Electronic Supplementary Material (ESI) for Chemical Science.

Supporting information

Dynamic Interaction of Fluoroquinolone with Magnesium Ions Monitored by Bacterial Outer Membrane Nanopores

Jiajun Wang[a]#*, Jigneshkumar Dahyabhai Prajapati[c]#, Ulrich Kleinekathöfer[c], Mathias Winterhalter[b]

[a] State Key Laboratory of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, 210023, Nanjing, China. Corresponding to **jiajunwang@nju.edu.cn**
[b] Department of Life Sciences and Chemistry, Jacobs University, 28759 Bremen, Germany
[c] Department of Physics and Earth Sciences, Jacobs University, 28759, Germany
# These authors contribute equally.

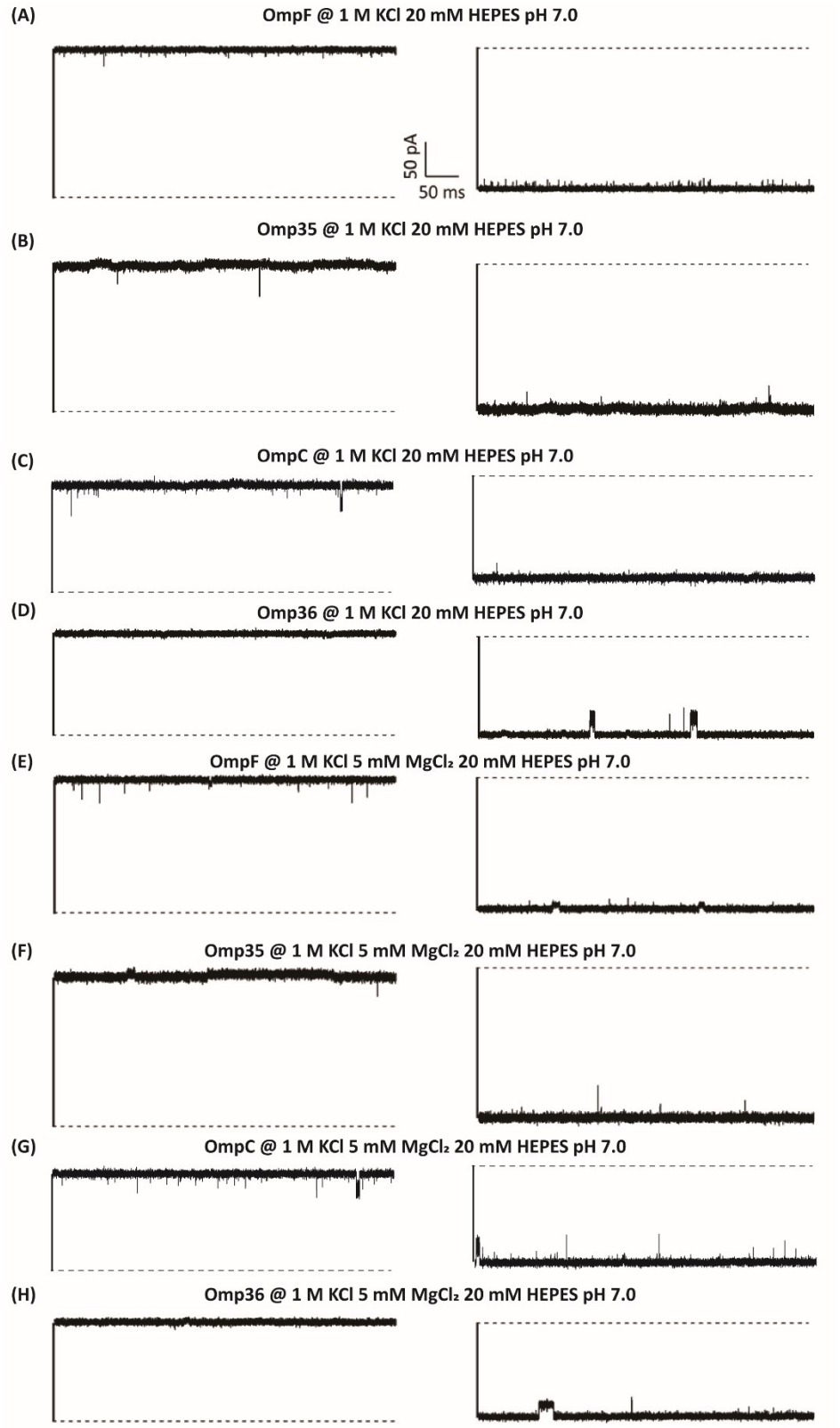

Figure S1. Intrinsic current recordings of single channels reconstituted into black lipid membranes (BLM) at ±50 mV. (A-D) OmpF, Omp35, OmpC and Omp36 using 1 M KCl and 10 mM HEPES at pH 7.0. (E-H) OmpF, Omp35, OmpC and Omp36 using 1 M KCl, 5 mM $MgCl_2$, and 10 mM HEPES at pH 7.0.

