Alkali–earth metal bridges formed in biofilm matrices regulate the uptake of fluoroquinolone antibiotics and protect against bacterial apoptosis

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22 ABSTRACT

23 Bacterially extracellular biofilms play a critical role in relieving toxicity of fluoroquinolone
24 antibiotic (FQA) pollutants, yet it is unclear whether antibiotic attack may be defused by a bacterial
25 one-two punch strategy associated with metal-reinforced detoxification efficiency. Our findings
26 help to assign functions to specific structural features of biofilms, as they strongly imply a
27 molecularly regulated mechanism by which freely accessed alkali–earth metals in natural waters
28 affect the cellular uptake of FQAs at the water-biofilm interface. Specifically, formation of
29 alkali-earth-metal (Ca^{2+} or Mg^{2+}) bridge between modeling ciprofloxacin and biofilms of
30 *Escherichia coli* regulates the trans-biofilm transport rate of FQAs towards cells (135-nm-thick
31 biofilm). As the addition of Ca^{2+} and Mg^{2+} (0–3.5 mmol/L, CIP: 1.25 $\mu\text{mol/L}$), the transport rates
32 were reduced to 52.4% and 63.0%, respectively. Computational chemistry analysis further
33 demonstrated a deprotonated carboxyl in the tryptophan residues of biofilms acted as a major bridge
34 site, of which one side is a metal and the other is a metal girder jointly connected to the carboxyl
35 and carbonyl of a FQA. The bacterial growth rate depends on the bridging energy at anchoring site,
36 which underlines the environmental importance of metal bridge formed in biofilm matrices in
37 bacterially antibiotic resistance.

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39 **Main finding of the work:** Alkali–earth metal bridges formed in biofilm matrices regulate the
40 uptake of fluoroquinolone antibiotics and protect against bacterial apoptosis

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42 **Keywords:** fluoroquinolone antibiotics; biofilms; alkali–earth metals; bridging energy;
43 computational chemistry; *escherichia coli*

44

45 **1. Introduction**

46 More and more attention has been paid to the challenging issue on environmental contamination
47 of antibiotics in recent years (Martinez, 2009; Pruden et al., 2006). A nationwide survey of
48 pharmaceutical compounds from USA showed that a number of antibiotics were detected in 27% of
49 139 rivers at concentrations up to 0.7 µg/L (Kolpin et al., 2002). And the annual usage of antibiotics
50 has been estimated to be between 1.0×10^5 and 2.0×10^5 tons globally, with more than 2.5×10^4
51 tons used each year in China (Xu et al., 2007). The cellular uptake of antibiotics via trans-biofilm
52 transport will inevitably increase the risk of microbial death, ecological disruption, amplification of
53 antibiotic resistance genes and even the creation of “superbugs” (Desnottes and Diallo, 1992;
54 Pruden et al., 2013). However, regulated mechanism occurring at water-biofilm interface, by which
55 bacteria exert extracellular biofilm barrier to defuse the antibiotic stress, has not been well
56 understand. This process has profound consequences for environmental stability.

57 Fluoroquinolone antibiotics (FQAs), which comprise an important and hard-degradable class of
58 synthetic pharmaceuticals, have been widely used and have been introduced into the environment
59 by a multitude of human and veterinary activities over the last 30 years in Europe and the United
60 States (Baquero et al., 2008; Mompelat et al., 2009). Bacteria generally hide into a biofilm matrix to
61 deal with the presence of these synthetic antibiotics (Wingender et al., 2012). One component of the
62 so-called bacterial one-two punch strategy is to use an extracellular biofilm permeability barrier to
63 impede the cellular uptake of antibiotic stressors. Such physical defenses in response to antibiotics
64 might be inefficient and energetically very costly (Martínez et al., 2007; Rice, 2006). For example,
65 previous studies have shown that sorption of FQAs to oxygen- and nitrogen-containing dissolved
66 organic matter (DOM) displays a nonlinear saturation pattern (with a Freundlich exponent $n =$
67 0.40–0.51) (Zhang et al., 2012), whereas their affinity to phospholipids exhibits a linear partitioning

68 pattern (partitioning coefficient = 20.0–79.4 L/kg) (Merino et al., 2003; Merino et al., 2002). These
69 findings implies that biofilms containing similar oxygen and nitrogen groups have a relatively low
70 capacity (Walter, 2012) to absorb FQAs and may result in high intracellular uptake due to the
71 low-efficiency of extracellular FQA interception. Furthermore, extracellular accretions that act as
72 entrapment agents will be energetically very costly, considering the demand for major saccharide
73 and protein moieties during the formation of biofilms. Nevertheless, little information is currently
74 available to determine how bacteria efficiently and economically deal with such stressors; this issue
75 requires proper investigation.

76 A positively charged metal bridging connection between electronegative biofilms and negatively
77 charged FQAs (i.e., their functional groups) may be the other component of the one-two punch
78 strategy for defending against antibiotic attack. Given the strong electrostatic attraction between
79 electronegative biofilms/FQAs and accessible alkali–earth metals, the formation of a stable
80 structure would anchor the FQAs in biofilms via a metal bridge. Whereas one side of the
81 metal-to-FQA connection (metal–carbonyl interaction) is supposedly understood (Aristilde and
82 Sposito, 2008), attractions between metals and extracellular biofilm components on the other side
83 are more multifarious and complicated. Thus, a quantitative description of an alkali–earth–metal
84 bridge based on these weak attractions is crucial for quantitatively expounding the association of
85 such weak interactions at the molecular level. Generally, on the basis of spectroscopic data, it is
86 considered that alcohol, carboxyl, phosphoric, and amino-acid residue groups in biofilm matrices
87 may be involved in interactions with such metals as Zn^{2+} , Co^{2+} , and Ca^{2+} (Ha et al., 2010; Sun et al.,
88 2009; Sundararajan et al., 2011; Xiong et al., 2002). These results only suggest that biofilms may
89 utilize their electronegative groups to interact with metals, but not know whether antibiotic attack
90 may be defused by a metal-reinforced detoxification efficiency occurring at water-biofilm interface.

And these spectroscopic data should be considered qualitative, as they do not specify which of the functional groups in biofilms are involved in metal binding. The listed spectroscopic techniques have been unable to access quantitative descriptions of imperceptibly weak interactions at the microscopic level. Therefore, based on the idea that FQA–biofilm interaction occurs via a metal bridge, including bridging energy and spectroscopic analyses, multiple approaches are necessary to substantiate an association between alkali–earth metals and the extracellular retardation of FQAs and biological growth.

In the present study, we investigated the association of bacterial extracellular biofilms with FQAs via an alkali–earth–Ca²⁺/Mg²⁺ bridge and determined the influence of metal bridges in biofilms on the extracellular interception and cellular uptake of FQAs. *In situ* confocal laser scanning microscopy (CLSM), X-ray photoelectron spectroscopy (XPS), Fourier transform infrared spectroscopy (FTIR), and ¹³C nuclear magnetic resonance (¹³C-NMR) were used to confirm the formation of alkali–earth–metal bridges in biofilms. A series of batch experiments was performed to explore the effect of alkali–earth–metal bridges in biofilm matrices on extracellular interception, trans-biofilm cellular uptake of a model antibiotic, and bacterial apoptosis. Subsequently, eight FQAs were used to determine the dependence of *E. coli* growth on the bridging energy at major sites through a set of fluorescence microtitration and computational chemistry analyses.

