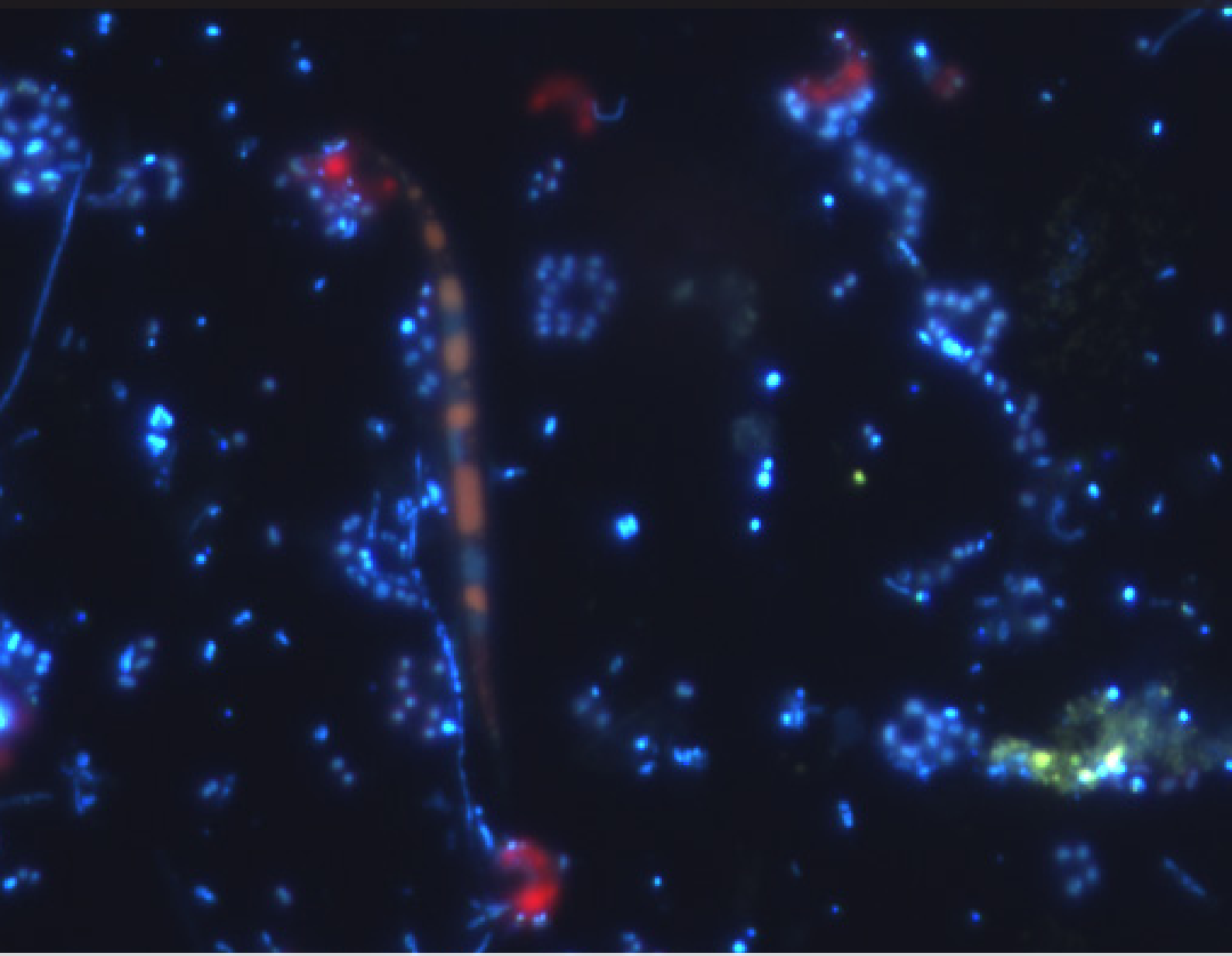
ANTIBIOTIC RESISTANCE IN AQUATIC SYSTEMS

EDITED BY: Satoru Suzuki, Amy Pruden, Marko Virta and Tong Zhang
PUBLISHED IN: Frontiers in Microbiology



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88945-131-9
DOI 10.3389/978-2-88945-131-9

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

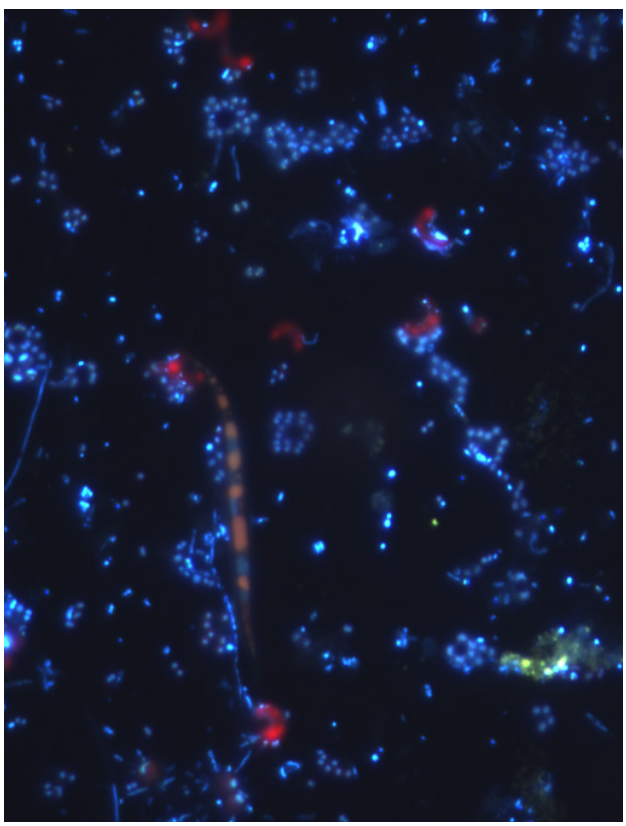
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: **researchtopics@frontiersin.org**

ANTIBIOTIC RESISTANCE IN AQUATIC SYSTEMS

Topic Editors:
Satoru Suzuki, Ehime University, Japan
Amy Pruden, Virginia Tech, USA
Marko Virta, University of Helsinki, Finland
Tong Zhang, The University of Hong Kong, Hong Kong



Cover image by Dr. Satoru Suzuki, Ehime University.

Rivers, lakes and the ocean receive antibiotic resistance genes from human environments. The aquatic environments are a huge reservoir and exchange stage of antibiotic resistance genes.

Citation: Suzuki, S., Pruden, A., Virta, M., Zhang, T., eds. (2017). Antibiotic Resistance in Aquatic Systems. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-131-9

Table of Contents

04 ***Editorial: Antibiotic resistance in aquatic systems***
Satoru Suzuki, Amy Pruden, Marko Virta and Tong Zhang

Chapter 1. Reservoirs

07 ***Detection of multi-drug resistant* Escherichia coli *in the urban waterways of Milwaukee, WI***
Anthony D. Kappell, Maxwell S. DeNies, Neha H. Ahuja, Nathan A. Ledeboer, Ryan J. Newton and Krassimira R. Hristova

19 ***Diverse and abundant multi-drug resistant* E. coli *in Matang mangrove estuaries, Malaysia***
Aziz Ghaderpour, Wing Sze Ho, Li-Lee Chew, Chui Wei Bong, Ving Ching Chong, Kwai-Lin Thong and Lay Ching Chai

32 ***Seawater is a reservoir of multi-resistant* Escherichia coli*, including strains hosting plasmid-mediated quinolones resistance and extended-spectrum beta-lactamases genes***
Marta S. Alves, Anabela Pereira, Susana M. Araújo, Bruno B. Castro, António C. M. Correia and Isabel Henriques

42 ***Sulfonamide and tetracycline resistance genes in total- and culturable-bacterial assemblages in South African aquatic environments***
Satoru Suzuki, Mitsuko Ogo, Tatsuya Koike, Hideshige Takada and Brent Newman

Chapter 2. Horizontal gene transfer

50 ***Survival of antibiotic resistant bacteria and horizontal gene transfer control antibiotic resistance gene content in anaerobic digesters***
Jennifer H. Miller, John T. Novak, William R. Knocke and Amy Pruden

61 ***Various pAQU plasmids possibly contribute to disseminate tetracycline resistance gene* tet*(M) among marine bacterial community***
Lisa Nonaka, Fumito Maruyama, Yuki Onishi, Takeshi Kobayashi, Yoshitoshi Ogura, Tetsuya Hayashi, Satoru Suzuki and Michiaki Masuda

73 ***Insight into the mobilome of* Aeromonas *strains***
Marta Piotrowska and Magdalena Popowska

Chapter 3. Resistance enhancement

89 ***The horizontal transfer of antibiotic resistance genes is enhanced by ionic liquid with different structure of varying alkyl chain length***
Qing Wang, Qian Lu, Daqing Mao, Yuxiao Cui and Yi Luo

97 ***The impact of triclosan on the spread of antibiotic resistance in the environment***
Daniel E. Carey and Patrick J. McNamara

frontiers in Microbiology

EDITORIAL
published: 25 January 2017
doi: 10.3389/fmicb.2017.00014

Editorial: Antibiotic Resistance in Aquatic Systems

Satoru Suzuki[1]*, *Amy Pruden*[2], *Marko Virta*[3] and *Tong Zhang*[4]

[1] Center for Marine Environmental Studies, Ehime University, Matsuyama, Japan, [2] Department of Civil and Environmental Engineering, Virginia Tech, Blacksburg, VA, USA, [3] Department of Food and Environmental Sciences, University of Helsinki, Helsinki, Finland, [4] Department of Civil Engineering, The University of Hong Kong, Hong Kong, Hong Kong

Keywords: antibiotic resistance, aquatic systems, wastewater treatment plant, aquaculture, horizontal gene transfer

Editorial on the Research Topic

Antibiotic Resistance in Aquatic Systems

The spread of antibiotic-resistant pathogens and their resistance traits is an epic global challenge, as recognized by various international bodies, including the G8 Science Ministry in 2013 and the Elmau summit in 2015 (https://www.g7germany.de/Webs/G7/EN/Home_en/home_node.html). While most attention continues to be devoted to the clinic and the need to develop new drugs, there is growing recognition of the need to understand the origin and ecology of antibiotic resistance in order to slow its spread and maximize the lifespan of our antibiotic arsenal (Wright, 2010). In particular, the aquatic environment can serve both as a natural reservoir of antibiotic resistance and a conduit for the spread of clinical resistance traits of major concern (Michael et al., 2013). Aquatic bodies, including lakes, rivers, streams and even coastlines, receive effluent from wastewater treatment plants (WWTP), runoff from agricultural activity, and other human inputs and influences that may either serve to elevate natural background levels of antibiotic resistance genes (ARGs) and stimulate their transfer into pathogens or other organisms, or as a conduit for the propagation of antibiotic resistant pathogens and clinical ARGs of concern (Michael et al., 2013). Current pathogen risk models are not equipped to factor in unique challenges that antibiotic resistance poses, including the potential that non-pathogenic antibiotic resistant bacteria (ARB) can serve as a reservoir to transfer their ARGs to pathogens or the role of selective agents, such as antibiotics and metals, in amplifying this potential. The challenge of controlling the spread of antibiotic resistance can have a different face in developed or developing countries, depending on local policies, practices, technologies and constraints.

The articles in this e-book include new evidences of the origin, spread, and fate of ARB and ARGs in aquatic systems, focusing on water systems from wastewater, freshwater to seawater. These various media have gained attention as potential sources, sinks, or conduits in the potential to spread antibiotic resistance.

It is well-known that heavy use of antibiotics and synthetic antimicrobial agents contribute to the selection pressure. The fate and impact of antibiotics used both in humans and livestock are of particular concern. Municipal and on-farm wastewater treatment is critical for controlling pollution and pathogens, but is not tailored specifically to the control of antibiotic resistance. WWTPs are mainly aimed at reducing solid and nutrient loads to surface waters, but this does not guarantee biodegradation of trace chemical pollutants or genetic elements. Although advanced disinfection facilities can greatly reduce the danger of waterborne diseases (United States Environmental Protection and Agency, 2004), antibiotics and ARGs can still be released to the environment in disinfected effluents (Michael et al., 2013; Rizzo et al., 2013; Berkner et al., 2014; Carey and McNamara). Recent works report the improvement of disinfection in terms of ARG

Edited by:
David W. Graham, Newcastle University, UK

Reviewed by:
Jose Luis Balcazar, Catalan Institute for Water Research, Spain
Eddie Cytryn, Agricultural Research Organization, Volcani Center, Israel
Maurizio Labbate, University of Technology Sydney, Australia

***Correspondence:**
Satoru Suzuki
ssuzuki@ehime-u.ac.jp

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *07 October 2016*
Accepted: *04 January 2017*
Published: *25 January 2017*

Citation:
Suzuki S, Pruden A, Virta M and Zhang T (2017) Editorial: Antibiotic Resistance in Aquatic Systems. Front. Microbiol. 8:14. doi: 10.3389/fmicb.2017.00014

removal (Munir et al., 2011; McKinney and Pruden, 2012; Guo et al., 2013; Yuan et al., 2015; Zhuang et al., 2015).

The potential for horizontal gene transfer (HGT) in WWTPs is a matter of importance, and there is debate regarding the favorable conditions and the actual HGT rates. In this e-book, Miller et al. explore the extent to which influent ARB and ARG composition in raw sludge influences the fate of ARB in the digested sludge community and potential for ARG transfer. Enhancement of HGT by the ionic liquid (IL) 1-butyl-3-methylimidazolium hexafluorophosphate showed that longer carbon chain enhanced HGT (Wang et al.). IL has been thought as an environmentally-friendly solvent, but recently water solubility, environmental toxicity and stability of IL are considered to be risk (Pham et al., 2010). The enhancement of HGT is a new effect of IL. Vanadium is also reported to have enhancement effect of HGT in environment (Suzuki et al., 2012). Such studies demonstrate that, besides antibiotics, other chemicals enhancing HGT could play a role to spread ARGs in water environments, including between pathogenic bacteria and environmental bacteria. In case of coastal sea, although the contaminated waters are significantly diluted, fecal-derived bacteria, such as *E. coli*, remain viable ARG reservoirs of concern (Alves et al.; Kappell et al.; Ghaderpour et al.). ARB and ARGs are known to occur in aquatic environments without antibiotic contaminations (Port et al., 2014). ARBs and ARGs flow into rivers, groundwater and marine environments by influx of WWTP effluent as mentioned above. Additionally, there is marked potential aquaculture practices (Tamminen et al., 2011) and open ocean conditions (Rahman et al., 2008) to contribute to the selection and spread of ARBs and ARGs. Mobile genetic elements (MGEs) of aquatic species conveying multidrug resistance genes are reported in this book (Nonaka et al.). These elements might pose a risk to human health if the MGEs transfer to other bacteria (Piotrowska and Popowska), especially human pathogens. Thus, this topic highlights insight obtained from various water environments and their interfaces.

The role of antibiotics and antimicrobials in ARB selection is of keen interest and evidence suggests that even very low concentration of antibiotics can be effective to select and maintain resistance traits in bacteria (Gullberg et al., 2011). Marine bacterial plasmids pAQUs (Nonaka et al.) appear to disseminate ARGs among marine organisms, which can be stably retained in the bacterial community, even after the antibiotics are removed (Bien et al., 2015). Exogenous ARGs retained in the environment can potentially be horizontally transferred among the native bacterial community.