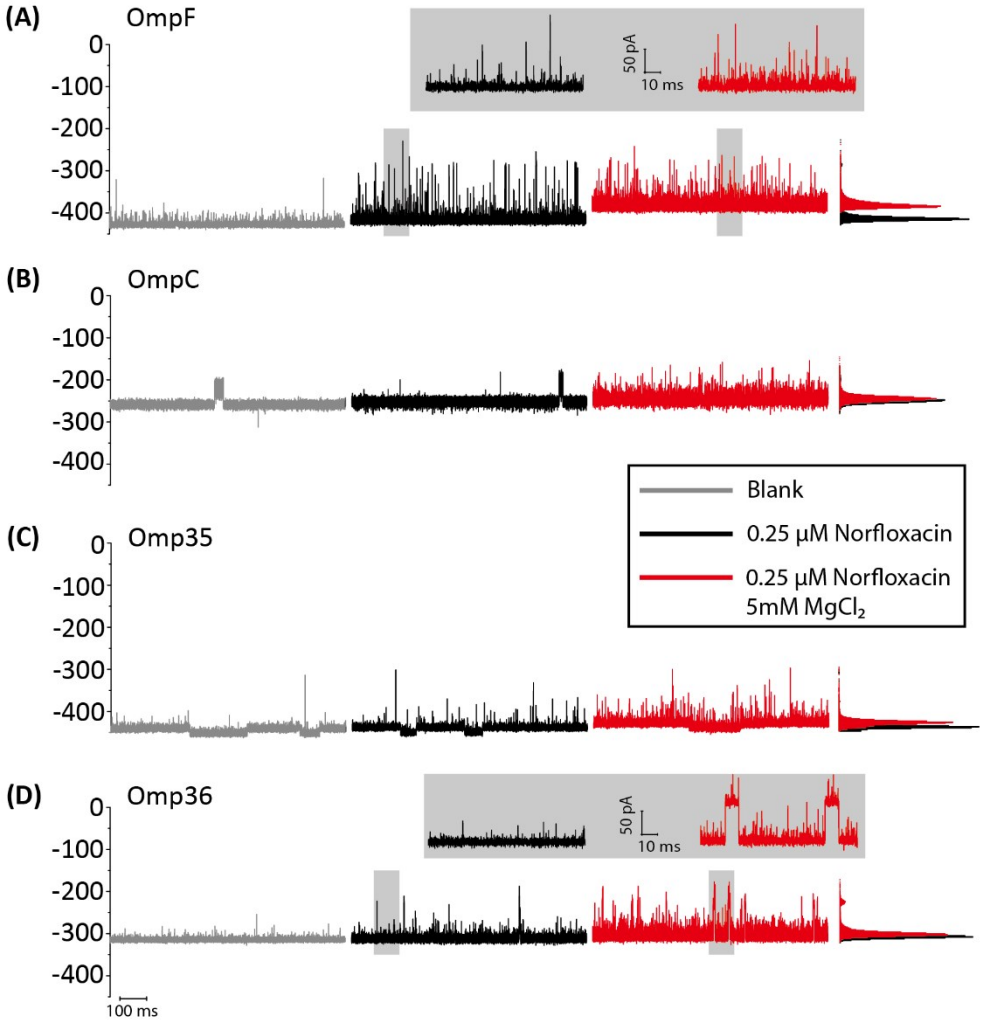

Figure S2. Single channel recordings of single porin interactions with norfloxacin (*cis* or extracellular side addition) buffered with 1 M KCl and 10 mM MES at pH 7.0 (black). The interaction with additional $MgCl_2$ is shown in red. In the histogram the current distributions are compared in the absence and presence of divalent ions. The insets display typical recordings for the interaction in the cases of OmpF and Omp36.