2. Materials and methods

2.1. Materials

Eight fluoroquinolone antibiotics (FQAs, ≥99.0%) were purchased from Sigma-Aldrich Co., Ltd, USA, namely ciprofloxacin (CIP), norfloxacin (NOR), enrofloxacin (ENR), ofloxacin (OFL), lomefloxacin (LOM), levofloxacin (LEV), pefloxacin (PEF), and fleroxacin (FLE). Their physicochemical properties, including molecular weight (MW), the dissociation constant for

114 carboxyl groups (pK_{a-COOH}), and the octanol–water partition coefficient ($\log K_{ow}$), are listed in the
115 **Table S1**. Magnesium sulfate ($MgSO_4 \cdot 7H_2O$, $\geq 99.0\%$) and calcium sulfate ($CaSO_4$, $\geq 99.0\%$) were
116 purchased from Sinopharm Chemical Reagent Co., Ltd, China. Milli-Q water ($18.2\text{ M}\Omega \times \text{cm}$,
117 Millipore, USA) was used for all experiments.

118 Other chemicals (as listed below, purity $> 99.0\%$) that were purchased from Nanjing Chemical
119 Reagent Co., Ltd. were used to prepare modified chloride-free culture medium (Kang et al., 2014).
120 The medium contained $K_2HPO_4 \cdot H_2O$ (28 mmol/L), $KH_2PO_4 \cdot 3H_2O$ (2.2 mmol/L), NH_4NO_3 (18.7
121 mmol/L), $CaSO_4$ (0.001 mmol/L), K_2SO_4 (2.0 mmol/L), $MgSO_4 \cdot 7H_2O$ (1.0 mmol/L), peptone (10
122 g/L), and a trace element solution (10 mL/L). The trace element solution contained $Na_2EDTA \cdot H_2O$
123 (5.0 g/L), $Fe_2(SO_4)_3$ (0.37 g/L), ZnO (0.05 g/L), $CuSO_4 \cdot 5H_2O$ (0.015 g/L), $Co(NO_3)_2 \cdot 6H_2O$ (0.01
124 g/L), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.01 g/L), and H_3BO_3 (0.01 g/L). The pH of the medium was adjusted
125 to 7.4 with sulfuric acid (98.0%). When necessary, solid agar medium was prepared with 1 L of
126 chloride-free medium and 15 g of agar powder. The sulfuric acid (0.01 mmol/L H_2SO_4) and sodium
127 hydroxide (0.02 mmol/L $NaOH$), used for pH adjustment in batch experiment and fluorescence
128 microtitration, were purchased from Nanjing Chemical Reagent Co., Ltd. (Nanshi, China).

129 2.2. Biofilm manipulation

130 Our previous method was used to manipulate the extracellular biofilms (Fang et al., 2002; Kang
131 et al., 2014; Liu and Fang, 2002). In brief, *Escherichia coli* (DH5 α) was initially cultured in 20 mL
132 of chloride-free medium at 37°C for a 12-h recovery of growth. The bacterial suspension (5 mL)
133 was then transferred to fresh chloride-free medium (1.0×10^3 mL) and grown for an additional 48 h
134 to reach the stable growth phase. *E. coli* cells were collected by low-speed centrifugation (3×10^3 g,
135 6 min, 4°C) followed by washing with Milli-Q water to obtain pure, pristine *E. coli* (high-biofilm *E.*
136 *coli*).

137 Low-biofilm *E. coli* was obtained by the removal of biofilms from the *E. coli* surface using a
138 sonication/centrifugation procedure (Kang et al., 2014). Specifically, one-half of the (high-biofilm)
139 *E. coli* pellet was suspended to an initial volume of 500 mL and then processed by a low-intensity
140 ultrasonic process for 7.0 minutes at an intensity of 2.5 W/cm² and a frequency of 40 KHz at 4°C.
141 The *E. coli* suspension was then centrifuged for 20 min at $2.0 \times 10^4 g$ and 4°C. The settled pellets
142 were collected as low-biofilm *E. coli* samples. The supernatant, which was filtered through a
143 0.22-μm membrane (Anpel, Shanghai, China), represented an aqueous biofilm solution and was
144 stored at 4°C for later chemical analyses. Elemental analysis was performed on freeze-dried
145 biofilms using an X-ray photoelectron spectrometer (Thermo Fisher Scientific, Waltham, MA,
146 USA), and the percentage contents of C (38.96), O (29.88), N (30.14), and S (1.02) were
147 determined. The dry weight of the extracted aqueous biofilms (25.7 mg/L) was measured by an
148 oven-drying method (2 h at 105°C) (Comte et al., 2006). The major proteins (345.1 mg/g) and
149 polysaccharides (173.8 mg/g), and a small quantity of nucleic acid (0.33 mg/g), were measured
150 according to previously reported techniques (Burton, 1956; Dubois et al., 1956; Lowry et al., 1951).
151 The low concentration of nucleic acid in the biofilms indicated negligible cell lysis during the
152 biofilm extraction procedure.

153 2.3. Interception and uptake of FQA and *E. coli* apoptosis

154 High- and low-biofilm *E. coli* cells that were subjected to different biofilm manipulations were
155 suspended in 1.0 L of ultrapure water to obtain approximately 4.0×10^7 cells/mL of each type of *E.*
156 *coli*. A liquid suspension of bacteria (40 mL) was placed into a 50-mL conical flask equipped with a
157 permeable silica-gel stopper prior to the addition of aqueous alkali–earth–metal stock solutions (200
158 mmol/L, alkali–earth Ca²⁺ or Mg²⁺) and an aqueous CIP stock solution (1 mmol/L) to the desired
159 concentrations (metals: 0–3.5 mmol/L; CIP: 1.25 μmol/L). Notably, the concentrations of the added

160 alkali–earth metals were within the range of natural oceanic water (Ca^{2+} : 53.75 mmol/L; Mg^{2+} : 10.1
161 mmol/L) and fresh water (Ca^{2+} : about 1.75 mmol/L; Mg^{2+} : about 1.77 mmol/L) (Adkins et al., 2002;
162 Bowen, 1979). Here, the added concentration of CaSO_4 was far below its solubility limit (14.2–15.4
163 mmol/L at 10–40°C) (Harvie et al., 1984). The static suspension experiment was performed in the
164 dark for 4 h at 120 rpm and 37°C.