It is well-known that the majority of aquatic environmental bacteria are unculturable or yet-to-be cultured (Amann et al., 1995; Takami et al., 1997; Bloomfield et al., 1998). This characteristic is distinct from that of human and animal pathogens, which have been the subject of the development of standard methods for isolation and monitoring. Consequently, conventional culture-dependent methods for monitoring ARBs and ARGs only reveal 0.1% or less of the true aquatic bacterial community (Amann et al., 1995; Takami et al., 1997; Bloomfield et al., 1998). Suzuki et al. (2013) and Suzuki et al. found that the defining features of ARGs tend to be distinct between culturable and yet-to-be cultured bacteria. The recent advent of next-generation DNA sequencing and metagenomics poises the scientific community on the verge of a major advance in understanding the behavior of ARGs in the environment (D'Costa et al., 2006; Port et al., 2014). However, deep sequencing and the capacity to perform high-throughput bioinformatics analyses are required to match ARGs with their bacterial hosts. Thus, there is still a long way to go before we have full understanding of the factors driving the behavior of ARGs in aquatic realm.

Articles in this e-book provide new insight into the dynamics of ARGs in a diverse range of aquatic environments and their interfaces, including distribution and diversity of ARGs in various countries, horizontal gene transfer, and the dynamic processes involved in governing ARB and ARG fate. These papers should expand our knowledge and understanding of connections between resistance traits in human- and animal-related bacteria and aquatic ecosystems.

Although this e-book does not include, gene flux among various aquatic compartments, such as sediments, biofilms and periphyton, should be paid attention. ARGs are exchanged between organisms, but it does not drive mass movement and local exposures. As an example, movement of ARGs is reported between water column and biofilms (Engemann et al., 2008). The geological wide flux of ARGs is occurred by natural water movement (Knapp et al., 2012), and water use and food are suspected also as ARGs transport factors (Suzuki and Hoa, 2012). The study from global ecological viewpoint is needed to reveal the ARGs fate and dynamics in environments.

AUTHOR CONTRIBUTIONS

SS: Planning, manuscript editing and making editorial manuscript. AP: Manuscript editing and making editorial manuscript. MV: Manuscript editing. TZ: Manuscript editing and reviewing editorial manuscript.

FUNDING

This work is partly supported by Grant-in-Aid KAKENHI 25257402 and 22241014.

REFERENCES

Amann, R. I., Ludwig, W., and Schleifer, K. H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev*. 59, 143–169.

Berkner, S., Konradi, S., and Schönfeld, J. (2014). Antibiotic resistance and the environment - there and back again. *EMBO Rep*. 15, 740–744. doi: 10.15252/embr.201438978

Bien, T. L., Sato-Takabe, Y., Ogo, M., Usui, M., and Suzuki, S. (2015). Persistence of multi-drug resistance plasmids in sterile water under very low concentrations of tetracycline. *Microbes Environ*. 30, 339–343. doi: 10.1264/jsme2.ME15122

Bloomfield, S. F., Stewart, G. S., Dodd, C. E., Booth, I. R., and Power, E. G. (1998). The viable but non-culturable phenomenon explained? *Microbiology* 144, 1–3.

D'Costa, V. M., McGrann, K. M., Hughes, D. W., and Wright, G. D. (2006). Sampling the antibiotic resistome. *Science* 311, 374–377. doi: 10.1126/science.1120800

Engemann, C. A., Keen, P. L., Knapp, C. W., Hall, K. J., and Graham, D. W. (2008). Fate of tetracycline resistance genes in aquatic systems: migration from the water column to peripheral biofilms. *Environ. Sci. Technol.* 42, 5131–5136. doi: 10.1021/es800238e

Gullberg, E., Cao, S., Berg, O. G., Ilbäck, C., Sandegren, L., Hughes, D. et al. (2011). Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathog.* 7:e1002158. doi: 10.1371/journal.ppat.1002158

Guo, M. T., Yuan, Q. B., and Yang, J. (2013). Ultraviolet reduction of erythromycin and tetracycline resistant heterotrophic bacteria and their resistance genes in municipal wastewater. *Chemosphere*, 93, 2864–2868. doi: 10.1016/j.chemosphere.2013.08.068

Knapp, C. W., Lima, L., Olivares-Rieumont, S., Bowen, E., Werner, D., and Graham, D. W. (2012). Seasonal variations in antibiotic resistance gene transport in the Almendares River, Havana, Cuba. *Front. Microbiol.* 3:396. doi: 10.3389/fmicb.2012.00396

McKinney, C. W., and Pruden, A. (2012). Ultraviolet disinfection of antibiotic resistant bacteria and their antibiotic resistance genes in water and wastewater. *Environ. Sci. Technol.* 46, 13393–13400. doi: 10.1021/es303652q

Michael, I., Rizzo, L., McArdell, C. S., Manaia, C. M., Merlin, C., Schwartz, T., et al. (2013). Urban waste water treatment plants as hotspots for the release of antibiotics in the environment: a review. *Water Res.* 47, 957–993. doi: 10.1016/j.watres.2012.11.027

Munir, M., Wong, K., and Xagoraraki, I. (2011). Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water Res.* 45, 681–693. doi: 10.1016/j.watres.2010.08.033

Pham, T. P., Cho, C. W., and Yun, Y. S. (2010). Environmental fate and toxicity of ionic liquids: a review. *Water Res.* 44, 352–372. doi: 10.1016/j.watres.2009.09.030

Rahman, H. M., Nonaka, L., Tago, R., and Suzuki, S. (2008). Occurrence of two genotypes of tetracycline (TC) resistance gene *tet*(M) in the TC-resistant bacteria in marine sediments of Japan. *Environ. Sci. Technol.* 42, 5055–5061. doi: 10.1021/es702986y

Port, J. A., Cullen, A. C., Wallace, J. C., Smith, M. N., and Faustman, E. M. (2014). Metagenomic frameworks for monitoring antibiotic resistance in aquatic environments. *Environ. Health Perspect.* 122, 222–228. doi: 10.1289/ehp.1307009

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M. C., et al. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. *Sci. Total Environ.* 447, 345–360. doi: 10.1016/j.scitotenv.2013.01.032

Suzuki, S., and Hoa, P. T. (2012). Distribution of quinolones, sulfonamides, tetracyclines in aquatic environment and antibiotic resistance in Indochina. *Front. Microbiol.* 3:67. doi: 10.3389/fmicb.2012.00067

Suzuki, S., Kimura, M., Agusa, T., and Rahman, H. M. (2012). Vanadium accelerates horizontal transfer of *tet*(M) gene from marine *Photobacterium* to *Escherichia coli*. *FEMS Microbiol. Lett.* 336, 52–56. doi: 10.1111/j.1574-6968.2012.02653.x

Suzuki, S., Ogo, M., Miller, T. W., Shimizu, A., Takada, H., and Siringan, M. A. (2013). Who possesses drug resistance genes in the aquatic environment?: sulfamethoxazole (SMX) resistance genes among the bacterial community in water environment of Metro-Manila, Philippines. *Front. Microbiol.* 4:102. doi: 10.3389/fmicb.2013.00102

Takami, H., Inoue, A., Fuji, F., and Horikoshi, K. (1997). Microbial flora in the deepest sea mud of Mariana Trench. *FEMS Microbiol. Lett.* 152, 279–285.

Tamminen, M., Karkman, A., Lõhmus, A., Muziasari, W. I., Takasu, H., Wada, S., et al. (2011). Tetracycline resistance genes persist at aquaculture farms in the absence of selection pressure. *Environ. Sci. Technol.* 45, 386–391. doi: 10.1021/es102725n

United States Environmental Protection and Agency (2004). *Primer for Municipal Wastewater Treatment Systems. EPA832-R-04-001*. Washington: DC: United States Environmental Protection and Agency, 25. Available online at: http://www.epa.gov/npdes/pubs/primer.pdf

Wright, G. D. (2010). Antibiotic resistance in the environment: a link to the clinic? *Curr. Opin. Microbiol.* 13, 589–594. doi: 10.1016/j.mib.2010.08.005

Yuan, Q.-B., Guo, M.-T., and Yang, J. (2015). Fate of antibiotic resistant bacteria and genes during wastewater chlorination: implication for antibiotic resistance control. *PLoS ONE* 10:e0119403. doi: 10.1371/journal.pone.0119403

Zhuang, Y., Ren, H., Geng, J., Zhang, Y., Zhang, Y., Ding, L. et al. (2015). Inactivation of antibiotic resistance genes in municipal wastewater by chlorination, ultraviolet, and ozonation disinfection. *Environ. Sci. Pollut. Res.* 22, 7037–7044. doi: 10.1007/s11356-014-3919-z

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 29 April 2015
doi: 10.3389/fmicb.2015.00336

Detection of multi-drug resistant *Escherichia coli* in the urban waterways of Milwaukee, WI

*Anthony D. Kappell[1], Maxwell S. DeNies[1], Neha H. Ahuja[1], Nathan A. Ledeboer[2,3], Ryan J. Newton[4] and Krassimira R. Hristova[1]**

[1] Department of Biological Sciences, Marquette University, Milwaukee, WI, USA, [2] Department of Pathology, Medical College of Wisconsin, Milwaukee, WI, USA, [3] Dynacare Laboratories, Milwaukee, WI, USA, [4] School of Freshwater Sciences, Great Lakes WATER Institute, University of Wisconsin–Milwaukee, Milwaukee, WI, USA

Urban waterways represent a natural reservoir of antibiotic resistance which may provide a source of transferable genetic elements to human commensal bacteria and pathogens. The objective of this study was to evaluate antibiotic resistance of *Escherichia coli* isolated from the urban waterways of Milwaukee, WI compared to those from Milwaukee sewage and a clinical setting in Milwaukee. Antibiotics covering 10 different families were utilized to determine the phenotypic antibiotic resistance for all 259 *E. coli* isolates. All obtained isolates were determined to be multi-drug resistant. The *E. coli* isolates were also screened for the presence of the genetic determinants of resistance including *ermB* (macrolide resistance), *tet*(M) (tetracycline resistance), and β-lactamases (bla_{OXA}, bla_{SHV}, and bla_{PSE}). *E. coli* from urban waterways showed a greater incidence of antibiotic resistance to 8 of 17 antibiotics tested compared to human derived sources. These *E. coli* isolates also demonstrated a greater incidence of resistance to higher numbers of antibiotics compared to the human derived isolates. The urban waterways demonstrated a greater abundance of isolates with co-occurrence of antibiotic resistance than human derived sources. When screened for five different antibiotic resistance genes conferring macrolide, tetracycline, and β-lactam resistance, clinical *E. coli* isolates were more likely to harbor *ermB* and bla_{OXA} than isolates from urban waterway. These results indicate that Milwaukee's urban waterways may select or allow for a greater incidence of multiple antibiotic resistance organisms and likely harbor a different antibiotic resistance gene pool than clinical sources. The implications of this study are significant to understanding the presence of resistance in urban freshwater environments by supporting the idea that sediment from urban waterways serves as a reservoir of antibiotic resistance.

Keywords: antibiotic resistance genes, antibiotic resistance bacteria, Great Lakes, sediment, environment, *E. coli*

OPEN ACCESS

Edited by:
Amy Pruden, Virginia Tech, USA

Reviewed by:
Celia Manaia, Universidade Católica Portuguesa, Portugal
Ying Yang, The University of Hong Kong, China

****Correspondence:***
Krassimira R. Hristova, Department of Biological Sciences, Marquette University, 530 N. 15th Street, Wehr Life Sciences Building, 208, P. O. Box 1881, Milwaukee, WI 53201-1881, USA
krassimira.hristova@marquette.edu

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *17 December 2014*
Accepted: *05 April 2015*
Published: *29 April 2015*

Citation:
Kappell AD, DeNies MS, Ahuja NH, Ledeboer NA, Newton RJ and Hristova KR (2015) Detection of multi-drug resistant Escherichia coli in the urban waterways of Milwaukee, WI. Front. Microbiol. 6:336. doi: 10.3389/fmicb.2015.00336

Introduction

The increasing number of multiple-antibiotic resistant pathogens has become a serious threat to human health (Centers for Disease Control and Prevention [CDC], 2013; Review on Antimicrobial Resistance [RAR], 2014; World Health Organization [WHO], 2014). Over the past two decades researchers have expanded their focus from the clinical settings to also include

the natural environment as a reservoir of antibiotic resistance (Kümmerer, 2004; Baquero et al., 2008; Martinez et al., 2009; Allen et al., 2010; Galán et al., 2013; Michael et al., 2013; Wellington et al., 2013). The resistome of fecal bacteria from human and animal sources released into the environment impart antibiotic resistances genes to the non-resistant indigenous microorganisms (Aminov, 2011; Tacão et al., 2014). The subsequent transfer of antibiotic resistance genes from the indigenous microorganisms to human-associated bacteria may take place (Devirgiliis et al., 2011; Figueira et al., 2011). Simultaneously, positive selective pressure for antibiotic resistance genes in the environment may be stimulated by the presence of antibiotics or other contaminants (Silveira et al., 2014; Varela et al., 2014; Gao et al., 2015). These facts highlight the need to identify the potential sources of antibiotic resistant bacteria in environments used by human populations (Rosewarne et al., 2010; Gomez-Alvarez et al., 2012).

Escherichia coli are currently used by the Environmental Protection Agency (EPA, USA) as an indicator organism for fecal contamination and bacterial impairment for watersheds. *E. coli* is a natural member of intestinal microbiome of humans and other animals (reviewed in Harwood et al., 2014). The major sources of fecal contamination in various watersheds include human (Bernhard and Field, 2000), agricultural animals (Shanks et al., 2008), pets (Ervin et al., 2014), and wild animals (Somarelli et al., 2007; Guber et al., 2015), such as gulls (Alves et al., 2014; Araújo et al., 2014). The major source of fecal contamination of urban waterways of Milwaukee, WI was determined to be human (Newton et al., 2011, 2013).

Multi-drug resistant (MDR) *E. coli* and other *Enterobacteriaceae* isolates are characterized by non-susceptibility (or non-sensitivity) to at least one agent in three or more antibiotic categories (Magiorakos et al., 2012). Antibiotic resistance surveillance data show that *E. coli* has high resistance for older generation human and veterinary antibiotics including ampicillin, streptomycin, and tetracycline and the increasing resistance to newer antibiotics such as fluoroquinolones and cephalosporins (Tadesse et al., 2012).

E. coli had been recognized as a contributor to the dissemination of antibiotic resistance genes in natural environments (Henriques et al., 2006; Zhao and Dang, 2012; Alm et al., 2014; Alves et al., 2014). The gene encoding resistance to tetracycline class antibiotics, *tet*(M), which is predominately found on transposons within enterococci have also been found on plasmids within *E. coli* (Jurado-Rabadán et al., 2014), and possibly in *E. coli* from a natural river basin (Hu et al., 2008). The *ermB* gene encoding resistance to macrolides, lincosamides, and streptogramin have been identified on transposons and plasmids within or transferable to *E. coli* (Poyart et al., 1995; Poirel et al., 2011). The genes bla_{OXA}, bla_{SHV}, and bla_{PSE} are grouped in the most common types of β-lactamases belonging to *Enterobacteriaceae* (Bush and Jacoby, 2010).

Freshwater environments are recognized as reactors for the evolution and dissemination of antibiotic resistance (Alm et al., 2014; Czekalski et al., 2014; Marti et al., 2014; Vaz-Moreira et al., 2014), however, processes occurring in urban freshwater environments are less understood. In addition, the presence of antibiotic resistance *E. coli* in urban waterways represents a health issue in areas that are used for recreation activities.