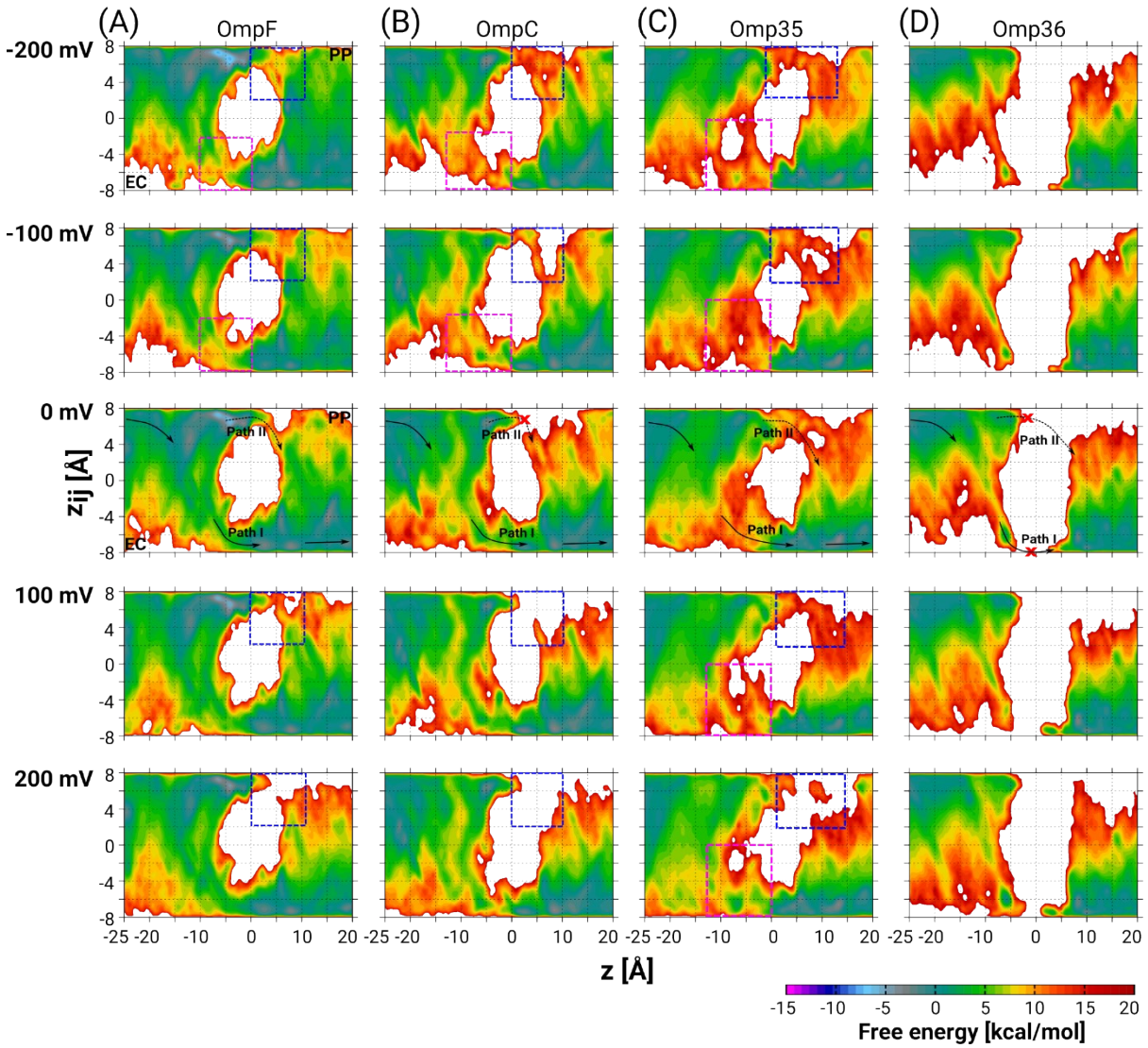

Figure S3. 2D FESs as function of the CVs z and z_{ij} based on TABD simulations. The FESs for the norfloxacin permeation through the four OM porins, i.e., OmpF (A), OmpC (B), Omp35 (C) and Omp36 (D), in absence of any ionic solution and in the presence of external voltages, i.e., at -200 (first row), -100 (second row), 0 (third row), 100 (fourth row) and 200 (fifth row) mV. The positive potential is applied on the PP side which is analogous to the *trans* side in experiments. The panels in the third row are same as those shown in Fig. 4. The most significant changes observed on the FESs in presence of external voltages are highlighted using blue and magenta boxes for Path I and II, respectively.

