Environmental Pollution xxx (2016) 1–12

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

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

Fuxing Kang [a], Qian Wang [b], Weijun Shou [a], Chris D. Collins [c], Yanzheng Gao [a, *]

[a] Institute of Organic Contaminant Control and Soil Remediation, College of Resources and Environmental Sciences, Nanjing Agricultural University, Jiangsu 210095, China
[b] State Key Laboratory of Lake Science and Environment, Nanjing Institute of Geography and Limnology, Chinese Academy of Sciences, Jiangsu 210008, China
[c] Soil Research Centre, University of Reading, Whiteknights, Reading RG6 6DW, UK

ARTICLE INFO

Article history:
Received 6 July 2016
Received in revised form
6 September 2016
Accepted 11 September 2016
Available online xxx

Keywords:
Fluoroquinolone antibiotics
Biofilms
Alkali–earth metals
Bridging energy
Computational chemistry
Escherichia coli

ABSTRACT

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

1. Introduction

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

Fluoroquinolone antibiotics (FQAs), which comprise an important and hard-degradable class of synthetic pharmaceuticals, have been widely used and have been introduced into the environment by a multitude of human and veterinary activities over the last 30 years in Europe and the United States (Baquero et al., 2008; Mompelat et al., 2009). Bacteria generally hide into a biofilm matrix to deal with the presence of these synthetic antibiotics (Wingender et al., 2012). One component of the so-called bacterial one-two punch strategy is to use an extracellular biofilm permeability barrier to impede the cellular uptake of antibiotic stressors. Such physical defenses in response to antibiotics might be

☆ This paper has been recommended for acceptance by Baoshan Xing.

* Corresponding author.
E-mail addresses: gaoyanzheng@njau.edu.cn, gaosoil@163.com (Y. Gao).

http://dx.doi.org/10.1016/j.envpol.2016.09.029
0269-7491/

inefficient and energetically very costly (Martínez et al., 2007; Rice, 2006). For example, previous studies have shown that sorption of FQAs to oxygen- and nitrogen-containing dissolved organic matter (DOM) displays a nonlinear saturation pattern (with a Freundlich exponent $n = 0.40–0.51$) (Zhang et al., 2012), whereas their affinity to phospholipids exhibits a linear partitioning pattern (partitioning coefficient = 20.0–79.4 L/kg) (Merino et al., 2002, 2003). These findings implies that biofilms containing similar oxygen and nitrogen groups have a relatively low capacity (Walter, 2012) to absorb FQAs and may result in high intracellular uptake due to the low-efficiency of extracellular FQA interception. Furthermore, extracellular accretions that act as entrapment agents will be energetically very costly, considering the demand for major saccharide and protein moieties during the formation of biofilms. Nevertheless, little information is currently available to determine how bacteria efficiently and economically deal with such stressors; this issue requires proper investigation.

A positively charged metal bridging connection between electronegative biofilms and negatively charged FQAs (i.e., their functional groups) may be the other component of the one-two punch strategy for defending against antibiotic attack. Given the strong electrostatic attraction between electronegative biofilms/FQAs and accessible alkali–earth metals, the formation of a stable structure would anchor the FQAs in biofilms via a metal bridge. Whereas one side of the metal-to-FQA connection (metal–carbonyl interaction) is supposedly understood (Aristilde and Sposito, 2008), attractions between metals and extracellular biofilm components on the other side are more multifarious and complicated. Thus, a quantitative description of an alkali–earth–metal bridge based on these weak attractions is crucial for quantitatively expounding the association of such weak interactions at the molecular level. Generally, on the basis of spectroscopic data, it is considered that alcohol, carboxyl, phosphoric, and amino-acid residue groups in biofilm matrices may be involved in interactions with such metals as Zn^{2+}, Co^{2+}, and Ca^{2+} (Ha et al., 2010; Sun et al., 2009; Sundararajan et al., 2011; Xiong et al., 2002). These results only suggest that biofilms may utilize their electronegative groups to interact with metals, but not know whether antibiotic attack 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–Ca^{2+}/Mg^{2+} 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 ^{13}C nuclear magnetic resonance (^{13}C 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 flerofloxacin (FLE). Their physicochemical properties, including molecular weight (MW), the dissociation constant for carboxyl groups ($pK_{a\text{-}COOH}$), and the octanol–water partition coefficient ($\log K_{ow}$), are listed in **Table S1**. Magnesium sulfate ($MgSO_4 \cdot 7H_2O$, ≥99.0%) and calcium sulfate ($CaSO_4$, ≥99.0%) were purchased from Sinopharm Chemical Reagent Co., Ltd, China. Milli-Q water (18.2 MΩ × cm, Millipore, USA) was used for all experiments.

Other chemicals (as listed below, purity > 99.0%) that were purchased from Nanjing Chemical Reagent Co., Ltd. were used to prepare modified chloride-free culture medium (Kang et al., 2014). 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 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 g/L), and a trace element solution (10 mL/L). The trace element solution contained $Na_2EDTA \cdot H_2O$ (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 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 to 7.4 with sulfuric acid (98.0%). When necessary, solid agar medium was prepared with 1 L of chloride-free medium and 15 g of agar powder. The sulfuric acid (0.01 mmol/L H_2SO_4) and sodium hydroxide (0.02 mmol/L NaOH), used for pH adjustment in batch experiment and fluorescence microtitration, were purchased from Nanjing Chemical Reagent Co., Ltd. (Nanshi, China).

2.2. Biofilm manipulation

Our previous method was used to manipulate the extracellular biofilms (Fang et al., 2002; Kang et al., 2014; Liu and Fang, 2002). In brief, *Escherichia coli* (DH5α) was initially cultured in 20 mL of chloride-free medium at 37 °C for a 12-h recovery of growth. The bacterial suspension (5 mL) was then transferred to fresh chloride-free medium (1.0×10^3 mL) and grown for an additional 48 h to reach the stable growth phase. *E. coli* cells were collected by low-speed centrifugation (3×10^3 *g*, 6 min, 4 °C) followed by washing with Milli-Q water to obtain pure, pristine *E. coli* (high-biofilm *E. coli*).

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

Please cite this article in press as: Kang, F., et al., Alkali–earth metal bridges formed in biofilm matrices regulate the uptake of fluoroquinolone antibiotics and protect against bacterial apoptosis, Environmental Pollution (2016), http://dx.doi.org/10.1016/j.envpol.2016.09.029