The Milwaukee Harbor, an urbanized estuary, has a documented history of contamination from human activities. Located within the harbor is a wastewater treatment plant discharging the treated effluent within the outer harbor into the Lake. High incidence of antibiotic resistance in *E. coli*, an indication of sewage contamination also was detected in storm water from the Menomonee River, which flows through the city of Milwaukee and into the Lake Michigan harbor (Salmore et al., 2006). Human fecal pollution is constant within the contributing rivers and the Milwaukee Harbor and increased contamination during heavy rain events has been reported (Newton et al., 2013). There is also evidence of high levels of personal care products and pharmaceuticals, including antibiotics present within the Milwaukee Harbor (Blair et al., 2013a). A study by LaPara et al. (2011) found that the presence of tetracycline resistance determinants *tet*(A), *tet*(X), and *tet*(W) in Lake Superior surface waters receiving Waste Water Treatment Plant (WWTP) effluent near urban environments were correlated with the presence of fecal bacteria.

The objective of this study was to evaluate the abundance of multiple-antibiotic resistant bacteria present in the Milwaukee's urban waterways compared to the human derived bacterial community from Milwaukee, WI. Since the urban waterways of Milwaukee have the potential for positive selection of antibiotic resistance due to history of antibiotics present (Blair et al., 2013a), we hypothesize that the *E. coli* isolated from urban waterways in Milwaukee maintain a similar or greater incidence of antibiotic resistance compared to the human derived *E. coli* isolates. The relationship of the resistances identified in the microbial community of the urban waterways and the human derived microbial community were explored in order to test this hypothesis. *E. coli* were isolated from sediment within the inner and outer Milwaukee harbor, human derived sewage, and from a clinical laboratory servicing Milwaukee. A broad range of antibiotics covering different families (β-lactams, aminoglycosides, tetracyclines, quinolones/fluoroquinolones, sulfonamides, dihydrofolate reductase inhibitors, UDP-*N*-acetylglucosamine enolpyruvyl transferase inhibitor, rifampicin, and chloramphenicol) were used to determine the resistance of the *E. coli* isolates. The presence of genetic determinates of resistance for *ermB* (macrolide resistance), *tet*(M), and β-lactamases (bla_{OXA}, bla_{SHV}, and bla_{PSE}), were screened in the same *E. coli* isolates.

Materials and Methods

Study Site and Sample Collection

On March 22, 2012, four sediment grab samples at different locations were collected with a box corer from the Milwaukee Harbor Estuary. (A sampling map is shown in Supplementary Figure S1.) The Milwaukee Harbor Estuary is defined as the confluence of the Milwaukee, the Kinnickinnic, and the Menomonee Rivers into the Milwaukee Harbor. A subsample from each grab sample was collected in two sterile 50 mL conical centrifuge tubes and held at 4°C until filtering and plating. Strains isolated from

the Kinnickinnic River and the junction of the Kinnickinnic River with the Milwaukee and Menomonee Rivers in the inner Milwaukee Harbor are referred to as the inner harbor isolates ($n = 36$). The strains referred to as the outer harbor isolates ($n = 58$) are further downstream of the junction of the rivers and were from sediment collected near the effluent pipe of the Jones Island WWTP in the outer Milwaukee Harbor. Human derived sewage ($n = 66$) from the influent of the Jones Island WWTP was used for assessing microbial resistance of the human population. The influent WWTP water was placed in a pre-chilled 1 L bottle and kept at 4°C until filtering and plating. Disassociation of bacteria from sediment particles in the grab samples were performed as by Boehm et al. (2009). Briefly, grab samples (3 g) were diluted 1:10 in sterile buffered (pH 7.0) water and shook vigorously by hand for 2 min. The undiluted and serial diluted eluents from the sediment samples and the water samples from the influent of the WWTP were subjected to filtration through a 0.45 μm filter and the filter was placed on a modified membrane-thermotolerant *E. coli* Agar (modified mTEC) plate as in EPA Method 1603 (Environmental Protection Agency [EPA], 2002). As the EPA Method indicates the plates were initially incubated for 2 h at 35°C for recovery of injured cells followed by incubation at 44.5°C for 22 h. The colonies identified as *E. coli* based on pigmentation on the modified mTEC were recovered on Tryptic Soy Agar (TSA) media and incubated at 37°C for 18 h. *E. coli* strains of clinical consequence ($n = 99$) were obtained from Dynacare Laboratories (Milwaukee, WI, USA) on TSA slants. The clinical *E. coli* isolates were collected from various patient populations including outpatients and hospitalized patients from throughout the Milwaukee area and identity was confirmed by MALDI-TOF (Anderson et al., 2012; Buchan et al., 2012). All *E. coli* isolates were then grown in Tryptic Soy Broth (TSB) at 37°C for 18 h and stored at −20°C after the addition of glycerol to a final concentration of 10%. An additional 1 mL of culture was pelleted by centrifugation and stored at −20°C for DNA extraction.

Antibiotic Susceptibility Testing

The *E. coli* isolates were tested for susceptibility to 17 antibiotics by a 96-well broth dilution method in Muller–Hinton broth utilizing three antibiotic concentrations based on the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute [CLSI], 2011) guidelines. Four wells were used for each *E. coli* isolate consisting of three wells containing a serial half concentration dilution of the antibiotic and one well with no antibiotic as a positive control for growth. The 96-well plates containing 200 μL per well were incubated at 35°C for 18 h. The following antibiotics were used: ampicillin (AMP; Sigma, A0166; 8–32 μg mL^{-1}), gentamicin (GEN; Sigma, G1264; 4–16 μg mL^{-1}), streptomycin (STR; Sigma, S9137; 8–32 μg mL^{-1}), neomycin (NEO; Sigma, N6386; 16–64 μg mL^{-1}), tetracycline (TET; Sigma, T7660; 4–16 μg mL^{-1}), ciprofloxacin (CPR; Sigma, 17850; 1–4 μg mL^{-1}), chloramphenicol (CHL; Sigma, C1919; 8–32 μg mL^{-1}), trimethoprim (TRM; Sigma, 92131; 8–32 μg mL^{-1}), sulfamethoxazole (SFM; Sigma, S7507; 256–1024 μg mL^{-1}), fosfomycin (FOS; Sigma, P5396; 64–256 μg mL^{-1}), erythromycin (ERY; Sigma, E5389; 2–8 μg mL^{-1}), aztreonam (AZT; Sigma, A6848; 4–16 μg mL^{-1}), cefuroxime (CFX; Sigma, C4417; 8–32 μg mL^{-1}), meropenem (MER; Sigma, M2574; 1–4 μg mL^{-1}), moxifloxacin (MOX; Selleckchem, S1465; 1–4 μg mL^{-1}), rifampicin (RIF; Sigma, R7382; 1–4 μg mL^{-1}), and cefepime (CFP; US Pharmacopeial, 1097636; 8–32 μg mL^{-1}). Isolates were classified as sensitive when growth was sequestered to the well without antibiotic and resistant when growth was observed in all four wells. Intermediate level of resistance was concluded when growth was observed in wells containing antibiotic concentrations diluted from the maximum. Where appropriate the classification of non-sensitive was used to denote any strain with any level of resistance to an antibiotic which includes Resistant or Intermediate levels determined by the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute [CLSI], 2011).

Antibiotic Resistance Gene Detection

Escherichia coli strains regardless of resistances were screened by real-time PCR to detect genes conferring resistance comprized of *tet(M)*, *ermB*, bla_{OXA}, bla_{SHV}, and bla_{PSE}. Extractions of the total DNA of the isolates were performed with the Wizard Genomic DNA Purification Kit (Promega, A1120) on 1 mL of culture in TSB incubated at 37°C for 18 h. The PCR reactions were performed in a MyiQ or CFX Connect Real-Time PCR Detection Systems (Bio-rad, USA). Primers used are presented in Supplementary Table S1. The reaction mixture of 20 μL consisted of Standard *Taq* Buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3; NEB, USA), 500 nM of each primer, 500 μM dNTPs, 0.5 U of *Taq* DNA Polymerase (NEB, USA), 0.1x SYBR Green I Nucleic Acid Gel Stain (Lonza, Cat#50513) and 25 ng of genomic DNA. PCR thermocycling conditions consisted of an initial denaturation step of 5 min at 95°C followed by 40 cycles of 95°C for 30 s, the gene specific annealing temperature (Supplementary Table S1) for 30 s, and 72°C for 30 s concluding with a melt curve analysis. Presence of the gene was determined by the cycle threshold and melt curve analysis of the real-time PCR results compared to a positive standard and no template controls. Amplifiable DNA was determined utilizing the 1369F and 1492R Bacterial 16S Primers (Suzuki et al., 2000).

Positive standards for PCR were generated by utilizing the PCR reaction mixture and conditions as used for screening without SYBR Green. DNA template for the PCR reactions to create standards was pooled DNA from clinical strains, not used in this study, with documented multiple resistances. PCR products at the expected product size determined by 2% agarose gel electrophoresis were excised from the gel, purified utilizing a QIAquick Gel Extraction Kit (Qiagen, USA), and cloned into the plasmid pUC19 (Yanisch-Perron et al., 1985). For cloning into pUC19, the plasmid was fully digested with SmaI endonuclease enzyme (NEB, USA) and the enzyme heat inactivated at 65°C for 30 min. The SmaI digested pUC19 was treated in a 50 μL reaction with Standard Taq Buffer, 0.5 U of Taq DNA Polymerase (NEB, USA), and 100 μM dTTP to generate 3′ T overhangs to aid in cloning the PCR products with 3′ A overhangs. The PCR products for the generation of standards were ligated into pUC19

utilizing T4 Ligase by manufacture's recommended conditions (NEB, USA). Chemically competent TOP10 cells (Invitrogen, USA) were transformed with the ligation reaction and plated onto TSA augmented with 200 $\mu g\ mL^{-1}$ ampicillin. Colony PCR was utilized to screen for isolates containing pUC19 with the insertions utilizing the M13 forward and reverse primers. Isolates demonstrating successful insertion of the PCR product into pUC19 were propagated and plasmids utilized for positive standards were isolated by I-Blue Mini Plasmid Kit (IBI, USA) and confirmed by Sanger sequencing.

Of the 295 positive amplification from the screening PCRs, 146 (49%) were purified utilizing the QIAquick PCR Purification Kit (Qiagen, USA) and sequenced utilizing their respective primers. All sequenced amplicons were confirmed to be the targeted genes (Results summarized in Supplementary Table S2).

Statistical Analysis

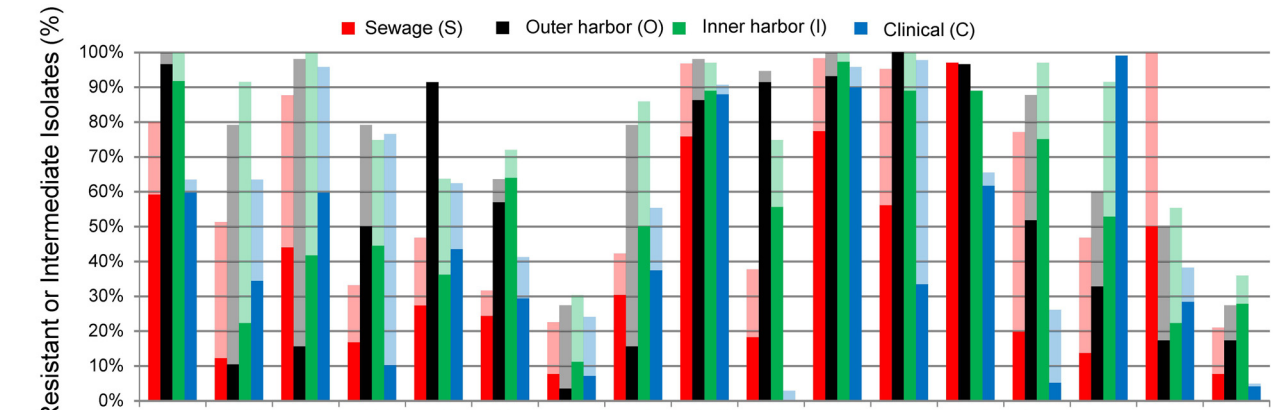
Antibiotic resistance phenotypic profiles and gene presence was converted into numerical code. For each antibiotic: 1 signified susceptibility, 2 for intermediate resistance, and 3 represented resistance. The presence of a gene was signified as 1 and absence as 0. Principal component analysis (PCA) was performed using the package FactoMineR (Lê et al., 2008) from the open source statistical program R (R Core Team, 2012). Multivariate statistical analyses were performed using routines in the R package 'vegan' (Oksanen et al., 2013). Bray–Curtis similarities were calculated for antibiotic resistance levels and gene presence or absence using vegdist function in vegan package as input for multivariate ANOVA (MANOVA) analyses using the permutational alternative (Anderson, 2001) to standard parametric MANOVA provided by vegan's 'adonis' function. Multiple pairwise tests were conducted using Tukey's honestly significant differences (HSD) at the 5% family wise level of significance. The 'glm' function with the Poisson distribution and Analysis of Deviance for Generalized Liner Model utilizing the Chi-square test to determine significant differences between the antibiotic variable vectors and the source vectors.

Proportional Z test was utilized to identify significant differences between count data which is represented as percentages such as antibiotic resistances and presence of genes by location. Mann–Whitney U test was used for comparison of number of antibiotic resistance in the isolates from the locations.

Results

Antibiotic Resistance Profiles

The antibiotic resistance patterns of the *E. coli* isolates to 17 antibiotics are shown in **Figure 1**. Duplicate isolates were determined and eliminated based on phenotypic and genotypic results; however, no duplication was observed. Resistant-level phenotype, growth at the highest concentrations of antibiotic within the phenotypic assay, was detected for all antibiotics tested. All isolates ($n = 259$) showed a resistant-level phenotype for at least one antibiotic and non-sensitive level phenotype, growth at any concentration of antibiotic tested within the phenotypic assay, for at least six antibiotics tested (**Figure 2**; Supplementary Figure S2). All *E. coli* isolates were non-sensitive to at least one agent in three or more antibiotic categories as defined for multi-drug resistant (MDR) bacteria in Magiorakos et al. (2012). Three isolates, two from the inner harbor and one from the outer harbor were non-sensitive (intermediate or resistant level of phenotypic antibiotic resistance) to all 17 antibiotics tested. These three strains resistant to all antibiotics tested are potential extensively



		AMP	GEN	STR	NEO	TET	CPR	CHL	TRM	SFM	FOS	ERY	RIF	AZT	CFX	MER	MOX	CFP
S	O	S<O		S>O	S<O	S<O	S<O				S<0	S<O	S<O		S<O	S<O	S>O	
	I	S<I			S<I		S<I				S<I	S<I	S<I		S<I	S<I	S>I	S<I
	C		S<C								S>C		S>C	S>C	S>C	S<C	S>C	
O	I			O<I		O>I			O<I		O>I		O>I					
	C	O>C	O<C	O<C	O>C	O>C	O>C		O<C		O>C		O>C	O>C	O>C	O<C		O>C
I	C	I>C			I>C		I>C				I>C		I>C	I>C	I>C	I<C		I>C

FIGURE 1 | Percentage of *Escherichia coli* isolates from sewage (S, $n = 66$), outer harbor (O, $n = 58$), inner harbor (I, $n = 36$), and clinical setting (C, $n = 99$) indicating resistance (solid) or intermediate resistance (transparent) to 17 different antibiotics. Table indicates significant difference in antibiotic resistance (not including intermediate resistance) between locations ($p < 0.01$) by proportional Z test.