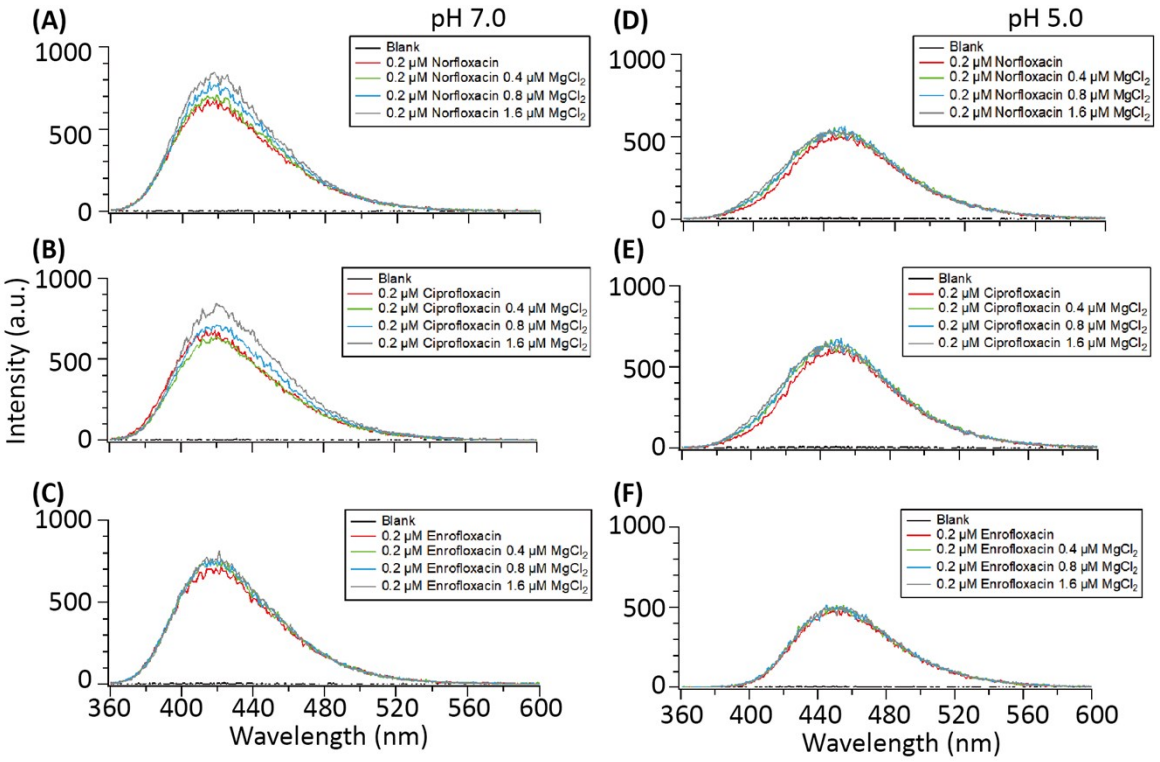

Figure S4. Fluorescence test (emission wavelength = 340 nm) of molecule-magnesium chelation (A) Norfloxacin at pH 7.0, (B) Ciprofloxacin at pH 7.0, (C) Enrofloxacin at pH 7.0. These experiments at pH 7.0 were performed using 1 M KCl and 20 mM HEPES as buffer. (D) Norfloxacin at pH 5.0, (E) Ciprofloxacin at pH 5.0, (F) Enrofloxacin at pH 5.0. The experiments at pH 5.0 were performed using 1 M KCl and 20 mM potassium acetate as buffer.

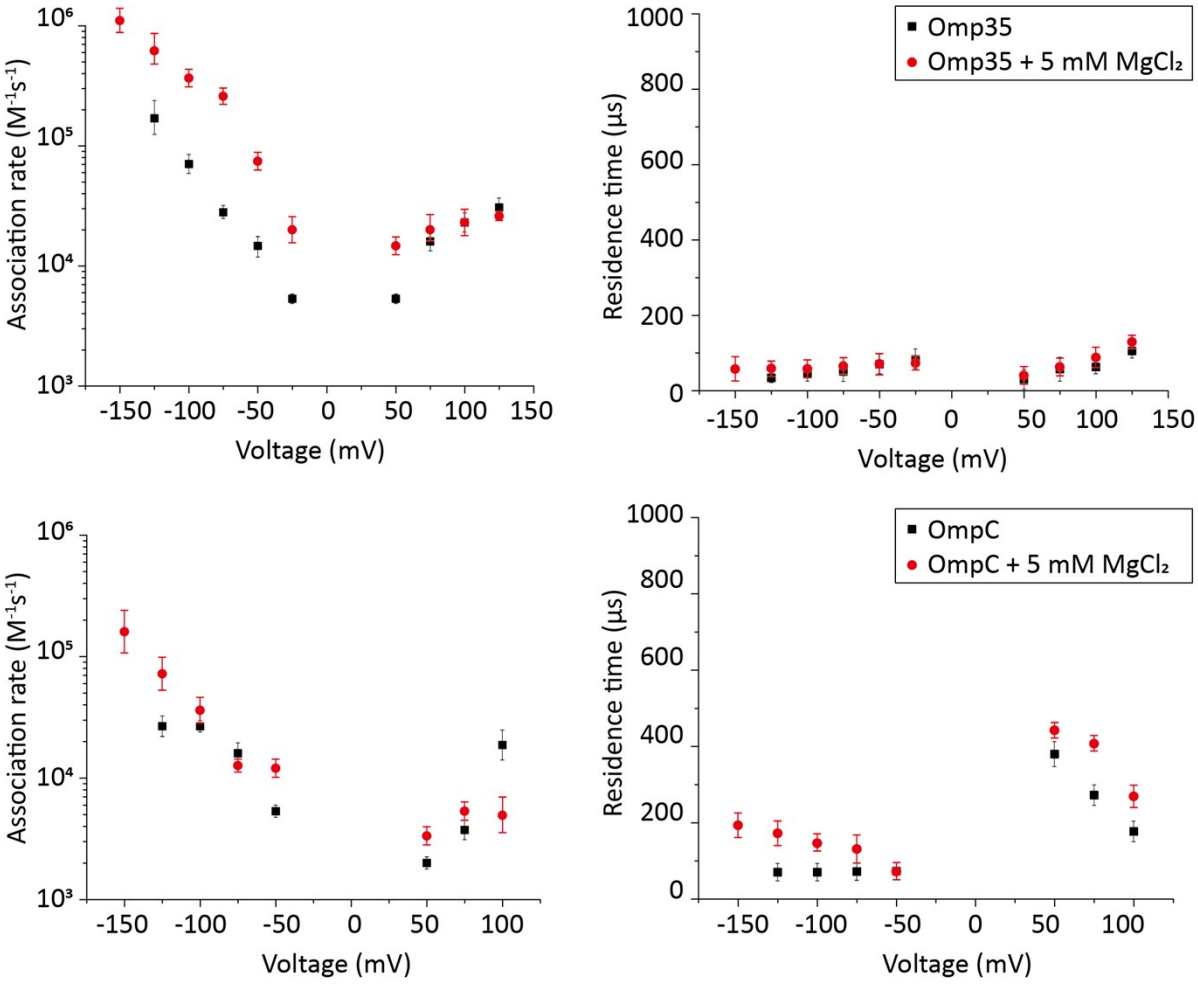

Figure S5. Interactions of norfloxacin with Omp35 as well as with OmpC. 0.25 mM Norfloxacin was added at the the *cis* (ground) side of the porin. A single protein analysis was performed to determine the association rates as well as the dwell times.

