An Ionic Liquid Facilitates the Proliferation of Antibiotic Resistance Genes Mediated by Class I Integrons

Yi Luo,† Qing Wang,† Qian Lu,† Quanhua Mu,† and Daqing Mao*,‡

†College of Environmental Science and Engineering, Ministry of Education Key Laboratory of Pollution Processes and Environmental Criteria, Nankai University, Tianjin 300071, China
‡School of Environmental Science and Engineering, Tianjin University, Tianjin 300072, China

ABSTRACT: Ionic liquids (ILs), as “environmentally friendly” replacements for industrial volatile organic solvents, have been widely and recently applied in the chemical industry. However, few data have been collected regarding the toxicity and potential environmental effects of ILs, which are fairly important for preparing for their potential release into the environment. In this study, the IL 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIm][PF6]) was tested for its ability to promote the proliferation and dissemination of antibiotic resistance genes (ARGs) in environmental bacteria. In freshwater microcosms, [BMIm][PF6] (0.5 g/L) significantly enhanced the abundance of the sulI gene (500-fold greater than in untreated controls). Meanwhile, [BMIm][PF6] significantly increased the abundance of class I integrons, which play a key role in ARG dissemination. A positive correlation ($p < 0.01$) between the intI1 and sulI genes suggested that [BMIm][PF6]-facilitated sulI propagation was mediated by class I integrons. This idea was supported by sequencing, which showed the sulI locus in the $3'$ region of class I integrons. Class I integron transfer experiments between different indigenous strains of Alcaligenes sp. (SMX$^3$) and Acinetobacter sp. (Stir$^2$) were conducted to show that the horizontal transfer frequency of class I integrons was up to 88-fold higher in the presence of an IL. An IL increased cell membrane permeability as evidenced by flow cytometry, thereby assisting sulI gene transfer mediated by class I integrons. This is the first report that ILs facilitate the proliferation of ARGs in environmental bacteria and thus increase risks to public health.

INTRODUCTION

Widespread antibiotic resistance and antibiotic resistance genes (ARGs) can pose serious health risks.1−3 The selective pressure generated by the use of antibiotics is now considered the major factor responsible for the emergence, persistence, and propagation of ARGs.4,5 However, recent studies have consistently demonstrated elevated levels of ARGs in the absence of antibiotic use.6−7 Additionally, researchers have found that reagents other than antibiotics, such as metals,8−10 detergents,11 pesticides,12 and even nanomaterials,13 can promote the transmission of ARGs.14,15 Generally, ARGs are located on mobile genetic elements such as plasmids, transposons, and integrons. Integrons are particularly adapted, as “genetic platforms”, to transfer and disseminate antibiotic resistance by capturing and integrating one or more gene cassettes and converting them into functionally expressed genes to permit rapid acquisition of diverse resistance genes.15

Ionic liquids (ILs) are organic salts with low melting points and excellent chemical and thermal stability that are being considered as “environmentally friendly” replacements for industrial volatile organic compounds.16−18 As novel materials, ILs have the potential to be widely applied in the energy industry,19 in chemical and electrochemical industries,20 for pharmaceutical extractions,21 and in other applications. Some other applications of ILs include the dimerization of butenes and their use as liquid pistons and as storage media for hazardous gases and dye-sensitized solar cells.22 ILs have been commercially applied to industrial processes (BASIL by BASF) since 2002. The most common ILs are manufactured on a multiton scale.23,24 There has not yet been any report of ILs in the environment,25 however, to prepare for the possible environmental release of ILs, recent efforts have been directed toward better understanding IL toxicity, biodegradability, fate, and potential environmental effects.18,26 ILs (quaternary ammonium and pyridinium compounds) have critical inhibitory effects on the growth of a variety of bacteria and fungi,27−30 and ILs alter the physiological and respiratory activities of microorganisms.31 Specifically, ILs can contribute to cell membrane disruption, cause uneven cell wall structure, and alter cell membrane composition by attacking lipid structures.18,32 Most interestingly, the isolated strain Enterobacter...
lignolyticus, which is resistant to an IL [1-ethyl-3-methylimidazolium chloride ([C₆mim][Cl])] showed altered cell membrane composition, including an increased number of cyclopropane fatty acids. Bacterial cell membranes constitute an important barrier against the horizontal transfer of genetic information between different species or genera of bacteria. Until now, the question of whether ILs promote horizontal gene transfer among environmental bacteria and facilitate the transmission and proliferation of ARGs has remained unanswered.