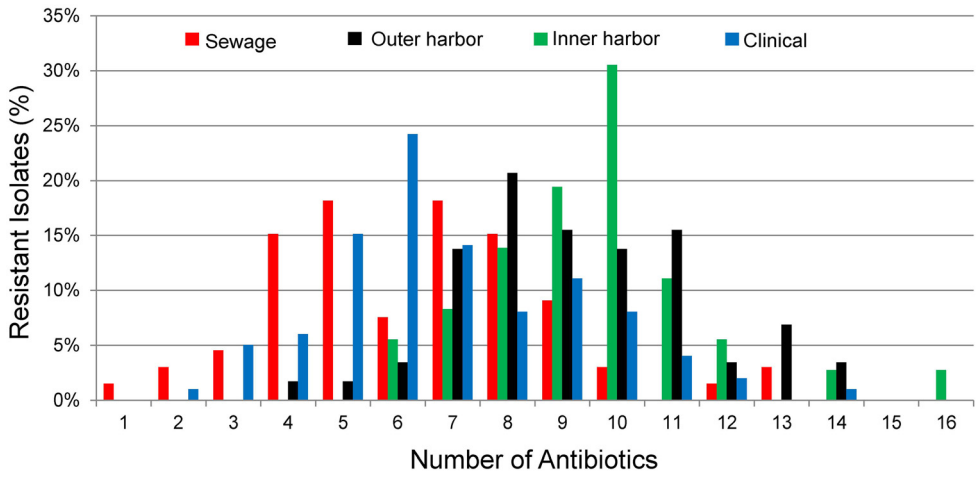


FIGURE 2 | Percentage of *E. coli* isolates from sewage ($n = 66$), outer harbor ($n = 58$), inner harbor ($n = 36$), and clinical setting ($n = 99$) showing number of antibiotic resistances (Resistant-level).

drug-resistant (XDR) bacteria showing non-sensitivity to 11 of the required 15 out of 17 antibiotic categories (Magiorakos et al., 2012). Antibiotic resistance was most prevalent for erythromycin, sulfamethoxazole, aztreonam, and ampicillin occurring in 72–88% of all isolates. The least prevalent antibiotic resistance in all isolates was for chloramphenicol, observed in less than 7%.

E. coli isolates collected in the inner or outer harbor of Milwaukee demonstrated significantly greater frequencies of resistances to eight antibiotics compared to the *E. coli* isolates collected from human derived sewage and of clinical concern ($p < 0.01$; **Figure 1**). These eight antibiotics included ampicillin, neomycin, tetracycline, ciprofloxacin, fosfomycin, rifampicin, cefuroxime, and cefepime. The isolates from the urban waterways additionally showed significantly greater frequencies of resistance to erythromycin compared to isolates from human derived sewage ($p < 0.01$) and aztreonam compared to clinical isolates ($p < 0.01$). Of the antibiotics tested, the isolates in the outer harbor collected near the WWTP effluent showed significantly greater frequencies of tetracycline, fosfomycin, and rifampicin resistance compared to the inner harbor isolates ($p < 0.01$).

Since all *E. coli* isolates tested were determined to be MDR bacteria, the distribution of the number of antibiotics the isolates were resistant was used to explore the differences between the urban waterway and human derived isolates. The number of antibiotic resistances in the *E. coli* isolates was greater from the inner harbor (median = 9, range: 6–16 antibiotics) and outer harbor (median = 10, range: 4–14) of the urban waterways compared to the clinical isolates (median = 6, range: 2–12) and isolates from human derived sewage (median = 6.5, range: 1–13; **Figure 2**, $p < 0.001$). (The number of antibiotic resistance in the *E. coli* isolates demonstrating non-sensitive phenotypes similar to determine MDR are in Supplementary Figure S2.) There was no significant difference in the number of antibiotics an isolate was resistant between inner and outer harbor isolates ($p = 0.52$) nor human derived sewage and clinical isolates ($p = 0.17$).

Principal component analysis of the combined genotype and phenotypic resistance profiles for each isolate was used to explore the difference in the environmental resistome from which the *E. coli* isolates were derived (**Figure 3**). (**Figure 3** contains genotype and phenotype in separate PCA analysis as well.) PCA analysis demonstrated a gradient related to number of antibiotics the *E. coli* isolates were resistant to (**Figure 3**, dotted arrow). Permutational MANOVA analysis showed significant differences between isolates from the different sources ($p < 0.001$) and follow up pairwise comparison showed significant differences between all sources ($p < 0.01$) with the exception of the human derived sources (clinical and human derived sewage, $p = 0.74$).

Isolates collected from human derived sewage were positively correlated with moxifloxacin resistance ($p < 0.001$). The gene bla_{OXA} encoding a β-lactamase showed significant ($p < 0.001$) positive correlation with the clinical isolates. The vectors related to the phenotypic antibiotic resistance for meropenem, a mono-β-lactam ($p < 0.001$) were also significantly correlated with the clinical isolates. The inner harbor isolates positively correlated with antibiotic resistance for fosfomycin, UDP-*N*-acetylglucosamine enolpyruvyl transferase inhibitor ($p < 0.01$), cefuroxime, a second generation cephalosporin ($p < 0.001$), and the presence of the *tet*(M) ($p < 0.01$) gene encoding tetracycline resistance. The outer harbor isolates were positively correlated with resistance to tetracycline ($p < 0.001$), fosfomycin ($p < 0.001$) and cefuroxime (second generation cephalosporin; $p < 0.01$).

The PCA analysis suggested putative incidences of co-occurrences of resistances across sources. The co-occurrences of non-sensitive phenotypes (resistance and intermediate levels) for combinations of antibiotics were recurrent. **Table 1** shows the co-occurrences of non-sensitive phenotypes to 3–5 antibiotics demonstrated within 20% or more of the isolates from a location. The majority of co-occurrences of resistances were identified within the urban waterways. The presence of the co-occurrences in the urban waterways is expected due to the high incidences of resistance in the isolates (**Figure 1**) and the number of resistance

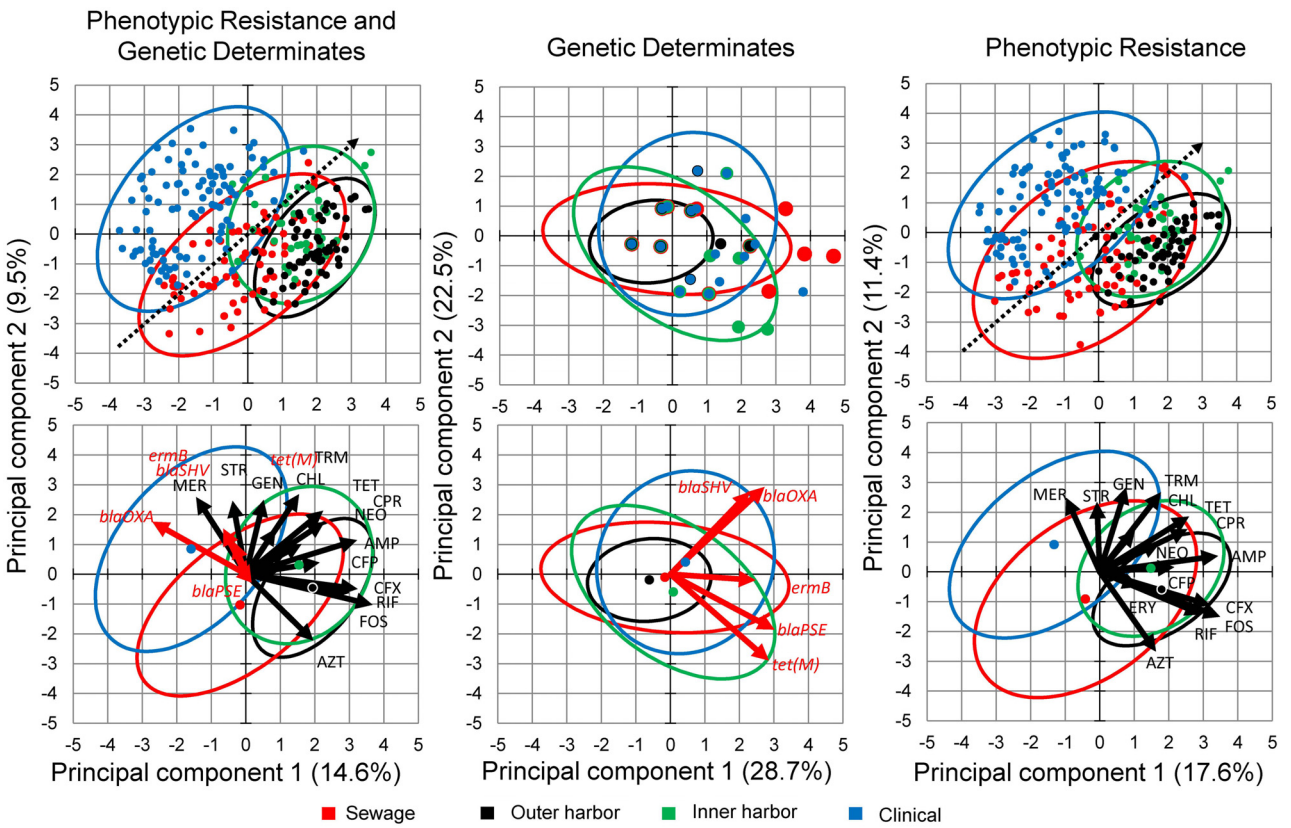


FIGURE 3 | Principal Component Analysis (PCA) biplots of *E. coli* isolates in terms of their phenotypic antibiotic resistance profiles and/or their genotype based on the presence of antibiotic resistance genes. Separate PCA were performed on data containing both the phenotypic and genotypic profiles **(Left)** and the genotypic **(Middle)** and phenotypic **(Right)** profiles separately. The green points represent the *E. coli* isolates from the inner Milwaukee Harbor, black points from the outer harbor, red points from human derived sewage, and blue from clinical isolates. Points in the upper panels are individual isolates and the points in the bottom panels represent the group center (average of individuals). Dotted line in the upper panels shows the gradient of number of resistance (multiple antibiotic resistances) in the isolates. The ellipses represent a 95% confidence interval of the respective points in the same color. In the right panel: Black arrows represent antibiotics (see Materials and Methods for abbreviations) utilized in the phenotypic antibiotic resistance screening. Erythromycin (ERY), Sulfamethoxazole (SFM), and Moxifloxacin (MOX) respective arrows may not be perceptible due to little influence in the ordination. Red arrows represent antibiotic resistance genes screened by PCR.

within the isolates (**Figure 2**). The co-occurrences of ampicillin, fosfomycin, rifampicin, and cefuroxime resistance were of the greatest abundance in urban waterway isolates compared to human derived sewage and clinical *E. coli* isolates. The co-occurrences of neomycin, sulfamethoxazole, erythromycin, and meropenem resistance were not significantly different between the urban waterways and clinical isolates indicating a potentially common resistance mechanism between the environmental and the clinical *E. coli* isolates.

In summary, a higher incidence of antibiotic resistance, prevalence of resistance to a greater number of antibiotics and a greater incidence of co-occurrences of resistance was identified within the urban waterway (inner and outer Milwaukee Harbor) *E. coli* isolates compared to the human derived isolates (sewage and clinical isolates) from Milwaukee.

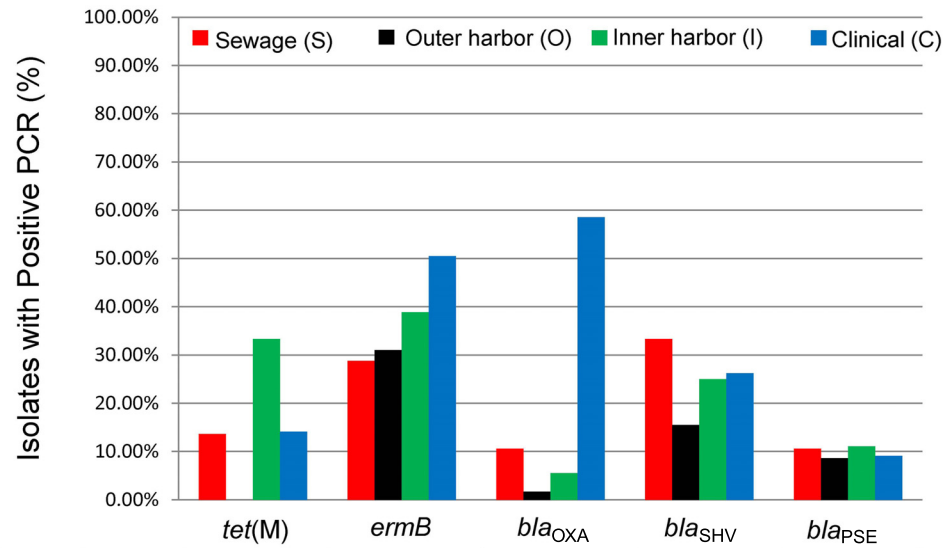
Detection of Antibiotic Resistance Genes

The presences of five antibiotic resistance genes: *ermB* (macrolide resistance), *tet*(M), and β-lactamases (bla_{OXA}, bla_{SHV}, and bla_{PSE}) were screened for in the *E. coli* isolates (**Figure 4**). More than 65% of all the *E. coli* isolates ($n = 259$) harbored at least one of the five resistance genes chosen for analysis. The presence of *ermB* was the most prevalent represented in 38% of the isolates. The β-lactamase encoding genes bla_{OXA} and bla_{SHV} were present in 26 and 25% of all strains, respectively. The resistance gene bla_{PSE} and *tet*(M) were the least prevalent at 10 and 14%, respectively. The clinical *E. coli* isolates showed significantly greater frequencies of the bla_{OXA} compared to the urban waterway isolates of the inner and outer harbor and the human derived sewage (**Figure 4**). The greater incidence of resistance to meropenem in the clinical *E. coli* isolates can be partially (58.2%) explained by the greater frequency of the bla_{OXA} gene in the isolates compared to less than 10% in the meropenem resistant *E. coli* isolates from urban waterways and human derived sewage (**Table 2**). The presence of any β-lactamase genes could explain 73% of the ampicillin resistant clinical *E. coli* isolates compared to only 21–40% of the ampicillin resistant *E. coli* isolates from the urban waterways and human derived sewage.

TABLE 1 | The percentage of *Escherichia coli* isolates from the urban waterways of the inner and outer Milwaukee Harbor and of human derived sources (sewage and clinical) with co-occurrence of antibiotic resistance.

Co-occurrence of antibiotic resistance[1]	Inner harbor (36)[2]	Outer harbor (58)	Sewage (66)	Clinical (99)	Significance ($p < 0.01$)[3]
GEN, STR, TRM, AZT, MER	58.3 (21)	37.9 (22)	12.1 (8)	22.2 (22)	O > S, I > S, I > C
AMP, FOS, RIF, CFX	75.0 (27)	82.8 (48)	25.8 (17)	2.0 (2)	O > S, I > S, O > C, I > C
NEO, SFM, ERY, MER	66.7 (24)	55.0 (29)	18.2 (12)	69.7 (69)	O > S, I > S, C > S, O > C
GEN, STR, CHL, MER	27.8 (10)	8.6 (5)	7.6 (5)	14.1 (14)	I > S, I > O
GEN, TET, SFM	58.3 (21)	70.7 (41)	28.8 (19)	39.4 (39)	O > S, I > S, O > C
TET, TRM, SFM	38.9 (14)	36.2 (21)	28.8 (19)	24.2 (24)	NS
STR, ERY, RIF	72.2 (26)	75.9 (44)	30.3 (20)	54.5 (54)	O > S, I > S, C > S, O > C

[1]*Criteria for inclusion was non-sensitive resistance (resistance or intermediate resistance level) for three or more antibiotics and presence in greater than 20% in one or more locations.*
[2]*Reported in percentage of isolates from location and number of isolates in parenthesis.*
[3]*Statistical significance was determined between locations by proportional Z test.*



S	O	S>O				
	I	S<I				
	C		S<C	S<C		
O	I	O<I				
	C	O<C	O<C	O<C		
I	C	I>C		I<C		

FIGURE 4 | Percentage of *E. coli* isolates from sewage ($n = 66$), outer harbor ($n = 58$), inner harbor ($n = 36$), and clinical setting ($n = 99$) harboring antibiotic resistance genes. Table indicates significant difference in antibiotic resistance (not including intermediate resistance) between locations ($p < 0.01$) by proportional *Z* test.

