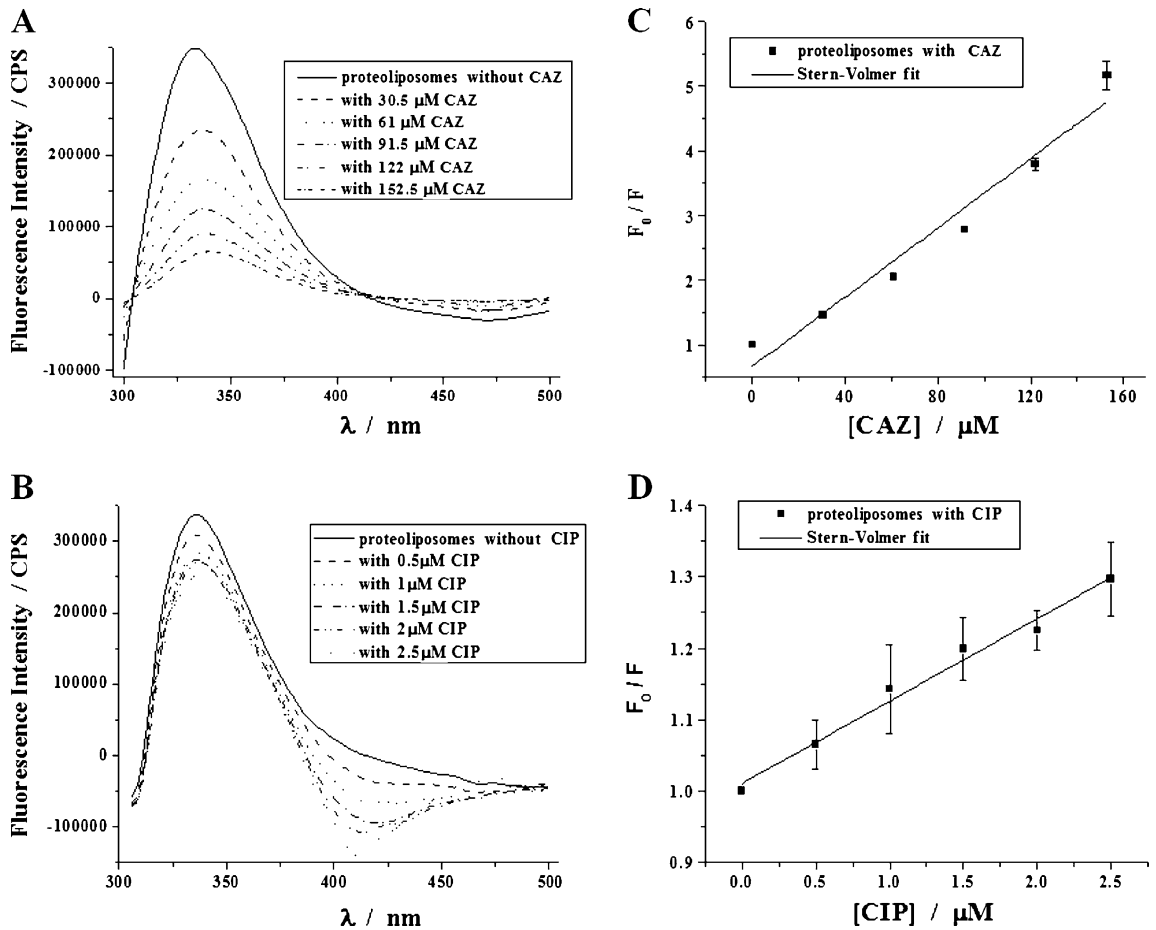

Fig. 5 Spectra of proteoliposomes reconstituted from *E.coli* no. 2662 strain. Fluorescence intensity for different CAZ (**a**) and CIP (**c**) concentrations. Stern-Volmer fit for OmpF-CAZ (**b**), and OmpF-CIP (**d**), respectively