165 A portion of the *E. coli* suspension was used to extract the CIP that was retained in extracellular
166 matrices and absorbed into the cell interiors. The CIP in extracellular matrices was extracted using
167 the biofilm-extraction method described above. The CIP antibiotic was obtained during the
168 separation of biofilms from the *E. coli* surface. For low-biofilm *E. coli*, CIP extraction from
169 extracellular matrices was also performed using the same procedures. Additionally, an ultrasonic
170 method of cellular disruption was used to obtain the intracellular components for CIP antibiotic
171 quantification (Turiel et al., 2006). An *E. coli* sample (5 mL) was subjected to ultrasonic disruption
172 at 450 W and 24 kHz ($\Phi 6$, Scientz, China). The period was 2.0 s with a 2.0-s interval in an
173 ice-water bath for 5 min, unless otherwise indicated. No bacterial colonies were observed in a plate
174 test (15 g/L agar in chloride-free medium), which suggested that the *E. coli* cells were completely
175 disrupted by the high-intensity ultrasound.

176 The CIP antibiotic in both extracted biofilms and disrupted *E. coli* cells was treated with a
177 vacuum freeze-drying technique (–65°C) that was followed by extraction using acetonitrile (7.0 mL)
178 with a 1% formic acid-water solution (0.5 mL) (Johnston et al., 2002). The solutions were
179 processed in a vortex mixer for 6 min and then centrifuged at 6,000 g and 4°C. This process was
180 performed thrice, and the supernatants were pooled for later chromatographic analysis. The CIP in
181 the supernatant was analyzed by high-performance liquid chromatography (HPLC) in an
182 instrumental setup that included a 20-mL automatic liquid sampler, a Zorbax XDB-C18 column

183 (Agilent, USA), and a fluorescence detector (FLD) (1260 series: Agilent, USA). The composition of
184 the mobile phase was constant (87/13, v/v, acetonitrile/aqueous H₃PO₄ buffer (0.025 mmol/L, pH
185 3.0, adjusted with triethylamine). The FLD was operated at 278-nm excitation and 440-nm emission.
186 The experiment demonstrated that the sequential method, including the CIP extraction and HPLC
187 analysis, gave a high recovery of approximately $98 \pm 3.5\%$, indicating no loss or metabolism of CIP
188 in the static suspension experiments.

189 After the static suspension experiment, another independent experiment was performed to
190 investigate *E. coli* growth. A portion of *E. coli* from the static suspension experiment was inoculated
191 into fresh chlorine-free medium (40 mL, 4.0×10^7 cells/mL) and incubated for 18 h at 37°C and
192 160 rpm. The cell densities (reflecting *E. coli* growth) were determined by a calibration curve for
193 optical density [cell density (cell/mL) = $(1.89 \times \text{OD}_{600\text{nm}} - 0.01) \times 10^8$], as described in a previous
194 study (Kang et al., 2014).

195 2.4. Spectroscopic analyses of alkali–earth–metal bridges

196 CLSM technology was used to observe *in situ* the enhanced FQA–biofilm association via metals.
197 A water-immersion lens (1,000 ×) was used to observe the sample (50 µL) on a slide. Image stacks
198 were created with a Zeiss LSM510 META that was controlled with AIM software (Jena, Germany).
199 The fluorescence signal of the CIP was recorded with an excitation/emission matrix of 278/440 nm.
200 The fluorescence signal of biofilms at this excitation/emission matrix could not be detected, but the
201 signal from CIP attached to biofilms was clearly visible. XPS analysis was performed to probe the
202 Ca2p and Mg2p signals from the bridges that were formed in *E. coli* biofilms (Thermo, USA). The
203 sampling depth was less than 10 nm, suggesting that the sampling site was located in the biofilms
204 and not on the cells (the biofilm thickness was approximately 135 nm).

205 TEM was used to observe the removal of the biofilm matrix from the *E. coli* surface. Dried *E.*

206 *coli* cells were placed onto carbon-coated copper grids for TEM observation with a brightfield
207 detector on a JEM-2100 (Tokyo, Japan). Additionally, after the static suspension experiment, the
208 CIP-containing biofilms that had been extracted from the *E. coli* surface were also dried at -65°C.
209 FTIR (Bruker, USA) and ¹³C-NMR (Bruker, Germany) analyses were performed to characterize the
210 functional groups of these dried biofilms through using previous method (Kang et al., 2014; Kang
211 and Zhu, 2013).

212 2.5. Fluorescence micro titration

213 Three-dimensional excitation-emission matrix (3DEEM) fluorescence spectroscopy combined
214 with micro titration (Kang et al., 2010; Kang et al., 2015) was used to quantitatively explore the
215 association of FQA with the amino acid residues of biofilms in the presence of alkali–earth metals.
216 In this test, the fluorescence peaks of tryptophan (Trp) residues in biofilms, which were located at
217 280/340 nm (EX/EM) in the 3DEEM, were traced by continuous microtitration/detection processes
218 (Chen et al., 2003; Kang et al., 2010). Specifically, a CaSO₄ or MgSO₄ stock solution (10 mmol/L)
219 was added in advance of the 20-mL aqueous biofilm solution (0.5 mg/L, dry weight basis) to obtain
220 a 250 μmol/L Ca²⁺ or Mg²⁺ solution. Afterwards, an aqueous CIP stock solution (10 μmol/L) was
221 titrated into each aqueous biofilm solution containing Ca²⁺ or Mg²⁺ using a chromatographic
222 injector (25-μL scale, Agilent, Santa Clara, CA, USA); this step was followed by magnetic stirring
223 for 20 min at 160 rpm, pH 7.0, and 25 °C. The fluorescence spectrum was recorded at an excitation
224 wavelength of 200–370 nm (5-nm bandwidth) and an emission wavelength of 280–500 nm (5-nm
225 bandwidth) at a speed of 3,000 nm/min (F96PRO, Lengguang). These microtitration/detection
226 procedures were repeated until there was no significant change in the fluorescence intensity. To
227 obtain comparative data, similar procedures were also performed to explore the association between
228 CIP and the biofilm matrix in the absence of alkali–earth metals. The fluorescence peak from

extracellular biofilms matrix (EX/EM: 280/340 nm) did not overlap with the FQA peak (EX/EM: 278/440 nm). Thus, the changes in the fluorescence intensity of Trp residues in the biofilms that were caused by the CIP antibiotic could be well described by the relationship between the fluorescence intensity and the quencher concentration (Boaz and Rollefson, 1950; Eftink, 1997; Lakowicz and Weber, 1973). The Stern–Volmer equation is given as follows (Valeur and Berberan-Santos, 2012):

$$\frac{F_0}{F} = 1 + K_q \tau_0 [Q] = 1 + K_{SV} [Q] \quad (1),$$

where F_0 and F are the relative fluorescence intensity of the chromophore in the absence and presence of the quencher, respectively; K_q is the bimolecular quenching rate constant; τ_0 is the average lifetime of the fluorophore in the absence of the quencher; $[Q]$ is the concentration of the quencher; and K_{SV} is the Stern–Volmer quenching constant. For the static quenching process, equation (2) was used to determine the association constant (K_A) and the number of binding sites (n) (Bi et al., 2004). The association constants with biofilms via metal bridging were determined for all eight FQAs as follows:

$$\log[(F_0 - F) / F] = \log K_A + n \log [Q]. \quad (2).$$

2.6. Quantum mechanical/molecular mechanical (QM/MM) modeling

Utilizing Gabedit software (Version 2.4.5) (Allouche, 2011), we structured the 20 standard amino acid residues: alanine, leucine, isoleucine, valine, proline, phenylalanine, methionine, tryptophan, glycine, serine, glutamine, threonine, cysteine, asparagine, tyrosine, aspartic acid, glutamic acid, lysine, arginine, and histidine. These residues were docked with the CIP antibiotic via an alkali–earth–metal bridge. The key bridging site in biofilms must meet two necessary conditions: a high abundance in biofilms and a high bridging energy between the residue-metal and the FQA. First, the backbones of oligopeptides were composed of the reported seven most common