The *E. coli* isolates of the inner harbor demonstrated greater frequencies of the *tet*(M) gene compared to the isolates of the outer harbor and derived from human sources (clinical and human derived sewage isolates). The presence of the *tet*(M) gene explained greater than 38.9% of the tetracycline resistant isolates detected in the inner harbor and less than 16.1% in the other tetracycline isolates from other sources.

The *ermB* gene was present in significantly greater frequency in clinical *E. coli* isolates than the *E. coli* isolates from the outer harbor and human derived sewage. The presence of *ermB* may explain 52% of the erythromycin resistance observed in the clinical isolates, while less than 40% of erythromycin resistance could be explained in the urban waterways and human derived sewage isolates.

The few genetic determinants detected in this study had little to no effect on the PCA analysis in the presence of the phenotypic data (**Figure 3**). Utilizing only the genetic determinations in PCA analysis and MANOVA did not show any significant difference between sources of *E. coli* isolates.

While the genetic determinates were able to explain some of the high incidences of resistance within the clinical *E. coli* isolates, direct phenotypic determination of antibiotic resistance showed

TABLE 2 | Percentage of antibiotic resistance in *E. coli* isolates from different locations explained by the presence of a gene able to confer the resistance.

Antibiotic	Gene[1]	Inner harbor[2]	Outer harbor	Sewage	Clinical
Penicillins (AMP)	SHV, PSE, OXA	36.1 (13/36)	20.7 (12/58)	39.6 (21/53)	73.0 (46/63)
Second generation Cephalosporin (CFX)	SHV, PSE	34.3 (12/35)	23.5 (12/51)	33.3 (17/51)	34.6 (9/26)
Fourth generation Cephalosporin (CFP)	SHV, PSE	53.9 (7/13)	12.5 (2/16)	14.3 (2/14)	60.0 (3/5)
Carbapenems (MER)	OXA	3.03 (1/33)	2.9 (1/35)	9.7 (3/31)	58.2 (57/98)
Monobactam (AZT)	SHV, PSE	34.4 (11/32)	19.6 (11/56)	32.8 (21/64)	35.4 (23/65)
Tetracycline (TET)	*tet*(M)	39.1 (9/23)	0.0 (0/53)	16.1 (5/31)	8.1 (5/62)
Macrolide (ERY)	*ermB*	38.9 (14/36)	31.0 (18/58)	29.2 (19/65)	51.6 (49/95)

[1]*Genes encoding β-lactamases are abbreviated to their types (i.e., bla_{SHV} as SHV).*
[2]*Percentages represent the number of strains from a location that have a non-sensitive resistance (resistance or intermediate resistance level) for an antibiotic and the presence of at least one gene listed in the table. In parenthesis: (number of isolates with gene/total with non-sensitive resistance).*

greater incidences of antibiotic resistance and greater number of resistance within the *E. coli* isolates from urban waterways.

Discussion

The emergence and dispersion of antibiotic resistance has reduced the susceptibility of pathogens to antibiotics in medical treatment. A detailed examination of the origin and role of antibiotic resistance in natural environments is necessary to understand the evolution and dissemination of antibiotic resistance genes in pathogens (Allen et al., 2010). The urban rivers merging into Milwaukee Harbor and ultimately Lake Michigan allowed for a representative assessment of the distribution of antibiotic resistance within these urban-influenced environments.

Prevalence of resistance to erythromycin, sulfamethoxazole, aztreonam, and ampicillin was detected in all studied sources. Occurrence of resistance and resistant determinates related to β-lactams, sulfonamides, and macrolides are not uncommon in urban settings (Hu et al., 2008; Munir et al., 2011; Rodríguez-Mozaz et al., 2014; Yang et al., 2014). The detection of macrolides and sulfonamides within the influent and effluent of a municipal wastewater treatment plant servicing the greater Milwaukee, WI area (Blair et al., 2013b) may indicate high historical usage of these antibiotics which may have led to establishment of resistant *E. coli* within the bacterial communities observed in this study. Similar levels of antibiotic resistance in the sediment of the urban waterways of Milwaukee compared to raw sewage have been detected previously (Salmore et al., 2006). The low prevalence of chloramphenicol resistance in this study is consistent with published data within the U. S. (Johnson et al., 2013) and supports the idea that this old-generation antibiotic has the potential to address the current need for new antibiotics despite the potential of negative side-effects (Falagas et al., 2008).

The high rate of MDR classification within the isolates used in this study may be due to choice of older individual antibiotics within the antibiotic classes as representatives to distinguish individual isolates based on phenotypic resistance. Previous studies have indicated lower percentages of MDR compared to this study (Baquero et al., 2008; Figueira et al., 2011; Ham et al., 2012). However, these studies used more recently deployed or fewer antibiotics compared to this study making it less likely to establish MDR.

The *E. coli* isolates from urban waterways demonstrated greater incidence of resistance to single antibiotics and multiple antibiotic resistance within a single isolate compared to human derived sources (sewage and clinical isolates). The observation of greater incidence of antibiotic resistance in *E. coli* from natural waters compared to a possible sources of fecal contamination is consistent with the observations of Alves et al. (2014), which showed a higher incidence of multi-resistant *E. coli* in coastal waters of an uninhabited island than the source of fecal contamination by seagull feces and human sewage from tourist. The higher level of resistance within the urban waterway *E. coli* isolates may be explained by the historically high abundance of antibiotics, in particular of macrolides, fluoroquinolones, and sulfonamides, quantified within the water and sediments of the Milwaukee Harbor (Blair et al., 2013a). These antibiotics may have synergistic selective effect on mechanisms of antibiotic resistance leading to increased selective pressure for antibiotic resistance within the microbial population (Davies et al., 2006; Andersson and Hughes, 2014). In other urban waterway environments receiving treated wastewaters a higher incidence and chronic presence of antibiotics and antibiotic resistance genes were detected downstream of released effluent (LaPara et al., 2011; Munir et al., 2011; Korzeniewska et al., 2013; Alves et al., 2014; Czekalski et al., 2014; Rodríguez-Mozaz et al., 2014).

The greater prevalence of the antibiotic resistance co-occurrences in urban waterways may point to a mobile resistome within the microbial population of urban waterway sediment. Previous studies tend to use a strict criteria for multiple resistance shared between isolates when exploring phenotypic similarities between locations and sources (Hu et al., 2008; Alves et al., 2014). For example, isolates showing resistance to four antibiotics would be grouped, while an isolate resistant to the same four antibiotics with additional resistances would be excluded from the grouping. This restriction may led to misinterpretation in the prevalence of specific multiple resistances shared between isolates. The prevalence of resistance to specific antibiotics or antibiotic classes within a microbial population may be explained by the presence of mobile genetic elements within the population encoding that antibiotic resistance (Thavasi et al., 2007). The existence of plasmids bearing extended spectrum β-lactamase (ESBL) and fosfomycin resistance determinants that can spread effectively in *Enterobacteriaceae* have been discovered and are of great clinical concern (Zhao et al., 2015). A high

prevalence of co-occurrence of resistance to fosfomycin, ampicillin, cefuroxime, and rifampicin and other patterns within the urban waterways of Milwaukee may be explained by the existence of mobile genetic elements encoding the resistances for these antibiotics within the microbial community. The detection of the antibiotic resistance genes *ermB*, *tet*(M), bla_{OXA}, bla_{SHV}, and bla_{PSE} (**Figure 1**) which are associated with natural plasmids (Brisson-Noel et al., 1988; Carattoli, 2013) and transposons (Bryan et al., 2004) within the *E. coli* isolates of the urban waterways supports the possible presence of these type of mobile genetic elements.

The greater incidence of resistance observed in the urban waterways (**Figure 1**) was not fully explained by the presence of the resistance genes tested (**Table 2**). The genes *ermB* and bla_{OXA} were present in a greater percentage of isolates in the clinical *E. coli* isolates. While the transfer of the resistance gene *ermB* from Gram-positive cocci to *E. coli* (Brisson-Noel et al., 1988) was previously detected, very few studies attempt to identify and quantify this gene in non-Gram-positives despite the ability to confer resistance (Goetting-Minesky and Fenno, 2010). The *ermB* gene has also been identified in *Bacteroides* (Shoemaker et al., 2001) and *Campylobacter coli* (Qin et al., 2013). To our knowledge, this study is the first to attempt to quantify the presence of *ermB* in *E. coli* from environmental waters. The emergence of bla_{OXA} carbapenemase producing *Enterobacteriaceae* is difficult to detect because of their relative low MICs (Seiffert et al., 2014). The detection of bla_{OXA} is consistent with expectations in the environment (Ojer-Usoz et al., 2014) and clinical isolates (Kaftandzieva et al., 2014). One possible explanation for the lower presence of the genes tested in *E. coli* from the urban waterways to that of the human derived sources may be related to survival in non-human environments. The selective pressure on the *E. coli* in the sediment of the urban waterways from various physical conditions including nutrient availability, temperature, and predation (Beversdorf et al., 2007) and chemicals including antibiotics, personal care products, and metals may select for phenotypes that have co-selection for mechanisms of antibiotic resistance other than the ones encoded by the studied genes (Graham et al., 2010). Co-selection can also be related to cross-resistance, such as multi-drug resistance pumps where the primary roles is thought to provide tolerance to toxic compounds in the environment (Poole, 2005; Martinez et al., 2009; Allen et al., 2010). An additional explanation of the higher incidence of antibiotic resistance within the urban waterways is the selection of antibiotic resistance within the bacteria prior to entering the environment. The sources of the antibiotic resistant *E. coli* in this study can include the WWTP (Korzeniewska et al., 2013) as the major contaminating source was previously determined to be human in the study area (Newton et al., 2013). Determining the resistances from the contaminating bacterial sources may yield information to determine if the resistances observed in this study are due to depositing of resistance or selection of resistance.

Conclusion

This study demonstrates urban waterway sediment as a relevant reservoir of *E. coli* strains containing multiple resistances to antibiotics and antibiotic resistance genes. The presence of known antibiotic resistance genes harbored on mobile genetic elements and a greater level of resistance compared to the local human derived *E. coli* suggests that sediment of the urban waterways may harbor an extensive mobile resistome. Further work is needed to explore the presence of a putative mobile resistome, its contribution to the reservoir of antibiotic resistance, and the potential of transfer to human commensal bacteria and pathogens.

Author Contributions

AK and KH conceived and designed the experiments. AK, MD, and NA performed the experiments. NL, RN, and KH contributed materials, reagents, strains, and tools for analysis. The manuscript was prepared by AK, RN, and KH.

Acknowledgments

This work was funded by the University of Wisconsin Sea Grant Institute under grants from the National Sea Grant College Program, National Oceanic and Atmospheric Administration, U.S. Department of Commerce, and from the State of Wisconsin. Federal grant number (NA14OAR4170092), project number (R/HCE-20). Partial financial support for this work was from Marquette University. Financial support was provided also to NA by the Biological Sciences Department, MU, and to MD by the Summer Research Program (NSF REU site DBI-0755047). The authors would like to thank Amanda Skowronski and Nicholas Callard, undergraduate students, and Meghan Fealey, Brittney Wyatt, and Michael J. Mashock, graduate students, from Marquette University for their contribution in gathering some of the data.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00336/abstract

References

Allen, H. K., Donato, J., Wang, H. H., Cloud-Hansen, K. A., Davies, J., and Handelsman, J. (2010). Call of the wild: antibiotic resistance genes in natural environments. *Nat. Rev. Microbiol.* 8, 251–259. doi: 10.1038/nrmicro2312

Alm, E. W., Zimbler, D., Callahan, E., and Plomaritis, E. (2014). Patterns and persistence of antibiotic resistance in faecal indicator bacteria from freshwater recreational beaches. *J. Appl. Microbiol.* 117, 273–285. doi: 10.1111/jam.12512

Alves, M. S., Pereira, A., Araújo, S. M., Castro, B. B., Correia, A. C., and Henriques, I. (2014). Seawater is a reservoir of multi-resistant *Escherichia coli*, including strains hosting plasmid-mediated quinolones resistance and

extended-spectrum beta-lactamases genes. *Front. Microbiol.* 5:426. doi: 10.3389/fmicb.2014.00426

Aminov, R. I. (2011). Horizontal gene exchange in environmental microbiota. *Front. Microbiol.* 2:158. doi: 10.3389/fmicb.2011.00158

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecol.* 26, 32–46. doi: 10.1111/j.1442-9993.2001.01070.pp.x

Anderson, N. W., Buchan, B. W., Riebe, K. M., Parsons, L. N., Gnacinski, S., and Ledeboer, N. A. (2012). Effects of solid-medium type on routine identification of bacterial isolates by use of matrix-assisted laser desorption ionization–time of flight mass spectrometry. *J. Clin. Microbiol.* 50, 1008–1013. doi: 10.1128/JCM.05209-11

Andersson, D. I., and Hughes, D. (2014). Microbiological effects of sublethal levels of antibiotics. *Nat. Rev. Microbiol.* 12, 465–478. doi: 10.1038/nrmicro3270

Araújo, S., Henriques, I. S., Leandro, S. M., Alves, A., Pereira, A., and Correia, A. (2014). Gulls identified as major source of fecal pollution in coastal waters: a microbial source tracking study. *Sci. Total Environ.* 470–471, 84–91. doi: 10.1016/j.scitotenv.2013.09.075

Baquero, F., Martínez J.-L., and Cantón, R. (2008). Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol.* 19, 260–265. doi: 10.1016/j.copbio.2008.05.006

Bernhard, A. E., and Field, K. G. (2000). A PCR assay to discriminate human and ruminant feces on the basis of host differences in *Bacteroides–Prevotella* genes encoding 16S rRNA. *Appl. Environ. Microbiol.* 66, 4571–4574. doi: 10.1128/AEM.66.10.4571-4574.2000

Beversdorf, L., Bornstein-Forst, S., and McLellan, S. (2007). The potential for beach sand to serve as a reservoir for *Escherichia coli* and the physical influences on cell die-off. *J. Appl. Microbiol.* 102, 1372–1381. doi: 10.1111/j.1365-2672.2006.03177.x

Blair, B. D., Crago, J. P., Hedman, C. J., and Klaper, R. D. (2013a). Pharmaceuticals and personal care products found in the Great Lakes above concentrations of environmental concern. *Chemosphere* 93, 2116–2123. doi: 10.1016/j.chemosphere.2013.07.057

Blair, B. D., Crago, J. P., Hedman, C. J., Treguer, R. J., Magruder, C., Royer, L. S., et al. (2013b). Evaluation of a model for the removal of pharmaceuticals, personal care products, and hormones from wastewater. *Sci. Total Environ.* 444, 515–521. doi: 10.1016/j.scitotenv.2012.11.103

Boehm, A. B., Griffith, J., McGee, C., Edge, T. A., Solo-Gabriele, H. M., Whitman, R., et al. (2009). Faecal indicator bacteria enumeration in beach sand: a comparison study of extraction methods in medium to coarse sands. *J. Appl. Microbiol.* 107, 1740–1750. doi: 10.1111/j.1365-2672.2009.04440.x

Brisson-Noel, A., Arthur, M., and Courvalin, P. (1988). Evidence for natural gene transfer from Gram-positive cocci to *Escherichia coli*. *J. Bacteriol.* 170, 1739–1745.