In this study, an IL was tested for its potential to affect the selection and dissemination of ARGs and their related class I integrons. The horizontal transfer of class I integrons between indigenous bacteria isolated from freshwater microcosms was examined to test the hypothesis that the common IL 1-butyl-3-methylimidazolium hexafluorophosphate ([BMI][PF6]) facilitates class I integron-mediated sulI gene propagation. Meanwhile, evidence of altered cell membrane permeability as shown by flow cytometry (FCM) was observed as a possible cause of IL-facilitated sulI gene proliferation. To the best of our knowledge, this is the first study to test if and how ILs promote the dissemination of ARGs in the environment.

## MATERIALS AND METHODS

### Setup of Freshwater Microcosms. The freshwater microcosm experiment based on the OECD 308 test was used to explore the effect of the IL [BMI][PF6] on the transmission and proliferation of ARGs. The water sample was collected from the Water Park in Tianjin, China, in September 2013. The water properties are described in the Supporting Information (Table S1). The microcosms were spiked with the IL [BMI][PF6] (>99% pure, Chinese Academy of Sciences), resulting in initial concentrations of 0, 0.001, 0.01, 0.1, 0.5, and 2.5 g/L. Each concentration was set up in triplicate in 500 mL flasks. All treatments lasted for 14 days at 28 °C. Cycloheximide (>95% pure, 100 mg/L) was added to all the microcosm treatments (each treatment was in triplicate) to prevent fungal growth (for further details, please see SI-1 of the Supporting Information). Periodic triplicate samples of each treatment were taken at 0, 2, 6, 10, and 14 days. Samples were stored at ~20 °C until DNA was extracted.

### DNA Extraction and Quantitative Polymerase Chain Reaction (qPCR). Samples (6 mL) from each treatment were centrifuged, and DNA extraction was performed using an E.Z.N.A. Bacterial DNA Kit according to the manufacturer’s instructions (OMEGA). The extracted DNA was further purified using a DNA Pure-Spin Kit (Vigorousbio, Beijing, China) to minimize PCR inhibition. qPCR assays were conducted in a Biometra T100 gradient cycler (Biometra), and qPCR analyses were performed on a Bio-Rad iQ5 instrument to quantify the bacterial 16S rRNA, intI, sulI, and sulII genes. The 16S rRNA, intI, sulI, and sulII primers, and details of PCR and qPCR procedures (including standard curves) are listed in the Supporting Information (SI-2 and SI-3) and in Tables S2 and S4.

### Isolation of intI-Positive Bacteria. Selective plates containing 100 mg/L sulfamethoxazole were used to isolate intI-positive strains from samples. Isolated single colonies were incubated in liquid LB medium, and plasmids were extracted using a plasmid extraction kit (Omega) according to the manufacturer’s instructions. The presence of intI and sulI genes was verified by PCR and DNA sequencing as detailed previously. Furthermore, gene cassettes and the 3’ regions of class I integrons were amplified with appropriate primer pairs (Table S2 of the Supporting Information) and verified by sequencing.

### Class I Integron Transfer Experiments. Two isolates, namely, an intI-positive bacterium (carrying sulfamethoxazole resistance but no streptomycin resistance, SMXR) and an intI-negative bacterium (carrying streptomycin resistance but no sulfamethoxazole resistance, StR), both of which were indigenous bacteria isolated from a water sample taken at the Water Park in Tianjin, were treated as the donor and the recipient, respectively, of class I integrone transfer experiments in [BMI][PF6]-treated microcosms. The class I integrons (BLAST with the intI gene, 100% identity, 145 matches with Klebsiella pneumoniae plasmid pRMH760 [GenBank entry KF976462.2]), sulI genes (100% identity, 787 matches with the plasmid mentioned above), and the linkage region between sulI genes and the 3’ region of class I integrons (99% identity, 767 matches with the plasmid mentioned above) were identified in donor bacteria by DNA sequencing, which confirmed that sulI genes were located on class I integrons in the donors. The donors and recipients in the class I integrone transfer experiments were Alkaligenes sp. and Acinetobacter sp., respectively, according to 16S rRNA sequence alignment (99% identity, 1466 matches).

The different characteristics of the bacterial colonies on LB plates were a white color (opaque) for the recipient and no color (translucent) for the donor. The morphological differences combined with 16S rRNA sequencing were used to identify the donor and recipient strains. After mating for 12 h, the recipients (SMXR and StR) were counted by the plate count method with LB plates (containing 100 mg/L sulfamethoxazole and 30 mg/L streptomycin). Meanwhile, the donor and recipient strains were separately plated onto identical LB plates as negative controls to rule out the spontaneous mutation of the donor and recipient bacteria. The results are presented as colony-forming units per milliliter of culture (cfu/mL). PCR and DNA sequencing were used to verify whether the class I integrons had been transferred into the recipient strain. For further details, see SI-5 of the Supporting Information.