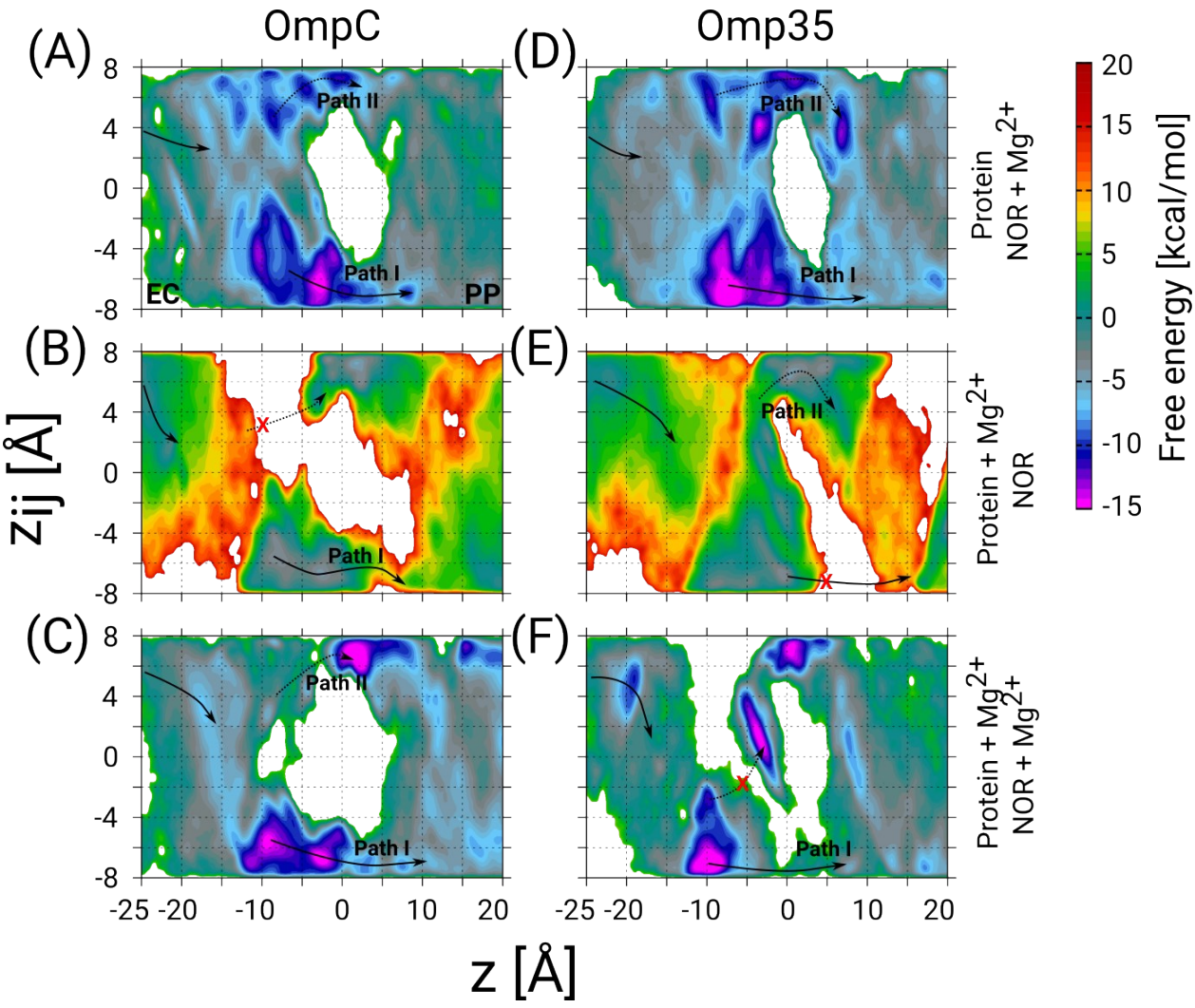

Figure S6. 2D FESs as function of the CVs z and z_{ij} based on TABD simulations for OmpC and Omp35 performed to get insights into the influence of Mg^{2+} ions on the norfloxacin permeation. The FESs for OmpC are obtained from simulations performed in three different scenarios, i.e., Mg^{2+} ion bound to (A) only norfloxacin, (B) only the porin, and (C) to both, norfloxacin and the porin. Panels D, E and F shows the same as panels A, B and C but for Omp35. As in Fig. 4 of the main text, the two possible paths with different orientation inside the constriction region are denotes as Path I and Path II with solid and dashed arrows, respectively. Broken paths are marked by red crosses.

Table S1. Presence or absence of the translocation pathways I and II based on the TABD simulations at different simulation conditions. For a given simulation, the plus and minus signs indicate the presence or absence of the particular path, respectively.