Bryan, A., Shapir, N., and Sadowsky, M. J. (2004). Frequency and distribution of tetracycline resistance genes in genetically diverse, nonselected, and nonclinical *Escherichia coli* strains isolated from diverse human and animal sources. *Appl. Environ. Microbiol.* 70, 2503–2507. doi: 10.1128/AEM.70.4.2503-2507.2004

Buchan, B. W., Riebe, K. M., and Ledeboer, N. A. (2012). Comparison of the MALDI Biotyper system using Sepsityper specimen processing to routine microbiological methods for identification of bacteria from positive blood culture bottles. *J. Clin. Microbiol.* 50, 346–352. doi: 10.1128/Jcm.05 02111

Bush, K., and Jacoby, G. A. (2010). Updated functional classification of β-lactamases. *Antimicrob. Agents Chemother.* 54, 969–976. doi: 10.1128/AAC.01009-09

Carattoli, A. (2013). Plasmids and the spread of resistance. *Int. J. Med. Microbiol.* 303, 298–304. doi: 10.1016/j.ijmm.2013.02.001

Centers for Disease Control and Prevention [CDC]. (2013). *Antibiotic Resistance Threats in the United States, 2013.* Washington, DC: US Department of Health and Human Services.

Clinical and Laboratory Standards Institute [CLSI]. (2011). *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-First Informational Supplement*. CLSI document M100-S21. Clinical and Laboratory Standards Institute, Wayne, PA.

Czekalski, N., Díez, E. G., and Bürgmann, H. (2014). Wastewater as a point source of antibiotic-resistance genes in the sediment of a freshwater lake. *ISME J.* 8, 1381–1390. doi: 10.1038/Ismej.2014.8

Davies, J., Spiegelman, G. B., and Yim, G. (2006). The world of subinhibitory antibiotic concentrations. *Curr. Opin. Microbiol.* 9, 445–453. doi: 10.1016/j.mib.2006.08.006

Devirgiliis, C., Barile, S., and Perozzi, G. (2011). Antibiotic resistance determinants in the interplay between food and gut microbiota. *Genes Nutr.* 6, 275–284. doi: 10.1007/s12263-011-0226-x

Environmental Protection Agency [EPA], U. S. (2002). *Method 1603: Escherichia coli (E. coli) in Water by Membrane Filtration using Modified Membrane-Thermotolerant Escherichica coli Agar* (*Modified mTEC*). Washington, DC: EPA, Office of Science and Technology.

Ervin, J. S., Van De Werfhorst, L. C., Murray, J. L., and Holden, P. A. (2014). Microbial source tracking in a coastal california watershed reveals canines as controllable sources of fecal contamination. *Environ. Sci. Technol.* 48, 9043–9052. doi: 10.1021/Es502173s

Falagas, M. E., Grammatikos, A. P., and Michalopoulos, A. (2008). Potential of old-generation antibiotics to address current need for new antibiotics. *Expert Rev. Anti. Infect. Ther.* 6, 593–600. doi: 10.1586/14787210.6.5.593

Figueira, V., Serra, E., and Manaia, C. M. (2011). Differential patterns of antimicrobial resistance in population subsets of *Escherichia coli* isolated from waste-and surface waters. *Sci. Total Environ.* 409, 1017–1023. doi: 10.1016/j.scitotenv.2010.12.011

Galán, J.-C., González-Candelas, F., Rolain J.-M., and Cantón, R. (2013). Antibiotics as selectors and accelerators of diversity in the mechanisms of resistance: from the resistome to genetic plasticity in the β-lactamases world. *Front. Microbiol.* 4:9. doi: 10.3389/fmicb.2013.00009

Gao, P., He, S., Huang, S., Li, K., Liu, Z., Xue, G., et al. (2015). Impacts of coexisting antibiotics, antibacterial residues, and heavy metals on the occurrence of erythromycin resistance genes in urban wastewater. *Appl. Microbiol. Biotechnol.* doi: 10.1007/s00253-015-6404-9 [Epub ahead of print].

Goetting-Minesky, M. P., and Fenno, J. C. (2010). A simplified erythromycin resistance cassette for *Treponema denticola* mutagenesis. *J. Microbiol. Methods* 83, 66–68. doi: 10.1016/j.mimet.2010.07.020

Gomez-Alvarez, V., Revetta, R. P., and Santo Domingo, J. W. (2012). Metagenomic analyses of drinking water receiving different disinfection treatments. *Appl. Environ. Microbiol.* 78, 6095–6102. doi: 10.1128/AEM.01018-12

Graham, D. W., Olivares-Rieumont, S., Knapp, C. W., Lima, L., Werner, D., and Bowen, E. (2010). Antibiotic resistance gene abundances associated with waste discharges to the Almendares River near Havana, Cuba. *Environ. Sci. Technol.* 45, 418–424. doi: 10.1021/es102473z

Guber, A. K., Fry, J., Ives, R. L., and Rose, J. B. (2015). *Escherichia coli* survival in, and release from, white-tailed deer feces. *Appl. Environ. Microbiol.* 81, 1168–1176. doi: 10.1128/AEM.03295-14

Ham, Y.-S., Kobori, H., Kang, J.-H., Matsuzaki, T., Iino, M., and Nomura, H. (2012). Distribution of antibiotic resistance in urban watershed in Japan. *Environ. Pollut.* 162, 98–103. doi: 10.1016/j.envpol.2011.11.002

Harwood, V. J., Staley, C., Badgley, B. D., Borges, K., and Korajkic, A. (2014). Microbial source tracking markers for detection of fecal contamination in environmental waters: relationships between pathogens and human health outcomes. *FEMS Microbiol. Rev.* 38, 1–40. doi: 10.1111/1574-6976.12031

Henriques, I. S., Fonseca, F., Alves, A., Saavedra, M. J., and Correia, A. (2006). Occurrence and diversity of integrons and β-lactamase genes among ampicillin-resistant isolates from estuarine waters. *Res. Microbiol.* 157, 938–947. doi: 10.1016/j.resmic.2006.09.003

Hu, J., Shi, J., Chang, H., Li, D., Yang, M., and Kamagata, Y. (2008). Phenotyping and genotyping of antibiotic-resistant *Escherichia coli* isolated from a natural river basin. *Environ. Sci. Technol.* 42, 3415–3420. doi: 10.1021/es7026746

Johnson, J. R., Drawz, S. M., Porter, S., and Kuskowski, M. A. (2013). Susceptibility to alternative oral antimicrobial agents in relation to sequence type ST131 status and coresistance phenotype among recent *Escherichia coli* isolates from US veterans. *Antimicrob. Agents Chemother.* 57, 4856–4860. doi: 10.1128/Aac.00650-13

Jurado-Rabadán, S., de la Fuente, R., Ruiz-Santa-Quiteria, J. A., Orden, J. A., de Vries, L. E., and Agersø, Y. (2014). Detection and linkage to mobile genetic elements of tetracycline resistance gene *tet*(M) in *Escherichia coli* isolates from pigs. *BMC Vet. Res.* 10:155. doi: 10.1186/1746-6148-10-155

Kaftandzieva, A., Trajkovska-Dokic, E., Kotevska, V., Cekovska, Z., and Jankoska, G. (2014). Genotypes of ESBL producing *Escherichia coli* and *Klebsiella pneumoniae* in relation to resistance to antimicrobial drugs. *PRILOZI* 35, 31–38. doi: 10.2478/prilozi-2014-0004

Korzeniewska, E., Korzeniewska, A., and Harnisz, M. (2013). Antibiotic resistant *Escherichia coli* in hospital and municipal sewage and their emission to the environment. *Ecotox Environ. Safe* 91, 96–102. doi: 10.1016/j.ecoenv.2013.01.014

Kümmerer, K. (2004). Resistance in the environment. *J. Antimicrob. Chemother.* 54, 311–320. doi: 10.1093/jac/dkh325

LaPara, T. M., Burch, T. R., McNamara, P. J., Tan, D. T., Yan, M., and Eichmiller, J. J. (2011). Tertiary-treated municipal wastewater is a significant point source of antibiotic resistance genes into Duluth-Superior Harbor. *Environ. Sci. Technol.* 45, 9543–9549. doi: 10.1021/es202775r

Lê, S., Josse, J., and Husson, F. (2008). FactoMineR: an R package for multivariate analysis. *J. Stat. Softw.* 25, 1–18.

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., et al. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* 18, 268–281. doi: 10.1111/j.1469-0691.2011.03570.x

Marti, E., Variatza, E., and Balcazar, J. L. (2014). The role of aquatic ecosystems as reservoirs of antibiotic resistance. *Trends Microbiol.* 22, 36–41. doi: 10.1016/j.tim.2013.11.001

Martinez, J. L., Sánchez, M. B., Martínez-Solano, L., Hernandez, A., Garmendia, L., Fajardo, A., et al. (2009). Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. *FEMS Microbiol. Rev.* 33, 430–449. doi: 10.1111/j.1574-6976.2008.00157.x

Michael, I., Rizzo, L., McArdell, C., Manaia, C., Merlin, C., Schwartz, T., et al. (2013). Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: a review. *Water Res.* 47, 957–995. doi: 10.1016/j.watres.2012.11.027

Munir, M., Wong, K., and Xagoraraki, I. (2011). Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water Res.* 45, 681–693. doi: 10.1016/j.watres.2010.08.033

Newton, R. J., Bootsma, M. J., Morrison, H. G., Sogin, M. L., and McLellan, S. L. (2013). A microbial signature approach to identify fecal pollution in the waters off an urbanized coast of Lake Michigan. *Microb. Ecol.* 65, 1011–1023. doi: 10.1007/s00248-013-0200-9

Newton, R. J., VandeWalle, J. L., Borchardt, M. A., Gorelick, M. H., and McLellan, S. L. (2011). Lachnospiraceae and Bacteroidales alternative fecal indicators reveal chronic human sewage contamination in an urban harbor. *Appl. Environ. Microbiol.* 77, 6972–6981. doi: 10.1128/AEM.05480-11

Ojer-Usoz, E., González, D., García-Jalón, I., and Vitas, A. I. (2014). High dissemination of extended-spectrum β-lactamase-producing Enterobacteriaceae in effluents from wastewater treatment plants. *Water Res.* 56, 37–47. doi: 10.1016/j.watres.2014.02.041

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., et al. (2013). *Vegan: Community Ecology Package. R Package Version* 2.0-8.

Poirel, L., Bonnin, R. A., and Nordmann, P. (2011). Analysis of the resistome of a multidrug-resistant NDM-1-producing *Escherichia coli* strain by high-throughput genome sequencing. *Antimicrob. Agents Chemother.* 55, 4224–4229. doi: 10.1128/AAC.00165-11

Poole, K. (2005). Efflux-mediated antimicrobial resistance. *J. Antimicrob. Chemother.* 56, 20–51. doi: 10.1093/jac/dki171

Poyart, C., Celli, J., and Trieu-Cuot, P. (1995). Conjugative transposition of Tn916-related elements from *Enterococcus faecalis* to *Escherichia coli* and *Pseudomonas fluorescens*. *Antimicrob. Agents Chemother.* 39, 500–506. doi: 10.1128/AAC.39.2.500

Qin, S., Wang, Y., Zhang, Q., Zhang, M., Deng, F., Shen, Z., et al. (2013). Report of ribosomal RNA methylase gene *erm*(B) in multidrug-resistant *Campylobacter coli*. *J. Antimicrob. Chemother.* 69, 964–968. doi: 10.1093/jac/dkt492

R Core Team. (2012). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing. Available at: http://www.R-project.org

Review on Antimicrobial Resistance [RAR]. (2014). *Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations, 2014*. London: Wellcome Trust.

Rodríguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-Melsió, A., et al. (2014). Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. *Water Res.* 69, 234–242. doi: 10.1016/j.watres.2014.11.021

Rosewarne, C. P., Pettigrove, V., Stokes, H. W., and Parsons, Y. M. (2010). Class 1 integrons in benthic bacterial communities: abundance, association with Tn402-like transposition modules and evidence for coselection with heavy-metal resistance. *FEMS Microbiol. Ecol.* 72, 35–46. doi: 10.1111/j.1574-6941.2009.00823.x

Salmore, A., Hollis, E., and McLellan, S. (2006). Delineation of a chemical and biological signature for stormwater pollution in an urban river. *J. Water Health* 4, 247–262.

Seiffert, S. N., Perreten, V., Johannes, S., Droz, S., Bodmer, T., and Endimiani, A. (2014). OXA-48 carbapenemase producing *Salmonella enterica* Kentucky of ST198 in a patient transferred from Libya to Switzerland. *Antimicrob. Agents Chemother.* 58, 2446–2449. doi: 10.1128/AAC.02417-13

Shanks, O. C., Atikovic, E., Blackwood, A. D., Lu, J., Noble, R. T., Santo Domingo, J., et al. (2008). Quantitative PCR for detection and enumeration of genetic markers of bovine fecal pollution. *Appl. Environ. Microbiol.* 74, 745–752. doi: 10.1128/AEM.01843-07

Shoemaker, N., Vlamakis, H., Hayes, K., and Salyers, A. (2001). Evidence for extensive resistance gene transfer among *Bacteroides* spp. and among *Bacteroides* and other genera in the human colon. *Appl. Environ. Microbiol.* 67, 561–568. doi: 10.1128/AEM.67.2.561-568.2001

Silveira, E., Freitas, A. R., Antunes, P., Barros, M., Campos, J., Coque, T. M., et al. (2014). Co-transfer of resistance to high concentrations of copper and first-line antibiotics among *Enterococcus* from different origins (humans, animals, the environment and foods) and clonal lineages. *J. Antimicrob. Chemother.* 69, 899–906. doi: 10.1093/jac/dkt479

Somarelli, J., Makarewicz, J., Sia, R., and Simon, R. (2007). Wildlife identified as major source of *Escherichia coli* in agriculturally dominated watersheds by BOX A1R-derived genetic fingerprints. *J. Environ. Manag.* 82, 60–65. doi: 10.1016/j.jenvman.2005.12.013

Suzuki, M. T., Taylor, L. T., and DeLong, E. F. (2000). Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5′-nuclease assays. *Appl. Environ. Microbiol.* 66, 4605–4614. doi: 10.1128/AEM.66.11.4605-4614.2000

Tacão, M., Moura, A., Correia, A., and Henriques, I. (2014). Co-resistance to different classes of antibiotics among ESBL-producers from aquatic systems. *Water Res.* 48, 100–107. doi: 10.1016/j.watres.2013.09.021

Tadesse, D. A., Zhao, S., Tong, E., Ayers, S., Singh, A., Bartholomew, M. J., et al. (2012). Antimicrobial drug resistance in *Escherichia coli* from humans and food animals, United States, 1950–2002. *Emerg. Infect. Dis.* 18, 741–749. doi: 10.3201/eid1805.111153

Thavasi, R., Aparnadevi, K., Jayalakshmi, S., and Balasubramanian, T. (2007). Plasmid mediated antibiotic resistance in marine bacteria. *J. Environ. Biol.* 28, 617–621.

Varela, A. R., André, S., Nunes, O. C., and Manaia, C. M. (2014). Insights into the relationship between antimicrobial residues and bacterial populations in a hospital-urban wastewater treatment plant system. *Water Res.* 54, 327–336. doi: 10.1016/j.watres.2014.02.003

Vaz-Moreira, I., Nunes, O. C., and Manaia, C. M. (2014). Bacterial diversity and antibiotic resistance in water habitats: searching the links with the human microbiome. *FEMS Microbiol. Rev.* 38, 761–778. doi: 10.1111/1574-6976.12062

Wellington, E. M., Boxall, A. B., Cross, P., Feil, E. J., Gaze, W. H., Hawkey, P. M., et al. (2013). The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. *Lancet Infect. Dis.* 13, 155–165. doi: 10.1016/S1473-3099(12)70317-1

World Health Organization [WHO]. (2014). *Antimicrobial Resistance: Global Report on Surveillance 2014*. Geneva: World Health Organization.