### Flow Cytometry Detection of Cell Membrane Permeability Induced by [BMI][PF6]. To explore the mechanisms by which ionic liquids stimulate horizontal gene transfer, flow cytometry (FCM) (BD FACSCalibur) was employed to differentiate and quantify the [BMI][PF6]-exposed bacterial cells and control cells. In these experiments, the fluorescence intensity was a function of cell membrane permeability, with a higher fluorescence intensity signifying enhanced cell membrane permeability. The FCM instrument was equipped with an excitation wavelength of 488 nm. One milliliter of bacterial suspension ([BMI][PF6]-treated or control) was stained with 10 μL of propidium iodide (PI, 1 mg/mL) (OMEGA) and incubated in the dark for 8 min before measurement. The concentration of the bacterial suspension was always <10⁶ cells/mL. All data were processed with CellQuest Pro.

### RESULTS AND DISCUSSION

[BMIm][PF6] Promotes Increased Levels of sulI and intI Genes in Freshwater Microcosms. The presence of [BMI][PF6] (0.001–0.5 g/L) significantly enhanced the abundance of the sulI and intI genes by 2–3 orders of magnitude ([5.45 ± 0.55] × 10⁷ and (1.51 ± 0.04) × 10⁸ copies/mL of water, respectively) versus that of the untreated controls ([1.07 ± 0.31] × 10⁶ and (2.72 ± 0.11) × 10⁶ copies/mL) and proliferation of ARGs has remained unanswered.
Student groups and the control group (0 g/L group) were tested with the ratio of various ARGs and are particularly adapted to the microcosm were primarily attributable to facilitation by abundance (Figure S2 of the Supporting Information), suggesting that different mechanisms may exist for gene maintenance and amplification.

**[BMIm][PF6] Facilitates the Horizontal Transfer of Class I Integrons.** The horizontal transfer frequency was increased up to 88-fold [(8.38 ± 0.43) × 10^{-5} per recipient cell] in 0.5 g/L [BMIm][PF6]-treated cells (Figure 2) versus untreated cells [(9.57 ± 0.79) × 10^{-6} per recipient cell]. The observed transfer frequency facilitated by IL is lower than that facilitated by 0.5 g/L nanolamina, which was reported to increase the rate of horizontal transfer of the RP4 plasmid from *Escherichia coli* to *Salmonella* spp. by up to 200-fold compared with that of untreated cells.13 However, the value for 0.5 g/L [BMIm][PF6] is higher than that of 0.16 g/L VCl3, which was used in previous studies to promote horizontal transfer from marine *Photobacterium* to *E. coli* by protoplast transformation.10 

The observed transfer frequency facilitated by IL is lower than that facilitated by 0.5 g/L nanolamina, which was reported to increase the rate of horizontal transfer of the RP4 plasmid from *Escherichia coli* to *Salmonella* spp. by up to 200-fold compared with that of untreated cells.13 However, the value for 0.5 g/L [BMIm][PF6] is higher than that of 0.16 g/L VCl3, which was used in previous studies to promote horizontal transfer from marine *Photobacterium* to *E. coli* by protoplast transformation.10 

Although the abundance of the *sull* gene also increased with an increase in the [BMIm][PF6] concentration [(1.17 ± 0.51) × 10^6 copies/mL of water], it was only 22-fold higher than controls [(5.29 ± 0.43) × 10^6 copies/mL of water] (Figure 1), suggesting that different mechanisms may exist for *sull* and *sullI* gene maintenance and amplification.

**[BMIm][PF6]-Facilitated sull Propagation Is Mediated by Class I Integrons.** The changes observed for *sull* gene abundance with different concentrations of [BMIm][PF6] followed a trend similar to that for the *intI* gene. A positive correlation (p < 0.01) was found between *sull* and *intI* gene abundance (Figure S2 of the Supporting Information), implying that the proliferation and propagation of *sull* within the microcosm were primarily attributable to facilitation by class I integrons. It has been reported that class I integrons, as a “genetic platform”, can trigger the transfer and/or incorporation of various ARGs and are particularly adapted to the transfer and dissemination of antibiotic resistance.25,26 The *sull* genes were then verified to be linked to the 3’ regions of class I integrons in isolated strains by DNA sequencing. The sequenced *sull* genes had a DNA sequence that was 100% identical (787 bp matches) to that of the *sull* gene carried by *K. pneumonias* plasmid pRMH760 (GenBank entry KP976462.2). These data confirm that [BMIm][PF6]-facilitated *sull* gene propagation is mediated by class I integrons. In contrast, no correlation (p = 0.128) was found between the abundance of the *sullI* gene and that of the *intI* gene, implying that *sullI* gene propagation and proliferation may not be mediated by class I integrons.