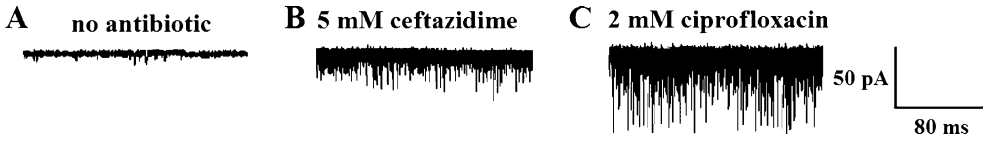

Fig. 6 Typical tracks of ion conductance through single trimeric OmpF channels from *E. coli* no. 2662 strain reconstituted into dip-tip bilayers (**a**) Control, (**b**) in the presence of 5 mM ceftazidime, and (**c**) in the presence of 2 mM ciprofloxacin. Membrane bathing solutions contained 150 mM KCl, 10 μM $CaCl_2$, 0.1 mM K-EDTA, 5 mM HEPES (pH 7.2). Applied voltage was −70 mV

their fluorescence would have increase. Our experimental findings concerning the decrease of CIP suggest a more complex interaction between CIP molecules and tryptophan residues, interactions that result in a quenching of CIP fluorescence also. The Stern-Volmer formalism was used also for CIP induced tryptophan quenching. In Fig. 5d is presented the fluorescence quenching ratio dependence against the CIP concentration the quenching constant being $(120\pm9)\times10^3$ M^{-1}. The quenching constant in the case of CIP is several times higher that for CAZ proving a stronger efficiency of quenching for CIP.

Patch-Clamp Recordings of Antibiotic Passage through OmpF from *E.coli* no. 2662 Strain

A stable planar lipid bilayer containing single trimeric OmpF was formed onto a patch-clamp pipette tip. After the formation of the lipid bilayer over the aperture of the pipette tip, we perfused the lipid bilayer with the buffer solution to remove remaining proteoliposomes to prevent multiple-channel insertions. In agreement with previous measurements [25] on liposomes studied by patch-clamp technique, single trimers in the bilayer showed channel conductance of 90 pS at −70 mV in a bathing solution of 150 mM KCl, 10 μM $CaCl_2$, 0.1 mM K-EDTA, 5 mM HEPES (pH 7.2). In our study, at transmembrane potentials below + 100 mV, the channels were open without any gating perturbations, similar to the data from the literature describing the voltage-dependence of OmpF [8]. The threshold potential (critical voltage) inducing closure of the channels was found to be around ±150 mV, very close to the values attributed for OmpF [8, 25, 27], but quite low as correspondent to OmpC, ± 200 mV [27]. Based on the values obtained for single trimeric channel conductance and for critical potential, we can distinguish more clearly OmpF from our recordings. Based on current signature upon addition of polyamines [11, 27], future studies can identify and extract the OmpC pattern from our records.

Porins activity in proteoliposomes reconstituted from outer membrane fragments of *E. coli* no. 2662 strain are show in Fig. 6a. We compared the permeation of cephalosporins (i.e. ceftazidime, Fig. 6b) and fluoroquinolones (i.e. ciprofloxacin, Fig. 6c) across porins from *E. coli* no. 2662 strain. Channel blockages were analyzed after adding antibiotic to the system. In the absence of antibiotic (Fig. 6a), ion currents through the channels were stable without fluctuations. Addition of antibiotics caused fluctuations of the ion current, indicating strong interactions of the antibiotics with the channel.

Different positive and negative voltages have been applied to the lipid bilayers containing the bacterial fragments from *E.coli* no. 2662 strain. For both antibiotics, the number of binding events is increased at more negative voltages compared to positive voltages. In Fig. 6, one can observe that the amplitude and number of fluctuations are greater in the case of ciprofloxacin (Fig. 6c) addition compared to ceftazidime (Fig. 6b). It should be pointed out that the ciprofloxacin concentration is only 2 mM, compared to ceftazidime concentration of 5 mM. It should be mentioned that ceftazidime concentration is higher than ciprofloxacin concentration, because at 2 mM ceftazidime no distinct ion conductance trace was recorded. Even if the ciprofloxacin concentration is below the values of ceftazidime, the events amplitude is significantly increased. These data are correlated with the quenching constants (K_{SV}) recorded by fluorescence measurements.

Discussions

In recent years, the emergence of multidrug-resistant *Gram-negative* pathogens, particularly extended-spectrum β-lactamase producing *Enterobacteriaceae*, has risen dramatically and caused a global healthcare problem [28]. Clinical data collected from Mercy University Hospital (MUH), Cork, Ireland indicate that resistance to third-generation cephalosporins, ceftazidime, and cefotaxime rose from 2% to 6% and 1% to 7%, respectively, and ciprofloxacin resistance rose from 5% to 15% [29].