System	Voltage [mV]	Path I	Path II
OmpF	-200	+	+
	-100	+	+
	0	+	+
	+100	+	+
	+200	+	-
OmpC	-200	+	+
	-100	+	+
	0	+	-
	+100	+	-
	+200	+	-
Omp35	-200	+	+
	-100	+	+
	0	+	+
	+100	+	+
	+200	+	-
Omp36	-200	-	-
	-100	-	-
	0	-	-
	+100	-	-
	+200	-	-

System	Chelation	Path I	Path II
OmpF	NOR+Mg^{2+}	+	+
	OmpF+Mg^{2+}	+	+
	both+Mg^{2+}	+	-
OmpC	NOR+Mg^{2+}	+	+
	OmpC+Mg^{2+}	+	-
	both+Mg^{2+}	+	+
Omp35	NOR+Mg^{2+}	+	+
	Omp35+Mg^{2+}	-	+
	both+Mg^{2+}	+	-
Omp36	NOR+Mg^{2+}	-	-
	Omp36+Mg^{2+}	-	-
	both+Mg^{2+}	-	-

1. Material and methods

1.1 Chemicals

For the electrophysiology experiments, 1,2-diphytanoly-*sn*glycero-3-phosphocholine (DPhPC) from Avanti polar lipids, Inc. (Alabaster, AL) was used to build membranes. Chloroform, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and potassium hydroxide (KOH) was obtained from Roth (Carl Roth GmbH, Karlsruhe, Germany). Potassium chloride (KCl), potassium acetate and norfloxacin were obtained from Sigma (Sigma-Aldrich, Steinheim, Germany). Ciprofloxacin was ordered from Applichem (Applichem GmbH, Germany) and enrofloxacin was provided by Fluka. Moreover, Octyl-Polyethylene glycol octyl ether (Octyl-POE) was obtained from Enzo Life Science (Germany). Purified Omp35 and Omp36 proteins from *E. aerogenes* were kindly provided by Jean-Marie Pages and Muriel Masi (Aix-Marseille Université, France), OmpF and OmpC were purified in house as previously published [1, 2]

1.2 Planar lipid bilayer formation and single channel characterization

The horizontal solvent containing lipid bilayers were formed on the MECA (Multi-electrode cavity array) chips (Ionera Technologies GmbH, Germany) using DPhPC as previously described by Baaken G. et al. [37]. 16 cavities each with a diameter of Ø50 µm were located on the MECA chip. Each cavity was painted automatically to form individual bilayers. The cavities were connected to the amplifier (Axon 200B, Molecular Devices, LLC., USA) from the bottom (*trans*) and faced a common bath solution which was connected to the ground (*cis*). The low-pass Bessel filter was set to 10 kHz, the sampling rate was set to 50 kHz. Briefly, 0.3 µL of 0.1 mg/ml DPhPC lipids dissolved in octane were added onto the MECA chip mounted in the automatic bilayer recording setup, Orbit 16 (Nanion Technologies GmbH, Germany). The planar lipid bilayers were formed in an automated fashion by controlling a Teflon coated magnetic bar, this process was computer controlled by the *OrbitControl* software (Nanion Technologies GmbH, Munich, Germany). Purified proteins were added to the *cis* side of the system, both the porin gating manner and conductance were used to determine the orientation of insertion.

Selectivity measurements require easy solvent access on both sides and thus the experiments were performed on a home made set-up. Both chambers of the bilayer apparatus of 2.5 mL each were separated by a 25 µm thick Teflon septum. An aperture in the septum with a diameter of 80-120 µm was pre-treated with hexadecane dissolved in n-hexane (1-5% (v/v)). In-order to remove the solvent, the chambers were dried for 20-25 min. To avoid asymmetric electrode effects, we used standard

Ag/AgCl calomel electrodes (Metrohm AG) having a porous bridge were used to detect the ionic current.

The ion current fluctuations in presence of a substrate was analyzed as previously discussed [32]. Using a one binding site, two barrier model we analysed the on-rate via counting the number of blockage events, namely $k_{on} = \frac{n}{3 \cdot [c]}$. On the other hand, the re-opening is a statistical event and correlated to the strength of the binding. A channel which is closed at $t = 0$ will have the probability $R(t)$ to open at time t. Within the above described simple binding model an exponential function $R(t) = e^{(-t/T)}$ was used. Fitting the life-time distribution of a closed channel by an exponential parameter τ (called residence time) yields the off-rate (dissociation rate) $k_{off} = \frac{1}{T}$. The equilibrium binding constant K (M^{-1}) of the substrate to the channel is given by the ratio of the rates $K = \frac{k_{on}}{k_{off}}$.

1.3 Fluorescence measurement of fluoroquinolones

Norfloxacin, ciprofloxacin and enrofloxacin were the three fluoroquinolones tested. All these fluoroquinolones were dissolved in 1 M KCl, 10 mM KAc, pH 5.0 and 1 M KCl, 10 mM HEPES, pH 7.0. The fluoroquinolone solutions were also buffered with 10 mM KAc, pH 5.0 and 10 mM HEPES, pH 7.0 for the negative controls. Each type of fluoroquinolone solution was separated into four sets, 1 M $MgCl_2$ stock solution was added to adjust the magnesium concentration. Final concentrations of 0.4 mM, 0.8 mM and 1.6 mM of magnesium were reached for each fluoroquinolone at each condition. The fluoroquinolone solutions containing different magnesium concentrations were measured at the appropriate excitation wavelength (400 nm) and emission wavelength (340 nm) using a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, USA) at room temperature.