252 amino acids in bacterial biofilms (Phe, Gly, Asn, Gln, Ser, Thr, and Tyr) (Nakashima and Nishikawa,
253 1992, 1994). The 20 standard amino acid residues (R) were then successively joined in pairs to both
254 sides of these backbones to structure the amino and carboxyl termini
255 ($\text{NH}_2\text{-R-Phe-Gly-Asn-Pro-Gln-Ser-Thr-Tyr-R-COOH}$). This unified model contains 20 short-chain
256 polypeptides (4940 atoms in all) that share a common central sequence and sample the 20 different
257 amino acid residues at each end. Second, the highest bridging energy between the Trp-metal and the
258 FQA was determined on the basis of the QM/MM computation. Additionally, the wave functions
259 from the computation were analyzed using the Multiwfn program. Bridging energies for the
260 combination of Trp with each of the eight FQAs via a metal girder were computed based on a
261 frequency analysis from the "FQA-Ca/Mg-Trp residue" model.

262 Our own multilayered, N-layered integrated molecular orbital and molecular mechanics
263 (ONIOM) scheme in Gaussian 09 was used to conduct the combined QM/MM computation
264 (Dapprich et al., 1999; Trucks et al., 2009). The entire molecular system was partitioned into two
265 layers: a QM system (CIP-metal-residue), which was treated with a high-level method, and an MM
266 system, consisting of the peripheral molecular system (backbone of oligopeptides), which was
267 treated with a low-level method. In this study, the MM system, as described by the Assisted Model
268 Building with Energy Refinement (AMBER) force field (Cornell et al., 1995), consisted of the
269 peripheral protein structures but excluded the CIP antibiotic, the metal girder, and the 20 amino acid
270 residues. The QM system consisted of the CIP antibiotic, the metal girder, and the amino acid
271 residues that were involved in the bridging reaction. The density function B3LYP was used to treat
272 the QM model system. The geometries of the model system were optimized with the 6-31G(d, p)
273 basis set (Petersson and Al-Laham, 1991; Petersson et al., 1988) with a long-range correction of
274 DFT-D3 (Trucks et al., 2009). All of the atoms in both layers were free to move in the geometry

275 optimization calculations. A Trp residue was determined as a major bridging site. The bridging
 276 energies at Trp residue-metals were determined for all eight FQAs. Solvent (water) effects were
 277 implicitly taken into consideration in all computations.

278 2.7. Bonding type and bridging energy

279 The wave functions from the computation were analyzed by the Multiwfn program (Lu and
 280 Chen, 2012). Many pieces of information were extracted by this software, including the reduced
 281 density gradient (RDG), the bridging energy, and the Lorentz oscillator. The Lorentz oscillators
 282 were used to predict the FTIR spectra, which were then compared with our experimental results.
 283 The bridging site at the Trp residue predicted by the FTIR computation was confirmed with the
 284 experimental FTIR, which supported the reliability of the model. Based on the functions of the
 285 CIP-metal-Trp residue combination, a topological analysis and a graphic illustration of the
 286 distribution of the electron density were also performed using the Multiwfn program (Lu and Chen,
 287 2012). The theoretical method for the discrimination of different bonding types can be explained by
 288 the quantum mechanical electron density ($\rho(r)$) (Johnson et al., 2010; Kang et al., 2015):

$$289 \quad \text{RDG}(r) = \frac{1}{2(3\pi^2)^{1/3}} \frac{|\nabla\rho(r)|}{\rho(r)^{4/3}}, \quad (3),$$

290 where $\rho(r)$, $|\nabla\rho(r)|$, and ∇ are the quantum mechanical electron density, the gradient operator, and
 291 the modular arithmetic of the gradient operator for the quantum mechanical electron density,
 292 respectively. The bonding types were identified and drawn with interactive isosurfaces around the
 293 metal bridge. In the colored contour planes between atoms, we used different colors to represent the
 294 bonding types between the metal and the CIP/residue. Blue, green and, red were used to represent
 295 strong coordinate bonds, weak vdWs bonds, and intermolecular repulsion, respectively. The
 296 bridging energies for the combination of Trp with each of the eight FQAs via a metal girder were
 297 computed based on a frequency analysis from the "FQA-Ca/Mg-Trp residue" model. The

calculation was as follows:

$$\text{Bridging energy } (\Delta G) = G_{\text{FQA-metal}\cdots\text{Trp}} - (G_{\text{FQA-metal}} + G_{\text{Trp}}), \quad (4),$$

where $G_{\text{FQA-metal}\cdots\text{Trp}}$, $G_{\text{FQA-metal}}$, and G_{Trp} are the Gibbs free energies (G) of the FQA-metal $\cdots$ Trp (dotted line represents the docking point), the FQA-metal bridge head, and the Trp residue in the polypeptide, respectively.

3. Results and discussion

3.1. Interactions of a model FQA, CIP, with biofilms are enhanced by a metal bridge

Fig. 1a–d presents a group of CLSM images that demonstrate the metal-enhanced CIP-biofilm association on the surface of *E. coli*. In the absence of alkali–earth metals and CIP, a fluorescence signal was not observed, as is apparent in **Fig. 1a**. After the addition of only CIP (1.25 $\mu\text{mol/L}$), some faint and scattered rods are visible in **Fig. 1b**, indicating a weak association of CIP with *E. coli* biofilms. In the presence of the CIP antibiotic (1.25 $\mu\text{mol/L}$) and alkali–earth Ca^{2+} (2.0 mmol/L), many bright rods are visible in **Fig. 1c**, suggesting the Ca^{2+} -enhanced attachment of CIP molecules to extracellular biofilms. Similarly, evidence from the CIP (1.25 $\mu\text{mol/L}$) and alkali–earth Mg^{2+} (2.0 mmol/L) conditions reveals numerous bright rods (**Fig. 1d**), confirming the Mg^{2+} -enhanced attachment. These results confirm that alkali–earth metals can enhance the association of CIP with biofilms.