Our study was focused on the ESBL *E. coli* strain no. 2662 isolated from an invasive infection (blood culture) occurred in a patient with implanted cardio-vascular device admitted for surgery, selected for its resistance to 3rd generation cephalosporins by a non-enzymatic mechanism. The outer membrane fragments reconstituted into proteoliposomes contain different types of porins as previously described in the literature [27, 30]. The outer membrane fragments extracted from *E. coli* no. 2662 (containing functional porins) were incorporated in proteoliposomes. It was proved that the outer membrane fragments are well-incorporated into the proteoliposomes

by FRET (Fig. 4) and that the porins are functional upon incorporation by patch-clamp recordings (Fig. 6).

Our approach is more extensive than the usual transport studies through a purified/reconstituted porin into liposomes, taking into consideration all the porins from the external outer membrane of *E.coli* strains. Even if a clear distinction between porins can't be done accurately from recordings, the clinical relevance of our study is more important pointing out for a global interpretation of the mechanisms of multidrug-resistance in extended-spectrum beta-lactamase *E.coli* strains.

OmpF channels represent the main pathway used by antibiotics to reach their target and proteoliposomes can be used for a better understanding of the antibiotic translocation, but also for the bacterial mechanisms. Several studies indicate the role played by OmpF in the translocation of different antibiotics, including beta-lactams [31–34] and quinolones [34, 35].

Our study indicates that antibiotics (CAZ/CIP) are able to quench the porines' tryptophan residues, the quenching being described by a linear dependence against the antibiotic concentration.

Data from the literature are suggesting two different mechanisms of interaction between OmpF and CIP, either the quenching of tryptophan fluorescence by a non-fluorescent OmpF-antibiotic complex formation [36, 37] or the decrease in tryptophan fluorescence by the energy transfer between OmpF and CIP [20]. In our study, the tryptophan fluorescence is quenched without an increase of CIP fluorescence proving that the FRET doesn't occurs. A question remains: what mechanism of quenching is involved in the observed processes? Other studies of antibiotic transport (i.e. ampicillin) through OmpF have revealed the quenching of tryptophan fluorescence as a dynamic process [36]. A possible response may be obtained evaluating the bimolecular constant. According to Lakowicz, the bimolecular constant is related to quenching constant by lifetime of the fluorophore: $K_{SV}=q^{*}\tau$. Using for tryptophan an usual value for the average value of lifetime, $<\tau>\approx 3$ ns, the bimolecular constant value is $q\approx 10^{13}\ M^{-1}s^{-1}$ for CAZ and $q\approx 4*10^{13}\ M^{-1}s^{-1}$ for CIP. On the other hand, a typical value for bimolecular constant in the case of free diffusion driven quenching (dynamic quenching) is of $10^{10}\ M^{-1}s^{-1}$ for diffusion in water [22]. In our cases both the fluorophore (tryptophan) and the quencher (antibiotic) have restricted movement resulting in a lower expected value for the bimolecular constant. Such high value for bimolecular constant can be explained by assuming a rather static mechanism of quenching [22]. For this reason we consider that our results support the hypothesis of a transient non-fluorescent complex formation at the passage of antibiotic molecule through porin channel.

Studies on a cephalosporin binding protein reveal the quenching of tryptophan residues fluorescence [38] and the binding constants were in the range of $(0.8–12)\times 10^{3}\ M^{-1}$. In a chip-based setup, the association rate constant k_{on} [34] was calculated at a negative constant voltage of −50 mV for OmpF-CAZ ($3\times 10^{3}\ M^{-1}$) and for OmpF-CIP ($14\times 10^{3}\ M^{-1}$). Our values are similar with the data from the literature, but greater values obtained for both binding constants should be associated with the presence of multiple porins inserted into the proteoliposomes bilayers. In addition, these values may be due to an increased phenomenon of multidrug-resistance in Romanian hospitals.