Yang, Y., Li, B., Zou, S., Fang, H. H., and Zhang, T. (2014). Fate of antibiotic resistance genes in sewage treatment plant revealed by metagenomic approach. *Water Res.* 62, 97–106. doi: 10.1016/j.watres.2014.05.019

Yanisch-Perron, C., Vieira, J., and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mpl8 and pUC19 vectors. *Gene* 33, 103–119. doi: 10.1016/0378-1119(85)90120-9

Zhao, J.-Y., and Dang, H. (2012). Coastal seawater bacteria harbor a large reservoir of plasmid-mediated quinolone resistance determinants in Jiaozhou Bay, China. *Microb. Ecol.* 64, 187–199. doi: 10.1007/s00248-012-0008-z

Zhao, J.-y., Zhu, Y.-q., Li, Y.-n., Mu, X.-d., You, L.-p., Xu, C., et al. (2015). Coexistence of SFO-1 and NDM-1 β-lactamase genes and fosfomycin resistance gene *fosA3* in an *Escherichia coli* clinical isolate. *FEMS Microbiol. Lett.* 362, 1–7. doi: 10.1093/femsle/fnu018

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 29 September 2015
doi: 10.3389/fmicb.2015.00977

Diverse and abundant multi-drug resistant *E. coli* in Matang mangrove estuaries, Malaysia

*Aziz Ghaderpour[1,2], Wing Sze Ho[1], Li-Lee Chew[1,2], Chui Wei Bong[1,2], Ving Ching Chong[1,2], Kwai-Lin Thong[1,2] and Lay Ching Chai[1,2]**

[1] Faculty of Science, Institute of Biological Science, University of Malaya, Kuala Lumpur, Malaysia, [2] Institute of Ocean and Earth Sciences, University of Malaya, Kuala Lumpur, Malaysia

*E.*coli, an important vector distributing antimicrobial resistance in the environment, was found to be multi-drug resistant, abundant, and genetically diverse in the Matang mangrove estuaries, Malaysia. One-third (34%) of the estuarine *E. coli* was multi-drug resistant. The highest antibiotic resistance prevalence was observed for aminoglycosides (83%) and beta-lactams (37%). Phylogenetic groups A and B1, being the most predominant *E. coli*, demonstrated the highest antibiotic resistant level and prevalence of integrons (integron I, 21%; integron II, 3%). Detection of phylogenetic group $B2_3$ downstream of fishing villages indicates human fecal contamination as a source of *E. coli* pollution. Enteroaggregative *E. coli* (1%) were also detected immediately downstream of the fishing village. The results indicated multi-drug resistance among *E. coli* circulating in Matang estuaries, which could be reflective of anthropogenic activities and aggravated by bacterial and antibiotic discharges from village lack of a sewerage system, aquaculture farms and upstream animal husbandry.

Keywords: *E. coli*, Matang mangrove estuaries, antibiotic resistance, phylogenetic groups

OPEN ACCESS

Edited by:
Rustam Aminov, Technical University of Denmark, Denmark

Reviewed by:
Veljo Kisand, University of Tartu, Estonia
Daniela Ceccarelli, University of Maryland, USA

***Correspondence:**
Lay Ching Chai, Faculty of Science, Institute of Biological Sciences, University of Malaya, 50603 Kuala Lumpur, Malaysia
lcchai@um.edu.my

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *10 April 2015*
Accepted: *02 September 2015*
Published: *29 September 2015*

Citation:
Ghaderpour A, Ho WS, Chew L-L, Bong CW, Chong VC, Thong K-L and Chai LC (2015) Diverse and abundant multi-drug resistant E. coli in Matang mangrove estuaries, Malaysia. Front. Microbiol. 6:977. doi: 10.3389/fmicb.2015.00977

Introduction

Escherichia coli (*E. coli*), a component of the common intestinal microbiota in humans and warm-blooded animals, is an important indicator of fecal contamination in aquatic environments and food. Certain strains of *E. coli* are able to cause gastrointestinal and extraintestinal infections in humans. The pathogenic *E. coli* that cause gastrointestinal infections include enterohemorrhagic *E. coli* (EHEC), also referred to as Shiga toxin-producing *E. coli* (STEC) or verocytotoxic *E. coli* (VTEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) (Galvin et al., 2010; Koczura et al., 2013).These pathogenic *E. coli*, together with other virulent strains of *E. coli* could cause extraintestinal infections in humans such as pyelonephritis, urinary tract infection, cystitis, neonatal meningitis, and bacteremia (Johnson and Stell, 2000; Galvin et al., 2010; Koczura et al., 2013).

E. coli is divided into four main phylogenetic groups A, B1, B2, and D, based on the presence and absence of, *chu*A, a gene that is responsible for heme transport in enterohemorrhagic O157:H7 *E. coli*; *yja*A, an unknown functional gene which is identified in the recent complete genome sequence of *E. coli* K-12; and TSPE4.C2, an anonymously designated DNA fragment which is the non-coding region in *E. coli* strains (Clermont et al., 2000). These four phylogenetic groups of *E. coli* appear to have distinctive genetic and phenotypic characteristics that are associated with different ecological

niches (Bergthorsson and Ochman, 1998; Johnson et al., 2001; Gordon and Cowling, 2003; Gordon, 2004; Walk et al., 2007). It is interesting to note that most of the *E. coli* strains that are able to endure environmental stresses in the aquatic environment belong to the B1 phylogenetic group (Walk et al., 2007). In addition, *E. coli* strains that belong to the phylogenetic groups B2 and D harbor more virulent genes than those belonging to A and B1 (Johnson et al., 2001). The extraintestinal pathogenic *E. coli* strains are mostly phylogenetic group B2 and to a lesser extent of group D; while the commensal *E. coli* strains are commonly categorized into phylogenetic group A and B1 (Maynard et al., 2004; Vignaroli et al., 2012; Koczura et al., 2013; Pereira et al., 2013). Escobar-Páramo et al. (2004) found that most ETEC, EHEC, and EIEC belong to the phylogenetic groups A and B1; while EPEC, EAEC, and DAEC are usually not linked to any phylogenetic grouping.

The occurrence of *E. coli* in the environment, particularly in aquatic systems, is a global public health concern, especially when the water is used for human activities. The problem is recently exacerbated by the emergence of antimicrobial resistant *E. coli* strains in aquatic environments worldwide (Koczura et al., 2013; Pereira et al., 2013). Antibiotic resistant *E. coli* and other enteric bacteria, that survived the extensive antibiotic treatments in the gut of humans or animals, can enter aquatic systems through discharge from poultry and livestock production, and hospital and municipal wastewaters (Pruden et al., 2006; Pereira et al., 2013). Therefore, the rivers that are used for recreational activities, irrigation, and other purposes, can be efficient vehicles that disseminate the antibiotic resistant bacteria (Pruden et al., 2006; Su et al., 2012; Pereira et al., 2013).

Antibiotic resistance is mostly transferred among bacteria by mobile genetic elements such as integrons, plasmids and transposons. Integrons are well-organized gene expression systems that can integrate one or more non-functional gene cassettes and convert them into expressed genes (Hall and Collis, 1995; Recchia and Hall, 1995; Su et al., 2012). Integrons can easily spread the antibiotic resistance among bacterial species due to their association with plasmids. There are three types of integrons, class 1, 2, and 3 integrons and their classification is according to the integrase gene (*intI*) (Carattoli, 2003; Cambray et al., 2010; Su et al., 2012).

In addition to their use in human medicine, antibiotics are also widely used as therapeutics, prophylactics and metaphylactics in animals and aquaculture farms (Jaime et al., 2012). Twenty different antibiotics (e.g., erythromycin, tetracycline, oxalinic acid, oxytetracycline, amplicillin, norfloxacine, trimethoprim, sulfudimethoxine) and chemicals (e.g., calcium carbonate, potassium permanganate, formalin, and thiodane) are commonly used in aquaculture farms in Malaysia (Keh, 2003). Generally, antibiotics are mixed with feeds, or dissolved directly in water. However, fish are not able to metabolize the antibiotics, which are largely returned to the environment via faces that pose a potential threat to public health (Burridge et al., 2010; Jaime et al., 2012).

Several estuaries within Matang Mangrove Forest Reserve (MMFR), Perak were selected as study sites in this study. The MMFR bears the distinctive features of being one of the best managed mangrove forests in the world, an important nursery area sustaining the country's largest fisheries landings and site to the state's largest brackish water aquaculture farms (Ariffin and Nik Mustafa, 2013). The MMFR exemplifies significant economic benefits and ecological services that could be obtained through sustained forest management. However, the Matang area also demonstrates the increasing dilemma of multiple-use conflicts of land and water resources (Chong et al., 2010). Twenty eight of the 34 permanent settlements in the MMFR are fishing villages sited along the mangrove estuaries with a total population of 31,800 people (Lim and Mohamad Parit, 2001).

A recent study shows that the Matang mangrove estuaries may be facing a great challenge of anthropogenic pollution due to contamination by various potentially pathogenic bacteria including high counts of *E. coli* in both water and sediment (Ghaderpour et al., 2014). This kind of anthropogenic pollution could become a more significant public health issue if these bacteria are resistant to antibiotics. Therefore, in this study, we aimed to (i) investigate the genetic diversity of *E. coli* in Matang estuaries, (ii) determine the virulence factors of the estuarine *E. coli* isolates, and (iii) detect antibiotic resistant *E. coli* and their resistance mechanisms in Matang mangrove estuaries.

Materials and Methods

Study Sites and Sample Collection

The MMFR is located in the northwest coast of peninsular Malaysia (**Figure 1**). It is a silviculture production forest of 41,000 ha managed on a sustainable basis since 1902. Within the sheltered waterways of the forest reserve, nearly 8000 floating fish cages are present, while 385 shrimp and fish ponds cover a total area of 223 ha on the landward margin of the mangrove forests (Ariffin and Nik Mustafa, 2013). Cockle culture covers an extensive area of 4726 ha within and outside the Matang estuaries.

Water and sediment samplings were carried out in the various estuaries, particularly the Sangga Besar, Sepetang and Selinsing Rivers. Sangga Besar River is the main waterway (8 km) traversed by fishing boats between the fishing village at Kuala Sepetang and the coastal fishing grounds. Unlike the Sangga Besar, the Selinsing River is a long waterway (18 km) relatively less used by boats as well as having few floating fish farms at its river mouth. Eight sampling sites (A–H) were set up, from upstream to downstream of the estuary (**Figure 1**). Station A is situated at the upper estuary of the Sepetang River, while stations B to F are located along the Sangga Besar River. Two further sampling sites, G and H were set up at the upstream and downstream of the Selinsing River, respectively. Station A is located upstream of the Kuala Sepetang village, while Station B, C, and G are situated 1–7 km downstream of the village, receiving the untreated sewage and other anthropogenic pollution from the village. Station D and E are located near to cockle culture beds and fish cages, respectively. Station F is situated at the river mouth downstream of the fish cages close to other cockle culture areas on the coastal mudflat. Station H is located 15 km downstream from the Kuala Sepetang village on Selinsing River, with very little human activity (**Figure 1**).

Four water and sediment samples were collected at each sampling station in October 2011 (wet season) and May 2012

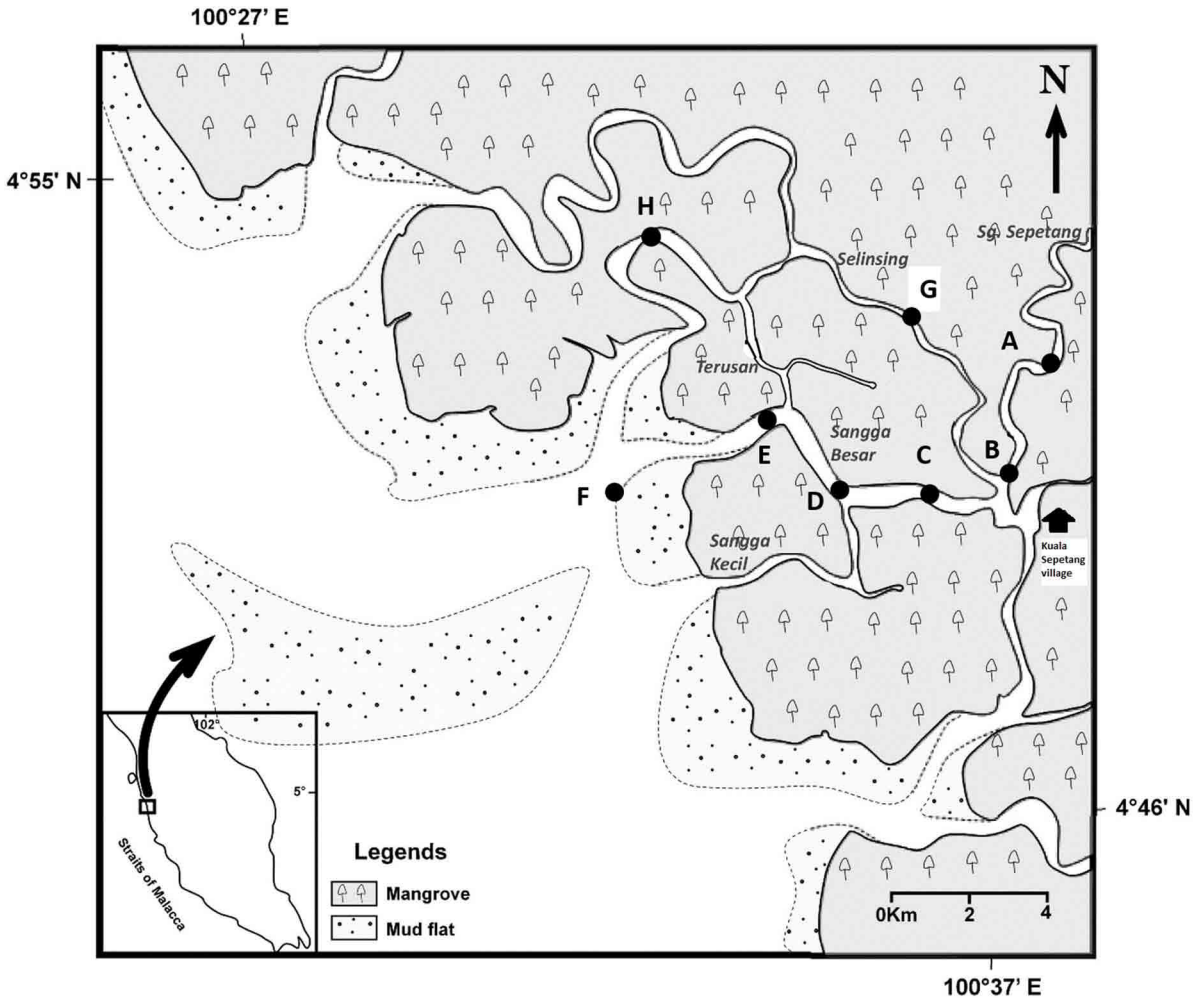


FIGURE 1 | Locations of eight sampling stations (A–H) along Sepetang, Sangga Besar and Selinsing rivers in Matang Mangrove Forest Reserve, Perak, Malaysia.

(dry season). Water samples (0.5 m depths) were collected in acid washed bottles. A 15 cm × 15 cm Ekman grab (Wildco, USA) was used to collect bottom sediment. The top 5 cm surface of the collected sediment samples was sampled using a spatula and kept in sterile bottles. All samples were stored in ice, and immediately transferred into the laboratory freezer kept at 4°C until the analyses were carried out within 24 h of sampling.