Fig. 1e and f presents the core-level Ca2p and Mg2p signals from XPS, respectively. In the reference CaSO_4 sample, the $\text{Ca}2p_{3/2}$ and $\text{Ca}2p_{1/2}$ peaks were located at 347.7 and 351.2 eV, respectively. After the addition of Ca^{2+} to aqueous *E. coli*, $\text{Ca}2p_{3/2}$ produced two peaks (primary 347.25 eV and secondary 346.7 eV; **Fig. 1e**) that shifted towards a lower energy by 0.45 and 3.95 eV, respectively. The $\text{Ca}2p_{1/2}$ peak shifted towards a lower energy by 0.1 eV. As CIP (1.25 $\mu\text{mol/L}$) was subsequently added, the signal continuously shifted towards a lower energy, from a primary

321 peak (Ca2p_{1/2}) of 350.6 eV and a secondary peak of 350.3 eV to lower primary (346.8 eV) and
 322 secondary peaks (346.4 eV; shoulder peak). The signal continuously shifted towards a lower energy,
 323 indicating that Ca²⁺ acted as an electron acceptor and attracted redundant electrons from the
 324 electron-donating biofilms and CIP (Gosselink et al., 2012; Stipp and Hochella, 1991). Combined
 325 with the CLSM result, these findings indicate that the Ca²⁺-enhanced association of CIP with
 326 biofilms on the surface of *E. coli* occurs through an alkali–earth Ca²⁺ bridge. Additionally, the
 327 Mg2p peak decreased to 50.2 eV when biofilms were added and further decreased to 49.7 eV upon
 328 subsequent CIP addition in comparison to the reference sample, MgSO₄ (51.1 eV) (**Fig. 1f**). Overall,
 329 the XPS peaks of alkaline-earth metals, mediated by biofilms (**Fig. 1e and f**) and CIP (**Fig. S4**),
 330 shifted towards a lower energy, demonstrating that the CIP–biofilm association is enhanced by
 331 alkali–earth metals and is related to the formation of a metal bridge between them.

332 3.2. Bridging site in biofilms

333 The CIP-to-biofilm connection via a metal bridge was analyzed by FTIR spectroscopy (black
 334 plots) to further explore the bridging site in biofilms. In the FTIR spectrum of biofilm alone (**Fig.**
 335 **2a**), the stretching vibrations of the C=O band at 1675 cm⁻¹ and N-H bending and C-N stretching
 336 vibrations at 1600 ± 10 cm⁻¹ are related to amino acid residues or peptides from proteins in
 337 extracellular biofilms. The band at 1430 cm⁻¹ denotes the stretching vibration of C-OH in
 338 carboxylates (Byler and Susi, 1986; Deacon and Phillips, 1980), whereas the bands near 1115 and
 339 1010 cm⁻¹ are ascribed to the stretching vibrations of hydroxyl and C-O-C in saccharides,
 340 respectively (Kang and Zhu, 2013). After the binding of CIP via Ca²⁺ (**Fig. 2b**) and Mg²⁺ bridges
 341 (**Fig. 2c**), the characteristics of the C=O band at 1675 cm⁻¹ became weaker compared with those of
 342 biofilms alone (**Fig. 2a**), suggesting that carbonyl groups in the amino acid residues of extracellular
 343 biofilms are responsible for the formation of the metal bridge. The band at 1430 cm⁻¹, which is

344 related to C-OH in carboxylates, also became much weaker (**Fig. 2b and c**). Considering the change
345 in the C=O band at 1675 cm^{-1} , these results suggest that the carboxylates in the amino acid residues
346 of biofilms are responsible for the alkali–earth–metal bridges.

347 **Fig. 2a–c** shows a group of computational FTIR spectra (red plots) that were plotted by the
348 Lorentz oscillator (blue plots) based on computation from short polypeptides. After associating Trp
349 carboxyl groups with CIP via Ca^{2+} (**Fig. 2b**) or Mg^{2+} (**Fig. 2c**), the bands at 1675 and 1430 cm^{-1}
350 became weaker (decrease in molar absorption coefficients). These results further confirm that
351 carboxyl groups of structured Trp residues may act as the main bridging sites associated with the
352 formation of alkali–earth–metal bridges in biofilms (further analysis in **Fig. 5**). In this study,
353 contribution of COOH in humics to the bridging may be ruled out, because humic and fulvic acids
354 were not observed in the highly sensitive three-dimensional excitation-emission matrix (3DEEM)
355 spectra (**Fig. S1**), which may be attributed to the lack of long-term humification (Milori et al.,
356 2002).

357 CIP-to-biofilm associations via metal bridges may be further analyzed by the ^{13}C NMR spectral
358 fingerprints of biofilms (**Fig. 2d**). Based on previous work (Wishart et al., 1995), the carboxyl
359 groups in biofilms may be assigned to Trp (176.1 ppm), Tyr (175.8 ppm), Phe (175.7 ppm), Gly
360 (174.9 ppm), Thr (174.7 ppm), Ser (174.4 ppm), Cys (oxidized and reduced states, 174.6 ± 0.2
361 ppm), and His (174.0 ppm) residues. In **Fig. 2d**, resonance intensity of Trp residues (176.1 ppm)
362 was dominant in all peaks; a comparison of this signal with different treatments revealed a marked
363 downfield shift ($\Delta\delta = 0.2$) of the resonances attributed to the carbon signals of carboxyl residues in
364 biofilms after treatment of biofilms with Mg/Ca-CIP. The downfield shift indicates that there are
365 some multinuclear species containing calcium/magnesium ions that are bridged through the
366 carboxylates of Trp (Barszcz et al., 2013), as Trp residues exhibited the highest abundance and the

367 most obvious change in all of the resonance signals.

368 3.3. Trans-biofilm uptake of FQA

369 **Fig. S2** shows the appearance of *E. coli* before and after biofilm manipulation, suggesting the
370 removal of biofilms from the surface of low-biofilm *E. coli*. **Fig. 3a** shows that the concentration of
371 CIP that was retained in high biofilms (135-nm thick in the **Fig. S2**) increased from $(5.2 \pm 0.3) \times$
372 10^{-3} to $2.1 \times 10^{-2} \mu\text{mol}/10^9$ cells as Ca^{2+} was added at concentrations up to 3.5 mmol/L. However,
373 the concentration of CIP in low biofilms increased only from $(5.2 \pm 0.3) \times 10^{-3}$ to 1.56×10^{-2}
374 $\mu\text{mol}/10^9$ cells. High biofilms on the *E. coli* surface caused greater retardation of CIP in the
375 extracellular matrix. **Fig. 3a** also shows that, in contrast, the intracellular CIP concentration in
376 high-biofilm *E. coli* gradually decreased from $(4.0 \pm 0.2) \times 10^{-3}$ to $2.5 \times 10^{-3} \mu\text{mol}/10^9$ cells when
377 Ca^{2+} was added at concentrations up to 3.5 mmol/L. The intracellular CIP concentration in
378 low-biofilm *E. coli* decreased to $3.7 \times 10^{-3} \mu\text{mol}/10^9$ cells with Ca^{2+} addition, but this value was still
379 greater than that in high-biofilm *E. coli*. These findings confirm that extracellular retardation in
380 biofilms, which is enhanced by alkali–earth– Ca^{2+} bridges, reduces cellular uptake of the CIP
381 antibiotic.