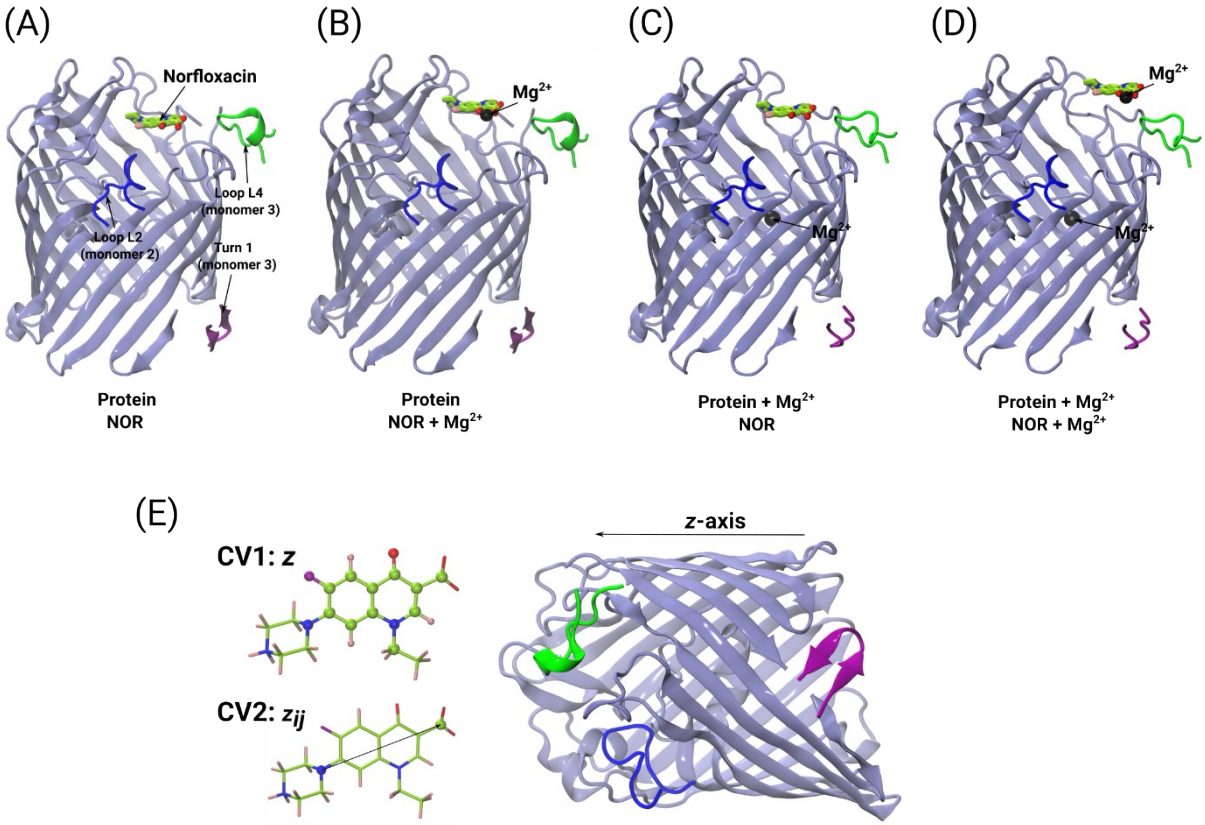

Figure S7. System setup for the TABD simulations. (A) A momoner of OmpF with norfloxacin placed on the extracellular side is shown in cartoon and stick representation, respectively. The loops L2 (blue), L4 (green) and turn 1 (magenta) from the neighbouring monomers are also highlighted. These fragments from the neighbouring monomers are considered due to the fact that some of their residues are positioned along the channel of monomer 1. Thereby they potentially influence the substrate permeation. (B, C, D) System setup used to study the influence of Mg^{2+} ions on norfloxacin permeation. Mg^{2+} ions are high shown as black beads which are placed on the norfloxacin molecule (B), inside the constriction region of the pore (C), and both, on the norfloxacin molecule and inside the constriction region of the pore (D). Description of the CVs, z and z_{ij}, used in the TABD simulations. The CV z is described by calculating the center of mass along the z-axis of the pore. The atoms shown as the spheres are used to calculate the CV z. The CV z_{ij} describes the difference between the z-component of two atoms shown as spheres.