In our patch-clamp recordings, the rate passage of ciprofloxacin is higher compared to ceftazidime (Fig. 6) and is well correlated with fluorescence binding constant values. Other studies indicate that the diffusion rates of quinolones (i.e. nalidixic acid, ofloxacin and ofloxacin's two optically active isomers) through OmpF channels in proteoliposomes exceed the rates of beta-lactams (i.e. cephaloridine, imipenem) [35]. Previous studies indicate a mechanism of action of the fluoroquinolones based on the inhibition of the catalytic activity of enzymes essential to the DNA replication, whereas cephalosporins inhibit the bacterial cell wall synthesis [37].

Following the antibiotic resistance, many bacterial strains undergo multiple mutations, one of the most important for the reduction of the permeability rate being located at the level of loop L3 [27]. This fact may account for a possible mechanism of multidrug-resistance in extended-spectrum beta-lactamase *E. coli* strains.

Conclusion

In conclusion, we propose a mechanism of bacterial resistance induced by changes in antibiotic rate diffusion through porins and also by a transient complex formation porin-antibiotic. Future studies should include extended analysis of these mechanisms for more multidrug-resistant *Gram-negative* pathogens and different classes of antibiotics.

Acknowledgements This work was supported by the grants CEEX 2-Cex-06-11-49/2006 and CEEX 168/2006 of the Romanian Ministry of Research. We are grateful to M.D. Daniel Dumitru Banciu for useful suggestions.

References

1. McManus MC (1997) Mechanisms of bacterial resistance to antimicrobial agents. Am J Health Sys Pharm 54:1420–1433
2. Simonet V, Mallea M, Fourel D, Bolla JM, Pages JM (1996) Crucial domains are conserved in Enterobacteriaceae porins. FEMS Microbiol Lett 136:91–97