382 **Fig. 3b** presents that with the addition of alkali–earth Mg^{2+} (0–3.5 mmol/L), the concentration
383 of CIP that was retained in high biofilms on the surface of *E. coli* increased from $(5.5 \pm 0.5) \times 10^{-3}$
384 to $2.9 \times 10^{-2} \mu\text{mol}/10^9$ cells, whereas the concentration in low biofilms increased less, from $(5.5 \pm$
385 $0.5) \times 10^{-3}$ to $1.8 \times 10^{-2} \mu\text{mol}/10^9$ cells. Correspondingly, the intracellular CIP concentration in
386 low-biofilm *E. coli* decreased to a final value of $2.7 \times 10^{-3} \mu\text{mol}/10^9$ cells, which was slightly higher
387 than that in high-biofilm *E. coli* ($2.4 \times 10^{-3} \mu\text{mol}/10^9$ cells). Similar findings reconfirm that high
388 biofilms on bacterial surfaces, which are enhanced by the formation of alkali–earth–metal bridges,
389 restrict the cellular uptake of CIP.

390 **Fig. 3c** shows the trans-biofilm transport rate of CIP into cells (r) with the addition of
 391 alkali–earth metals. As the Ca^{2+} concentration increased up to 3.5 mmol/L (CIP = 1.25 $\mu\text{mol/L}$), the
 392 r value for high-biofilm *E. coli* decreased from $(2.06 \pm 0.02) \times 10^{-4}$ to $1.09 \times 10^{-4} \mu\text{mol}/\mu\text{m}^2 \text{ h } 10^9$
 393 cells. In the low-biofilm *E. coli*, the r value was reduced from $(2.06 \pm 0.02) \times 10^{-4}$ to 1.75×10^{-4}
 394 $\mu\text{mol}/\mu\text{m}^2 \text{ h } 10^9$ cells. High biofilms act to lower the trans-biofilm transport rate of CIP, and
 395 alkali–earth–metal bridges formed in high biofilms will have greater potential for decreasing
 396 trans-biofilm antibiotic transport. In the presence of Mg^{2+} (0–3.5 mmol/L), the r value was reduced
 397 from $(2.10 \pm 0.01) \times 10^{-4}$ to $1.31 \times 10^{-4} \mu\text{mol}/\mu\text{m}^2 \text{ h } 10^9$ cells in high-biofilm *E. coli* and to $(2.10 \pm$
 398 $0.01) \times 10^{-4} \mu\text{mol}/\mu\text{m}^2 \text{ h } 10^9$ cells in low-biofilm *E. coli*. This finding further supports the notion
 399 that the alkali–earth–metal bridges formed in high biofilms are not as conducive to the trans-biofilm
 400 transport of CIP. These comparable results regarding extra- and intra-cellular distribution underline
 401 an important role for the alkali–earth–metal bridges in reducing the cellular uptake of FQAs.

402 On the basis of the extra- and intra-cellular distribution in **Fig. 3**, **Fig. 4** depicts the association
 403 of *E. coli* growth with the distribution of CIP. In presence of alkali–earth Ca^{2+} (0–3.5 mmol/L,
 404 initially added CIP = 1.25 $\mu\text{mol/L}$), the *E. coli* density was enhanced to 0.42×10^9 cells/mL for
 405 high-biofilm manipulation (retardation: $2.08 \times 10^{-2} \mu\text{mol CIP}/10^9$ cells; uptake: $2.13 \times 10^{-3} \mu\text{mol}$
 406 $\text{CIP}/10^9$ cells) and to 0.359×10^9 cells/mL for low-biofilm manipulation (retardation: 1.58×10^{-2}
 407 $\mu\text{mol CIP}/10^9$ cells; uptake: $3.49 \times 10^{-3} \mu\text{mol CIP}/10^9$ cells) (**Fig. 4a and b**). The highest growth
 408 density corresponded to the highest extracellular retardation and the lowest cellular uptake of CIP,
 409 which demonstrates that the formation of Ca^{2+} bridges on the surface of *E. coli* has a positive effect
 410 on the protection of *E. coli* cells. In the presence of alkali–earth Mg^{2+} (0–3.5 mmol/L, initially
 411 added CIP = 1.25 $\mu\text{mol/L}$), *E. coli* densities were enhanced to maximal levels of 0.388×10^9
 412 cells/mL for high-biofilm *E. coli* and 0.365×10^9 cells/mL for low-biofilm *E. coli* (**Fig. 4c and d**).

413 Accordingly, the CIP retardation values were 0.392×10^{-2} (high biofilms) and 0.365×10^{-2} μmol
414 CIP/ 10^9 cells (low biofilms), which corresponded to uptake values of 2.58×10^{-3} and $(4.08\text{--}4.18) \times$
415 10^{-3} μmol CIP/ 10^9 cells, respectively. The high-biofilm *E. coli* causes a higher density than the
416 low-biofilm *E. coli*. These results lead us to conclude that the formation of an metal bridge in
417 biofilms facilitates *E. coli* growth by enhancing extracellular retardation of CIP.

418 3.4. Bond types at key sites

419 **Fig. 5a** presents results for structured short polypeptides on the basis of the quantum
420 mechanical/molecular mechanical (QM/MM) computation. Metal girder-centered local images are
421 presented in **Fig. 5b, d, and f**. The electronegative carbonyl groups in FQAs interact with the
422 carboxyl groups of Trp residues in extracellular biofilms through metal girders. To intuitively
423 observe the bond types of the alkali–earth–metal bridges, we analyzed the gradient isosurfaces of
424 the interactive forces around the central alkali–earth metal girder (**Fig. 5b, d, and f**). The
425 corresponding plots of the reduced density gradient (RDG) versus the electron density multiplied by
426 the sign of the second Hessian eigenvalues are presented in **Fig. 5c, e, and g** (Johnson et al., 2010).
427 In the absence of metals, the gradient isosurfaces exhibited a rich H-bonding interaction between
428 CIP and biofilms (**Fig. 5b**). Two pairs of H bonds were observed, between a Trp residue in the
429 biofilm and the carbonyl in the carboxyl of CIP and between the carbonyl of a Trp residue in the
430 biofilm and a hydrogen in pyridine. The corresponding plots of the RDG versus the electron density
431 multiplied by the sign of the second Hessian eigenvalues revealed two spikes, as noted in **Fig. 5c**
432 (-0.01 to -0.04 au). a spike denoting their attraction (-0.03 to -0.04 au) corresponds to H bonding
433 between a Trp residue in biofilm and the carbonyl of the carboxyl group in CIP. Another spike, at
434 -0.01 to -0.02 au, shows the H bond between a carbonyl in the Trp residue of a biofilm and a
435 hydrogen atom in pyridine. Additionally, a spike at 0 to -0.01 au reveals a weak intramolecular H

436 bond between the pyridine and carbonyl in the molecular interior of the CIP antibiotic. These
437 observations were consistent with previously simulated result from molecular docking between
438 NOR and protein (Lu et al., 2010), suggesting that the CIP–biofilm interaction is based primarily on
439 H bonds.