**Figure 1.** Absolute abundances of the 16S rRNA, *intI*, *sull*, and *sullI* genes in freshwater microcosms treated with [BMIm][PF6] for 10 days. The concentration of [BMIm][PF6] had a significant effect on the absolute abundances of the *intI*, *sull*, and *sullI* genes (analysis of variance; p < 0.05), but it did not have a significant effect on the absolute abundance of the 16S rRNA gene (except for the 2.5 g/L group). Significant differences between individual concentration groups and the control group (0 g/L group) were tested with the Student–Newman–Keuls (S–N–K) test. *p < 0.05, and **p < 0.01.

**Figure 2.** Effect of [BMIm][PF6] on the donors (SMX^8), recipients (St^8), and recipients (SMX^8 and Str^8) of class I integron transfer between the donor intI-positive Alcaligenes sp. and the recipient *Acinetobacter* sp. mated for 12 h at pH 7.0 and 28 °C. The concentration of [BMIm][PF6] had a significant effect on the recipients (SMX^8 and Str^8) (analysis of variance; p < 0.05); however, the concentration of [BMIm][PF6] did not have a significant effect on the donors or recipients alone (except in the 2.5 g/L group). Significant differences between individual concentration groups and the control group (0 g/L group) were tested with the Student–Newman–Keuls (S–N–K) test. *p < 0.05, and **p < 0.01.
facilitate class I integron-assisted horizontal transfer of genetic information by increasing the permeability of the bacterial cell membrane. Previous work also verified that modification of the cell membrane composition in an Enterobacter lignolyticus strain makes the bacterium resistant to 1-ethyl-3-methylimidazolium chloride (an IL) by increasing the level of cyclopropane fatty acids in the cell membrane.32

The lower horizontal transfer frequency [(4.11 ± 0.63) × 10^-2] per recipient cell] facilitated by 2.5 g/L [BMIm][PF6] may be attributed to the toxic effect of very high concentrations of [BMIm][PF6] to microorganisms. This idea was supported by the coincident inhibition of bacterial abundance (or growth) of both donor and recipient strains (Figure 2). The acquisition of antibiotic resistance determinants by horizontal gene transfer plays a major role in the persistence and propagation of antibiotic resistance from exogenous bacteria to indigenous bacteria and among indigenous species.14,40 Among the novel selective pressures faced by environmental bacterial populations, the discharge of heavy metals, xenobiotic compounds, antibiotics, and organic solvents can have a strong effect on the environmental selection of ARGs.31 In this study, IL exerts a selective pressure to facilitate the spread of antibiotic resistance through horizontal gene transfer among indigenous species, promoting increasing levels of the sulI and intI genes. Unlike antibiotics, ILs are not subject to degradation and thus can represent a continuous selective pressure for the horizontal transfer of antibiotic resistance. This study shows that ILs significantly facilitate the horizontal transfer of class I integrons from intI-positive bacteria to other bacterial strains, contributing to ARG transfer between environmentally indigenous bacteria and therefore posing great risks to public health. These findings suggest that ILs proposed for use in industrial processes should be carefully evaluated for their ecological and environmental risks before they are discharged into the environment.

■ ASSOCIATED CONTENT

Supporting Information

PCR conditions and primers, standard curves, statistical analysis, freshwater properties of samples, class I integron transfer of intI-positive bacterium, and the effect of [BMIm]-[PF6] concentration on class I integron transfer. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
*E-mail: mao@tju.edu.cn. Phone: +86 (22) 87402072.

Author Contributions
Y.L. and Q.W. contributed equally to this work.

Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (Grants 21277075, 21037002, and 31270542) and The Ministry of education program for New Century Excellent Talents (NCET-11-0254).

■ REFERENCES


Figure 3. Cell membrane permeability of untreated cells (0 g/L) and [BMIm][PF6]-treated cells (0.5 g/L) as quantified using flow cytometry. Quadrant D: negative signal (normal cells). Quadrant C: PI-positive cells (with increased cell membrane permeability).