3. Jeanteur D, Lakey JH, Pattus F (1991) The bacterial porin superfamily: sequence alignment and structure prediction. Mol Microbiol 5:2153–2164
4. Hancock REW (1997) The bacterial outer membrane as a drug barrier. Trends Microbiol 5:37–42
5. Koebnik R, Locher KP, van Gelder P (2000) Structure and function of bacterial outer membrane proteins: barrels in a nutshell. Mol Microbiol 37:239–253
6. Nikaido H (2001) Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. Semin Cell Dev Biol 12:215–223
7. Samartzidou H, Delcour AH (1999) Distinct sensitivities of OmpF and PhoE porins to charged modulators. FEBS Lett 444(1):65–70
8. Brunen M, Engelhardt H (1993) Assymmetry of orientation and voltage gating of the *Acidovorax delafieldii* porin Omp34 in lipid bilayers. Eur J Biochem 212:129–135
9. Todt JC, Rocque WJ, Mcgroarty EJ (1992) Effects of pH on bacterial porin function. Biochemistry 31:10471–10478
10. Schindler H, Rosenbusch JP (1981) Matrix protein in planar membranes: clusters of channels in a native environment and their functional reassembly. Proc Natl Acad Sci USA 78:2302–2306
11. dela Vega AL, Delcour AH (1996) Polyamines decrease *Escherichia coli* outer membrane permeability. J Bacteriol 178:3715–3721
12. le Dain AC, Häse CC, Tommassen J, Martinac B (1996) Porins of *Escherichia coli*: unidirectional gating by pressure. EMBO J 15:3524–3528
13. Iyer R, Wu Z, Woster PM, Delcour AH (2000) Molecular basis for the polyamine-OmpF porin interactions: inhibitor and mutant studies. J Mol Biol 297(4):933–945
14. Apetrei A, Asandei A, Park Y, Hahm KS, Winterhalter M, Luchian T (2010) Unimolecular study of the interaction between the outer membrane protein OmpF from *E. coli* and an analogue of the HP(2-20) antimicrobial peptide. J Bioenerg Biomembr 42 (2):173–180
15. Clinical and Laboratory Standards Institute (CLSI) (2008) Perfomance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; Approved standard—third edition, CLSI document M31-A3, 28(8), Wayne, Pennsylvania, USA
16. New RRC (1990) Liposomes: a practical approach. Oxford University Press, Oxford, p 44
17. http://www.avantilipids.com/index.php?option=com_content&view=article&id=1384&Itemid=372
18. Thieffry M, Chich JF, Goldschmidt D, Henry JP (1988) Incorporation in lipid bilayers of a large conductance, cationic channel from mitochondrial membranes. EMBO J 7(5):1449–1454
19. Bacalum M, Radu M (2007) Insertion of proteins in the lipid bilayer of liposomes revealed by FRET. Rom J Biophys 17:129–138
20. Fernandez F, Neves P, Gameiro P, Loura LMS, Prieto M (2007) Ciprofloxacin interactions with bacterial protein OmpF: modelling of FRET from a multi-tryptophan protein trimer. Biochim Biophys Acta 1768:2822–2830
21. Wolber PK, Hudson BS (1979) An analytic solution to the Forster energy transfer problem in two dimensions. Biophys J 28:197–210
22. Lakowicz JR (2006) Principles of fluorescence spectroscopy, 3rd edn. Springer, New York
23. Gago M, Koper R, Gruszecki WI (2001) Spectrophotometric analysis of organisation of dipalmitoylphosphatidylcholine bilayers containing the polyene antibiotic amphotericin B. Biochim Biophys Acta 1511(1):90–98
24. Baslé A, Iyer R, Delcour AH (2004) Subconductance states in OmpF gating. Biochim Biophys Acta 1664(1):100–107
25. Delcour AH, Martinac B, Adler J, Kung C (1989) Voltage-sensitive ion channel of *Escherichia coli*. J Membr Biol 112 (3):267–275
26. Dubois V, Arpin C, Dupart V, Scavelli A, Coulange L, André C, Fischer I, Grobost F, Brochet JP, Lagrange I, Dutilh B, Jullin J, Noury P, Larribet G, Quentin C (2008) Beta-lactam and aminoglycoside resistance rates and mechanisms among Pseudomonas aeruginosa in French general practice (community and private healthcare centres). J Antimicrob Chemother 62(2):316–323
27. Delcour AH (2009) Outer membrane permeability and antibiotic resistance. Biochim Biophys Acta 1794:808–816
28. Coque TM, Baquero F, Canton R (2008) Increasing prevalence of ESBL-producing Enterobacteriaceae in Europe. Euro Surveill 13 (47):19–29
29. Mac Aogáin M, Mooij MJ, Adams C, Clair J, O'Gara F (2010) Emergence of extended-spectrum beta-lactamase and fluoroquinolone resistance genes among Irish multidrug-resistant isolates. Diagn Microbiol Infect Dis 67(1):106–109
30. Buehler LK, Kusomoto S, Zhang H, Rosenbusch JP (1991) Plasticity of *Escherichia coli* porin channels. J Biol Chem 266 (36):24446–24450
31. Nestorovich EM, Danelon C, Winterhalter M, Bezrukov SM (2002) Designed to penetrate: time-resolved interaction of single antibiotic molecules with bacterial pores. Proc Natl Acad Sci USA 99:9789–9794
32. Danelon C, Nestorovich EM, Winterhalter M, Ceccarelli M, Bezrukov SM (2006) Interaction of zwitterionic penicillins with the OmpF channel facilitates their translocation. Biophys J 90 (5):1617–1627
33. Hajjar E, Mahendran KR, Kumar A, Bessonov A, Petrescu M, Weingart H, Ruggerone P, Winterhalter M, Ceccarelli M (2010) Bridging timescales and length scales: from macroscopic flux to the molecular mechanism of antibiotic diffusion through porins. Biophys J 98(4):569–575
34. Mahendran KR, Kreir M, Weingart H, Fertig N, Winterhalter M (2010) Permeation of antibiotics through *Escherichia coli* OmpF and OmpC porins: screening for influx on a single-molecule level. J Biomolec Screen 15(3):302–307
35. Sawaia T, Yamaguchia A, Saikia A, Hoshino K (1992) OmpF channel permeability of quinolones and their comparison with β-lactams. FEMS Microbiol Lett 95(1):105–108
36. Mach T, Neves P, Spiga E, Weingart H, Winterhalter M, Ruggerone P, Ceccarelli M, Gameiro P (2008) Facilitated permeation of antibiotics across membrane channels—Interaction of the quinolone moxifloxacin with the OmpF channel. J Am Chem Soc 130:13301–13309
37. Nikaido H (2003) Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 67 (4):593–656
38. Ogawara H (1976) A specific cephalosporin-binding protein of *Citobacter Freundii*. Biochim Biophys Acta 420:155–164