440 The gradient isosurfaces exhibited a rich visualization of *n*–metal electron donor–acceptor
441 (*n*–metal EDA) interactions (**Fig. 5d**). Four planar isosurfaces (blue) indicate a strong attraction
442 between Ca^{2+} and oxygen atoms. The non-bonded interaction between a pair of oxygen atoms in
443 Trp residues, which is also indicated by the red isosurface, shows the repulsion between them. On
444 the CIP side (the left side of **Fig. 5d**), both of the oxygen atoms in the deprotonated carboxyl group
445 and its adjacent carbonyl in the aromatic nucleus together contacted the Ca^{2+} -bridge girder through
446 a blue oval-shaped gradient isosurface, indicating a strong *n*–metal EDA attraction. On the biofilm
447 side (the right side of **Fig. 5d**), a pair of oxygen atoms in a deprotonated carboxyl group of a
448 tryptophan residue were associated with a Ca^{2+} -bridge girder through another blue oval-shaped
449 gradient isosurface, further confirming the presence of another strong *n*–metal EDA interaction.
450 These results suggest that both carbonyl/deprotonated carboxyl groups in FQAs and deprotonated
451 carboxyl groups in Trp residues are collectively responsible for the formation of alkali–earth–metal
452 bridges.

453 **Fig. 5e** shows plots of RDG versus electron density multiplied by the sign of the second Hessian
454 eigenvalue (corresponding to **Fig. 5d**). Three low-gradient spikes, at 0.01–0.04 au, indicated
455 molecular repulsions between a pair of oxygen atoms in the deprotonated carboxyl group of a Trp
456 residue and between the carbon of the carboxyl and its adjacent carbon in the aromatic nucleus of
457 CIP (corresponding to the red color of the gradient isosurfaces in **Fig. 5d**). The spike at -0.018 au
458 revealed the weak vdWs attraction between one of the oxygen atoms in a deprotonated carboxyl

group and its adjacent carbonyl in the CIP molecule (corresponding to the green color of the gradient isosurfaces in **Fig. 5d**). A pair of spikes (-0.03~ -0.06 au) revealed the strong *n*-metal EDA attractions around the alkali-earth-metal bridge girder.

In addition to the weak vdWs attraction in the molecular interiors of CIP and Trp residues, a strong EDA attraction around the central Mg^{2+} girder was also indicated by the visual gradient isosurfaces (**Fig. 5f**). In **Fig. 5g**, an apparent low-gradient spike, which corresponds to the isosurface of an Mg^{2+} bridge, has a more negative value at -0.057 a.u, suggesting a stronger EDA interaction of electronegative oxygen atoms with Mg^{2+} than with Ca^{2+} . This quantitative result showing the greater negativity of $\text{sign}(\chi_2)\rho$ suggests that a stronger EDA interaction via an Mg^{2+} bridge might be better able to detain CIP in the extracellular matrices.

3.5. Dependency on bridging energy

Association constants between FQAs and the biofilm matrix via metals were obtained by a fluorescence micro titration (**Fig. S3**). **Fig. 6a** shows that the growth rate of *E. coli* increased linearly along with the enhancement of the association constant between FQAs and the biofilm matrix, which implies that the metal bridge-enhanced FQA-biofilm association protects against bacterial apoptosis. The slopes were 0.03 for Ca^{2+} -enhanced growth and 0.09 for Mg^{2+} -enhanced growth. The growth rate (the vertical coordinate) followed the following order: $\text{CIP} \approx \text{LOM} > \text{NOR} > \text{LVX} \approx \text{ENX} \approx \text{OFX} > \text{PEF} \approx \text{FLE}$ for Ca^{2+} , and $\text{CIP} \approx \text{NOR} > \text{ENX} > \text{LOM} > \text{OFX} > \text{LVX} > \text{PEF} \approx \text{FLE}$ for Mg^{2+} .

Plots of changes in the Gibbs free energy (ΔG) computed at a major Trp residue site versus the association constant obtained by fluorescence microtitration ($\log K_A$) were used to further explain the dependence of the bridging energy on the alkali-earth-metal bridge (**Fig. 6b**). It shows the computed ΔG values for the listed eight FQ antibiotics binding at a Trp residue via a metal bridge. A

group of strong linear correlations between $\Delta G_{\text{Trp}\cdots\text{Ca-FQAs}}/\Delta G_{\text{Trp}\cdots\text{Mg-FQAs}}$ and the corresponding association constants ($\log K_A$) ($R^2 \geq 0.67$; probability, $p \leq 0.01$) were noted. Importantly, a minus sign in the computed ΔG value arises only if a spontaneous reaction occurs. Thus, the linear correlations suggest that higher $\log K_A$ values (absolute value) correspond to greater ΔG values. With respect to Mg^{2+} bridging, this plot has a large slope (absolute value of 4.7), suggesting that variations in the FQA-Mg-to-biofilm combination may cause a large change in the bridging energy. All bridging energies were greater than 150 kJ/mol, indicating that all active sites experienced a spontaneously coordinated reaction (Gu et al., 1994), which was associated with EDA interactions.

Fig. 6c shows the linear correlations between ΔG and the rate of *E. coli* growth. Bridging energies combined with FQAs (the horizontal axis) followed a descending order (in abscissa), as follows: $\text{FLE} \approx \text{PEF} > \text{LVX} > \text{OFX} > \text{NOR} > \text{ENX} \approx \text{CIP} > \text{LOM}$ for added Ca^{2+} and $\text{FLE} \approx \text{PEF} > \text{LVX} > \text{OFX} \approx \text{LOM} > \text{ENX} > \text{NOR} \approx \text{CIP}$ for added Mg^{2+} . With the decrease in ΔG at major n -metal EDA interactions, growth rates exhibit a clear linear reduction. This finding indicates that a weaker CIP-Ca-biofilm combination may result in less retardation, which acts against the growth of *E. coli*. Additionally, the Ca^{2+} and Mg^{2+} bridges had the same slope (-0.02), suggesting that the growth rates of *E. coli* recovered by these two metals are dependent on the bridging energy at Trp residue in biofilms rather than on the particular metal species.

4. Conclusion

More and more attention has been paid to the challenging issue on environmental contamination of antibiotics in recent years. How microbes regulate the trans-biofilm uptake of FQAs is a fundamental question in environmental chemistry and biology. Traditional research thinks basically that bacterially extracellular biofilm matrices are known as passive defense barrier to impede the invasion of hazardous substance, but not know whether FQA attack may be efficiently defused by a

505 metal-reinforced detoxification mechanism occurring at water-biofilm interface. We recently found
506 that metal bridge formed in biofilm matrices anchors the FQAs, and efficiently defuses the FQA
507 attack through this reinforced detoxification mechanism. Here, we present that a deprotonated
508 carboxyl in the tryptophan residues of biofilms acted as a major bridge site, of which one side is a
509 metal and the other is a metal girder jointly connected to the carboxyl and carbonyl of a FQA.
510 Meanwhile, we further demonstrated that a metal bridge formed in extracellular biofilms acts
511 through an suppressive trans-biofilm transport to protect against bacterial apoptosis. Without this
512 metal bridge, extracellular biofilms exhibit higher permeability, larger antibiotic uptake towards
513 cells, and profoundly defective chemotaxis. Computation regarding bridging energy indicates that
514 this metal bridge is favorable for bacterial survival. This new perspective will enhance the
515 understanding of bacterially antibiotic resistance involved in the field of natural products.