1.4 System setup and MD simulations

The same setup as in our previous study was used for MD simulations of all four porins in presence of 1 M $MgCl_2$ salt.[3] Briefly, the trimer of each protein was inserted into a POPE lipid using VMD 1.9.3. The residues E296 (OmpF), D299 (OmpC), E247 (Omp35) and D307 (Omp36) were kept in their protonated form to avoid gating of loop L3 during the simulations.[4] Later on, the system was

solvated using TIP3P waters and 1 M $MgCl_2$ salt was added, leading to a system size of ~130,000 atoms in all cases. Following the minimization and equilibration steps,[4] 200 ns of unbiased MD simulations were performed for all four systems. All simulations were performed using GROMACS 5.1.2 [5] and the CHARMM36 force field.[6] The long range electrostatics interactions were calculated using the particle mesh Ewald method[7] with a grid spacing of 1 Å. The short range electrostatics and the van der Waals interactions were calculated using a cutoff of 12 Å. All bonds were constrained using the LINCS algorithm.[8]

1.5 System setup and TABD simulation

A monomer of the each porins aligned along the *z* axis was considered as the starting structure including additional loops and a turn from the adjacent monomers as described in our previous work (see Fig. S7A).[9] In a next step, four systems were built in order to perform the TABD simulations: (i) protein with norfloxacin molecule in absence of any ionic salts, (ii) protein with norfloxacin molecule complexed with one Mg^{2+} ion, (iii) protein with a Mg^{2+} ion placed inside the constriction region and an uncomplexed norfloxacin molecule, (iv) protein with a Mg^{2+} ion placed inside the constriction region and a norfloxacin molecule complexed with a Mg^{2+} ion (see Fig. S7A-D). In each system, the norfloxacin molecule (with or without Mg^{2+} ion) was placed at around 25 and 20 Å away from the constriction region in the extracellular and the periplasmic vestibules, respectively. For system (iii) and (iv), one Mg^{2+} ion was placed inside the constriction region and allowed to move freely in *x* and *y* dimensions and restricted to move in the *z* dimension within 10 Å region. This type of setup was established with the anticipation to reproduce the interaction of Mg^{2+} ions as observed during the molecular dynamics simulations (see Fig. 2C in main text).

In the present study we have used a so-called implicit model for the BD simulations,[9] where the entire protein is treated implicitly and only the antibiotic molecule and the Mg^{2+} ions were treated explicitly. The force field for the norfloxacin molecule was obtained from our previously optimized ciprofloxacin force field.[4] In order to maintain the complex of norfloxacin molecule with a Mg^{2+} ion, we have imposed additional bonds between the Mg^{2+} ion and the two oxygen atoms of the norfloxacin molecule. The additional force field parameters for the complexed structure were obtained from bacteriochlorophyll based on analogy.[10]

Prior to performing the TABD simulations, the electrostatic and reaction field potentials for the implicit porin atoms were calculated on a mesh grid with a grid spacing of 0.5 Å and stored in binary files. The dielectric constants for implicit protein, lipid bilayer and water were set to 2, 2 and 80, respectively, for computing the potentials. The energies and forces were derived from these potentials using third-order B-splines. Moreover, the alternative core-repulsive steric potential was applied during the simulations. During the simulations, the electrostatic interactions between the explicit

atoms, i.e., norfloxacin and Mg^{2+} ion, were damped using a C3 type [11] distance-dependent dielectric screening function which corresponds to a screening function for a point charge immersed in water. Furthermore, the self-diffusion constants for the hydrogen atoms of the norfloxacin molecule were manually adjusted to the value of the HB1 atom type of a protein. The self-diffusion constant for Mg^{2+} atom was obtained from a previous study.[12]

Next, we have performed TABD simulations[9] for all four system types for all four porins. In addition, for system (i) where no divalent ions were added to the systems, simulations are also performed including external potentials of -200, -100, 100 and 200 mV. During the simulations, the CV (collective variable) z which describes the position of the antibiotic along the pore axis was considered as a dynamic variable. Additionally, the CV z_{ij}, which represents the orientation of the antibiotic with respect to pore axis, was monitored in order to construct the 2D free energy surfaces (FESs). Both CVs are same as those used in our previous studies[4] for other fluoroquinolone permeation studies through the OmpC porin (see Fig. S7E). Moreover, the sampling of the molecule was restricted inside the monomer by imposing half-harmonic restraints along the x, y and z directions using a force constant of 0.25 kcal/mol/Å^2. The value of the artificial temperature was set to 1000 K for enhancing the sampling of the CV z. Moreover, the force constant and self-diffusion constant associated with the CV z was set to 15 kcal/mol/Å^2 and 0.0118 Å^2/ps, respectively. Next, we have performed a total of 20 independent TABD simulations for each systems, where the antibiotic molecule was started at initial positions from extracellular and periplasmic sides in 10 simulations each. Each simulation was performed for 2 μs which led to total simulation time of 40 μs for each system. All calculations were performed using a time step of 10 fs and 100 BD steps were used for every step in CV space. Moreover, the SHAKE algorithm was used to constraint the bond distance. An atom-based force-switching method with switching distances r_{on} and r_{off} of 16 and 20 Å, respectively, was used for the 5 non-bonded forces calculations. A Verlet list for non-bonded interactions was updated every 100 fs during simulations.

Values of both CVs (z and z_{ij}) were stored every 10 ps during the simulations for the construction of 2D FESs. Normalized histograms $N(z, z_{ij})$ were estimated as a function of the CVs z and z_{ij} with a grid size of 0.5 and 0.4 Å, respectively. Subsequently, the FESs were calculated using the expression [13, 14] $F(z,z_{ij}) = -k_B T\, ln(N(z,z_{ij})) + C$, where T = 1000 K and the constant C was added such that $F(z, z_{ij}) = 0$ at $z = -25$ Å and $z_{ij} = 8$ Å.

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