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Figure Captions

676 **Fig. 1.** *In situ* CLSM and corresponding XPS analyses of an alkali–earth–metal bridge formed in *E.*
 677 *coli* biofilms. (a): *E. coli* alone. (b): *E. coli* plus ciprofloxacin (CIP). (c): *E. coli* plus Ca^{2+} and CIP.
 678 (d): *E. coli* plus Mg^{2+} and CIP. XPS analyses of a Ca^{2+} bridge (e) and a Mg^{2+} bridge (f). The density
 679 of *E. coli* was approximately 1.0×10^8 cells/mL. The concentrations of the alkali–earth metals and
 680 CIP were 2.0 mmol/L and 1.25 $\mu\text{mol/L}$, respectively.

681

682 **Fig. 2.** FTIR and ^{13}C NMR spectra of biofilms before and after their association with CIP via
 683 alkali–earth Ca^{2+} and Mg^{2+} bridges. FTIR spectra: (a) biofilm alone; (b) biofilm- Ca^{2+} -CIP; (c)
 684 biofilm- Mg^{2+} -CIP. Black plots represent the experimental data. Blue bar charts and red plots
 685 represent the Lorentz oscillators and infrared (IR) spectra from the computation at the
 686 B3LYP/6-31G(d, p) level with a dispersion-corrected DFT-D3(BJ). Red arrows represent the
 687 absorption bands related to the bridging sites. (d) Experimental ^{13}C -NMR spectra from biofilm,
 688 biofilm- Ca^{2+} -CIP, and biofilm- Mg^{2+} -CIP. After the combination of biofilms with Mg/Ca-CIP, the
 689 two pink arrows represent two marked downfield shifts ($\Delta\delta = 0.2$) of the resonances that are
 690 attributed to the carbon signal of the carboxyl residues in biofilms.

691

692 **Fig. 3.** Extracellular retardation and cell uptake of CIP and its trans-biofilm transport rate (r) in
 693 high- and low-biofilm *E. coli*. (a) Ca^{2+} -affected retardation and uptake. (b) Mg^{2+} -affected
 694 retardation and uptake. (c) Trans-biofilm transport rate of CIP as a model FQA (r). In (a) and (b),
 695 the CIP concentration and cell density were initially 1.25 $\mu\text{mol/L}$ and 1.0×10^8 cells/mL,
 696 respectively. In (c), the trans-biofilm transport rate of CIP (r) was calculated based on
 697 $r = q / (S \times h)$, where S , q , and h are the interfacial area between bacterial cells and the biofilm layer,

698 the cellular uptake of CIP, and the statically suspension time (4 h), respectively. The superficial area
 699 of *E. coli* at the diffusion layer ($S = 2\pi r \times (L + 2\pi r)$) was calculated by randomly selecting 100 *E.*
 700 *coli* cells (diagram at the bottom-left corner of panel (c)). L and r are the length of the rod
 701 (subtraction of rod-head cabochons) and the radius of the rod head, respectively ($\pi = 3.14$). The
 702 diffusion layer was measured by TEM analysis (average value: $4.95 \pm 0.31 \mu\text{m}^2/\text{cell}$). Error bars
 703 represent standard deviations calculated from triplicate samples.

704

705 **Fig. 4.** Association of *E. coli* growth with extracellular retardation and cellular uptake of CIP. After
 706 a static suspension experiment (4 h), a portion of the bacterial culture was analyzed to obtain the
 707 extracellular retardation and cellular uptake of CIP, while another portion was incubated in fresh LB
 708 medium for 18 h to obtain a final *E. coli* density. The data for the extracellular retardation and
 709 cellular uptake of CIP antibiotic in the abscissa of the graphs can be found in Fig. 3a–d. The initial
 710 concentrations of the added alkali–earth metals ranged from 0 to 3.5 mmol/L. Each data point
 711 shown in the figures is the average of three measurements.

712

713 **Fig. 5.** Models of CIP interactions with Trp residues in biofilms. (a) Model obtained by QM/MM
 714 computation of CIP interacting with Trp residues in biofilms via metal bridges. Magnified gradient
 715 isosurfaces (b, d, and f) and the corresponding plots of the reduced density gradient versus the
 716 electron density multiplied by the sign of the second Hessian eigenvalues (c, e, and g). (b) and (c),
 717 CIP-biofilms. (d) and (e), CIP- Ca^{2+} -biofilms. (f) and (g), CIP- Mg^{2+} -biofilms. In (b), (d), and (f), the
 718 gradient isosurfaces are colored on a blue-green-red scale according to the values of $\text{sign}(\lambda_2)\rho$,
 719 which range from -0.08 to 0.08 au. Blue, green, and red indicate the strong attraction of an n -metal
 720 EDA interaction, vdWs interaction, and strong non-bonded overlap, respectively. Here, the sign of

721 $\tilde{\lambda}_2$ is used to distinguish bonded ($\tilde{\lambda}_2 < 0$, such as a π - π bonding) from non-bonded ($\tilde{\lambda}_2 > 0$)
722 interactions (a negative value of $\tilde{\lambda}_2$ represents strong bonding) (Panels (c), (e), and (g)). The lower
723 the negative value of $\tilde{\lambda}_2$, the stronger the attraction in the region. The O, C, H, N, and F in the
724 molecular structures are represented as red, cyan, white, blue, and brown spheres, respectively.
725 Solvent (water) effects were implicitly taken into consideration.

726

727 **Fig. 6.** Correlation of the Trp-Ca²⁺-FQA bridging energy at a major tryptophan (Trp) residue site in
728 biofilms (computational $\Delta G_{\text{Trp}\cdots\text{metal-FQAs}}$) with an association constant (K_A , log-transformed) and the
729 rate of *E. coli* growth. (a) Growth rate (T/T_0) versus $\log K_A$. (b) Computed $\Delta G_{\text{Trp}\cdots\text{metal-FQAs}}$ versus
730 $\log K_A$. (c) Growth rate (T/T_0) versus computed $\Delta G_{\text{Trp}\cdots\text{metal-FQAs}}$. The growth rate of *E. coli* was
731 calculated as the ratio of *E. coli* density in the presence of an alkali–earth–metal bridge (T) to the
732 density in the absence of alkali–earth metals (T_0) (growth ratio = T/T_0) after 18 h of incubation with
733 a constant concentration of FQAs (1.25 $\mu\text{mol/L}$, 37 °C, and 150 rpm). The association constant
734 ($\log K_A$) was obtained by fluorescence microtitration. The Gibbs free energy (ΔG) at the major EDA
735 interaction (Trp...metal-FQ) was computed at the B3LYP/6-31G (d, p) level with a
736 dispersion-corrected DFT-D3 (BJ). The change in ΔG was calculated by the difference between the
737 Trp-metal head and FQ. Solvent (water) effects were implicitly taken into consideration. Each data
738 point shown in panels (a) and (c) is the average of three measurements. R^2 : correlation coefficient; p :
739 probability.

740

Fig. 1.

744

745 **Fig. 2.**

Fig. 4.

754

755 **Fig. 5.**

758 **Fig. 6.**

Graphical Abstract