Isolation of *E. coli* and Detection of Their Housekeeping and Virulence Genes

Membrane filtration technique was used to isolate *E. coli*. Water (5 ml) and sediment (1 g in 9 ml of sterile 0.85% saline) samples were filtered through 0.45 mm nitrocellulose filters (47 mm diameter). The filters were then transferred on CHROMagar ECC (CHROMagar Inc., Paris, France) and incubated at 42 ± 0.5°C for 24 h. Blue colonies as presumptive *E. coli* were randomly picked and purified on Luria Bertani medium. After purification, presumptive *E. coli* isolates were kept in stab and in glycerol forms (Luria Bertani Broth with 50% glycerol) at 37°C and −20°C, respectively for the future analysis. The identity of the presumptive *E. coli* isolates was confirmed by PCR targeting the *pho*A gene, an *E. coli* housekeeping gene (Yu and Thong, 2009).

Two different multiplex PCR assays were carried out for all confirmed *E. coli* to identify the pathogenic *E. coli* (EHEC, EPEC, EAEC, ETEC, EIEC and DAEC). For both multiplex PCR, reaction mixture was carried out in 25 μL volume consisting of 1X green buffer (5X green GoTaq reaction buffer, pH 8.5), 0.5U of *Taq* DNA polymerase (Promega, Madison,Wis, USA), 1.65 mM $MgCl_2$, 0.3μM of the selected primers, 220 μM of each deoxynucleoside triphosphate (dNTP), and 5 μL of DNA template. PCR amplification and primers used in this study were previously described by Gómez-Duarte et al. (2009). Primers sequences are listed in Supplementary Table 1. Six positive control strains (*E. coli* 2060–004, E2348/69, JM221, E9034A, C1845, and EC-12) were used in this study and sterile water was included as negative control. The six positive

strains were provided by Oscar G. Gomez-Duarte, International Enteric Vaccines Research Program [IEVRP], University of Iowa Children's Hospital, Iowa City, IA (Gómez-Duarte et al., 2009).

Phylogenetic Grouping

A triplex PCR assay was used to determine the phylogenetic groups (A, B1, B2, and D) of *E. coli*. PCR conditions for presence or absence of *chu*A, *yja*A, and TSPE4-C2 were used as described by Clermont et al. (2000) and Carlos et al. (2010) (**Table 1**). Each reaction was performed in 25 μL volume containing 5 μL (appr 10 ng) DNA template, 120 μM each dNTP, 1X PCR buffer, 0.5 U *Taq* polymerase, 1.5 mM $MgCl_2$, 0.4 μM of yjaA primers, 0.24 μM each chuA, and TSPE4-C2 primers (Supplementary Table 1). Combination of presence or absence of *chu*A, *yja*A, and TSPE4-C2 was used for identification of phylogenetic grouping as follows: phylogenetic group A (A0, −/−/−; A1, −/+/−), phylogenetic group B (B1, −/−/+), phylogenetic group B2 ($B2_2$, +/+/−; $B2_3$, +/+/+) and phylogenetic group D (D1, +/−/−; D2, +/−/+) (Carlos et al., 2010).

Antibiotic Susceptibility Testing

Agar disc diffusion method on Mueller-Hinton Agar (Oxoid, Basingstoke, UK) was used to determine the antibiotic resistance patterns among *E. coli* isolates. A total of 15 antibiotics belonging to 7 classes were tested, including aminoglycosidase: gentamicin (CN, 10 μg), kanamycin (K, 30 μg), streptomycin (S, 25 μg), and neomycin (N, 30 μg); β-lactams: amoxicillin/clavulanic acid (AMC, 30μg), ampicillin (AMP, 10 μg), ceftriaxone (CRO, 30 μg), ceftiofur (EFT, 30 μg); quinolones: nalidixic acid (NA, 30 μg), oxolinic acid (OA, μg); fluoroquinolone: enrofloxacin (ENR, 5 μg), ciprofloxacin (CIP, 5 μg); tetracycline: tetracycline (TE, 30 μg); phenicols: chloramphenicol (C, 30 μg), and sulfamethoxazole/trimethoprim (SXT, 25 μg) (Oxoid Ltd.,Basingtoke, Hampshire, England). The bacteria were designated as resistant, intermediate and sensitive according to the Clinical Laboratory Standards Institute (CLSI)'s recommendations (CLSI, 2012). Bacteria strains that were resistant and intermediate were categorized as non-susceptible while the sensitive strains were categorized as susceptible.

Detection of Class 1, 2, and 3 Integrons

All *E. coli* isolates were screened for integrase genes. Three different sets of primers were used to detect class 1, 2, and 3 integrase genes (Supplementary Table 1). Primers IntI1-F, IntI1-R and 5′CS and 3′CS were used to detect *intI*1 gene and class 1 integron gene cassettes, respectively. For class two integrons, IntI2-F, IntI2-R, and attI2-F/orfX-R primers were used for amplifying *intI*2 and class 2 integron gene cassettes, respectively. The primer IntI3-F/IntI3-R was used to amplify *intI*3 gene.

The reaction mixture for all PCR assays was performed in 25 μL volume which consisted of 1X PCR green buffer, 1.4 mM $MgCl_2$, 120 μM of each dNTP, 0.06 μM of each primers, and 0.5 U of *Taq* DNA polymerase (Promega, Madison, Wisconsin, USA). The multiplex PCR amplification of integrase type 1 and 2, and class 1 integron gene cassettes was performed as previously described by Lim et al. (2009).

Confirmation of PCR Products by DNA Sequencing

PCR products were purified and submitted to a commercial company (First BASE Laboratories, Malaysia) for sequencing using BigDye® Terminator v3.1 cycle sequencing kit chemistry. Homologs search against the GenBank databases of nr/nt using BLASTN (http://blast.ncbi.nlm.nih.gov/blast) were carried out for all the DNA sequences obtained.

Genetic Diversity of *E. coli* Isolates Using REP-PCR

Genetic diversity of 148 *E. coli* isolates was analyzed by Repetitive Extragenic Palindromic-PCR (REP-PCR) using REP primer (Supplementary Table 1). Bacterial isolates were grown in 1 ml of sterile Luria Bertani broth overnight at 37°C. Bacterial suspension in 1.5 ml microfuge tubes were centrifuged and washed with sterile Phosphate buffered saline (PBS) and 1X TE (Tris-EDTA) buffer. Bacterial pellets were suspended in sterile water and were then heated at 99°C for 10 min and chilled on ice for 20 min. The extracted crude DNA was used for amplification with REP oligonucleotides (Operon Biotechnologies GmBH, Germany) as previously reported (Lim et al., 2009). REP-PCR assay was carried out in 25 μL volume which included of 1X buffer (5X buffer GoTaq reaction buffer, pH 8.5), 1U of *Taq* DNA polymerase (Promega, Madison, Wisconsin, USA), 2.5 mM $MgCl_2$, 200 μM of each dNTP 0.5 μM of the primer and 4 μL of DNA template. Temperature program for this assay consisted of initial denaturing at 94°C for 7 min, followed by 30 cycles of 30 s at 94°C, 1 min at 44°C, 8 min at 72°C and a final extension step 16 min at 65°C. PCR products were loaded in 1.5% agarose gels and stained in Gel red nucleic acid stain (Biotium Inc, USA). Banding patterns were analyzed by GelCompar II, version 2.5 (Applied Maths, Kortrijk, Belgium).

Statistical Analysis

Prevalence of the antibiotic resistance was determined using x/y 100% equation, where x is defined as number of resistant *E. coli* isolates to antibiotics, and y is total isolated number of *E. coli* from each sample. Principle component analysis (PCA) was used to depict the distribution of *E. coli* with respect to phylogenetic groups, integron genes, and antibiotics resistance among eight sampling stations in Matang mangrove estuary. To perform the PCA procedure, the number of isolates of *E. coli* containing the phylogenetic groups, integron genes and antibiotics resistance was square root transformed to meet the parametric assumptions. PCA was performed using CANOCO 4.5 software (Ter Braak and Smilauer, 2002). Pearson's chi-squared goodness-of-fit test was applied to evaluate the significant difference among phylogenetic groups based on the frequency data. Also, comparisons of associations between phylogroups and antibiotic resistance were done using the chi- square test when no more than 20% of the expected cell frequencies were <5 and all individual expected counts were ≥ 1, otherwise Fisher's exact test (two-tailed) was used. The degree of association between the presence of antibiotic resistance and presence of integron genes were tested using the correlation (R_n) of Ives and Gibbons for dichotomous normal-scale data in Zar (2010). Significance was tested using the

TABLE 1 | Antibiotic susceptibility characterization of 148 isolates of *E. coli* isolated from estuarine water and surface sediments of Matang mangrove estuary, Perak, Malaysia.

Characteristics	No. of Isolates	Antibiotics[a,b,c]															Antibiotic resistance pattern				
		I				II				III				IV	V	VI					
		N	S	K	CN	AMC	AMP	CRO	EFT	ENR	CIP	OA	NA	TE	C	SXT	≥1 antibiotic	≥3 antibiotics	≥6 antibiotics	≥9 antibiotics	All antibiotics
STATIONS																					
A	17	14 (82)	7 (41)	3 (18)	2 (12)	1 (6)	3 (18)	1 (6)	1 (6)	3 (18)	1 (6)	2 (12)	3 (18)	6 (35)	2 (12)	1 (6)	16 (94)	7 (41)	2 (12)	1 (6)	1 (6)
B	28	24 (86)	12 (43)	9 (32)	1 (4)	4 (14)	16 (57)	0 (0)	0 (0)	11 (39)	7 (25)	14 (50)	14 (50)	13 (46)	10 (36)	13 (47)	28 (100)	15 (54)	13 (47)	8 (29)	3 (11)
C	8	5 (63)	2 (25)	1 (13)	0 (0)	0 (0)	2 (25)	0 (0)	0 (0)	3 (38)	1 (13)	3 (38)	3 (38)	3 (38)	1 (13)	0 (0)	6 (75)	3 (38)	2 (25)	1 (13)	0 (0)
D	24	13 (54)	12 (50)	5 (21)	0 (0)	0 (0)	10 (42)	0 (0)	0 (0)	7 (29)	3 (13)	3 (13)	5 (21)	9 (38)	8 (33)	6 (25)	19 (79)	9 (38)	6 (25)	5 (21)	0 (0)
E	20	16 (80)	6 (30)	2 (10)	0 (0)	0 (0)	3 (20)	0 (0)	0 (0)	1 (5)	0 (0)	0 (0)	1 (5)	1 (5)	1 (5)	0 (0)	18 (90)	1 (5)	0 (0)	0 (0)	0 (0)
F	8	7 (88)	2 (25)	2 (25)	1 (13)	5 (63)	3 (38)	0 (0)	2 (25)	2 (25)	0 (0)	1 (13)	2 (25)	2 (25)	0 (0)	1 (13)	7 (87)	4 (50)	2 (25)	2 (25)	0 (0)
G	19	14 (74)	2 (11)	4 (21)	0 (0)	6 (32)	8 (42)	1 (5)	1 (5)	3 (16)	1 (5)	3 (16)	4 (21)	6 (32)	2 (11)	5 (26)	17 (90)	9 (48)	3 (16)	1 (5)	0 (0)
H	24	16 (67)	3 (13)	2 (8)	1 (4)	0 (0)	2 (8)	0 (0)	0 (0)	2 (8)	1 (4)	1 (4)	1 (4)	2 (8)	0 (0)	2 (8)	17 (71)	2 (8)	1 (4)	1 (4)	0 (0)
PHYLOGROUPES																					
A	38	29 (76)	10 (26)	8 (21)	2 (5)	5 (13)	16 (42)	1 (3)	1 (3)	13 (34)	5 (13)	11 (29)	11 (29)	12 (32)	8 (21)	7 (18)	34 (98)	15 (40)	11 (29)	6 (16)	1 (3)
B1	65	50 (77)	14 (22)	16 (25)	1 (2)	6 (9)	22 (34)	0 (0)	1 (2)	17 (26)	8 (12)	15 (23)	20 (31)	21 (32)	13 (20)	14 (22)	55 (85)	25 (39)	15 (23)	11 (17)	3 (5)
B2	7	3 (43)	5 (71)	0 (0)	0 (0)	2 (29)	1 (15)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (29)	0 (0)	1 (15)	6 (86)	2 (29)	0 (0)	0 (0)	0 (0)
D	38	27 (71)	10 (26)	4 (11)	2 (5)	3 (8)	9 (24)	1 (3)	2 (5)	2 (5)	1 (3)	1 (3)	2 (5)	7 (18)	3 (8)	3 (8)	34 (98)	8 (21)	3 (8)	2 (5)	0 (0)
SEASONAL																					
Wet	56	42 (75)	2 (4)	6 (11)	0 (0)	0 (0)	14 (25)	0 (0)	0 (0)	3 (5)	3 (5)	4 (7)	5 (9)	5 (9)	5 (9)	3 (5)	44 (79)	5 (9)	3 (5)	3 (5)	0 (0)
Dry	92	67 (73)	44 (48)	22 (24)	5 (5)	16 (17)	34 (37)	2 (1)	4 (4)	29 (32)	11 (12)	23 (25)	28 (30)	37 (40)	19 (21)	25 (27)	84 (91)	45 (49)	26 (28)	16 (17)	4 (4)
Total	148	109 (74)	46 (31)	28 (19)	5 (3)	16 (11)	47 (33)	2 (1)	4 (3)	32 (22)	14 (10)	27 (18)	33 (22)	42 (28)	24 (16)	28 (19)	128 (87)	50 (34)	29 (20)	19 (13)	4 (3)

[a] *Number of E. coli isolates (percentage over number of isolates,%).*

[b] *Antibiotic class. I, aminoglycosides; II, beta-lactams; III, quinolones/fluoroquinolones; IV, tetracyclines; V, phenicols; VI, sulpha group.*

[c] *Antibiotic. N, neomycin; S, streptomycin; K, kanamycin; CN, gentamicin; AMC, amoxicillin/clavulanic acid; AMP, ampicillin; CRO, ceftriazone; EFT, ceftiofur; ENR, enrofloxacin; CIP, ciprofloxacin; OA, oxolinic acid; NA, nalidixic acid; TE, tetracycline; C, chloramphenicol; SXT, sulfamethoxazole/trimetroprim.*

chi-square contingency test. All tests were performed at the 5% level of significance.

Results

Distribution of Phylogenetic Groups and Pathotypes of *E. coli* in Matang Mangrove Estuaries

A total of 175 presumptive *E. coli* bacteria were isolated from 64 water and sediment samples, during dry and wet seasons. Out of 175 presumptive *E. coli* isolates, 148 (85) were confirmed as *E. coli*, of which 17 were from station A, 28 from station B, 8 from station C, 24 from station D, 20 from station E, 8 from station F, 19 from station G and 24 from station H.

Among the 148 *E. coli* isolates, phylogenetic group B1 was the most prevalent ($n = 65$, 44%) followed by phylogenetic group A and D which had similar prevalence ($n = 38$; 26%). The phylogenetic group B2 was the least prevalent (5%) in Matang mangrove estuaries, and was only detected during the dry season. Phylogenetic groups B1 and D were present at all the stations located along Sangga Besar and Selinsing Rivers. Phylogenetic group B1 was the most frequent isolate from all stations except station E and H, where phylogenetic groups A and D predominated, respectively (**Figure 2**). Phylogenetic group D was more frequently isolated in the wet season (32%) than in the dry season (22%). The prevalence of group A was significantly higher at station E, located in Sangga Besar River ($\chi^2 = 16.526$, $df = 6$, $p = 0.011$); while station E recorded a significantly lower occurrence of *E. coli* subset D. Station H located downstream of Selinsing River had significantly higher occurrence of *E. coli* subset D ($\chi^2 = 20.105$, $df = 6$, $p = 0.005$) (**Figures 1**, **2**).

The genotypes of each phylogenetic group (A0/A1, $B2_2/B2_3$, and D1/D2) were not distributed homogenously among all stations. Genotype A0 comprised almost two thirds of

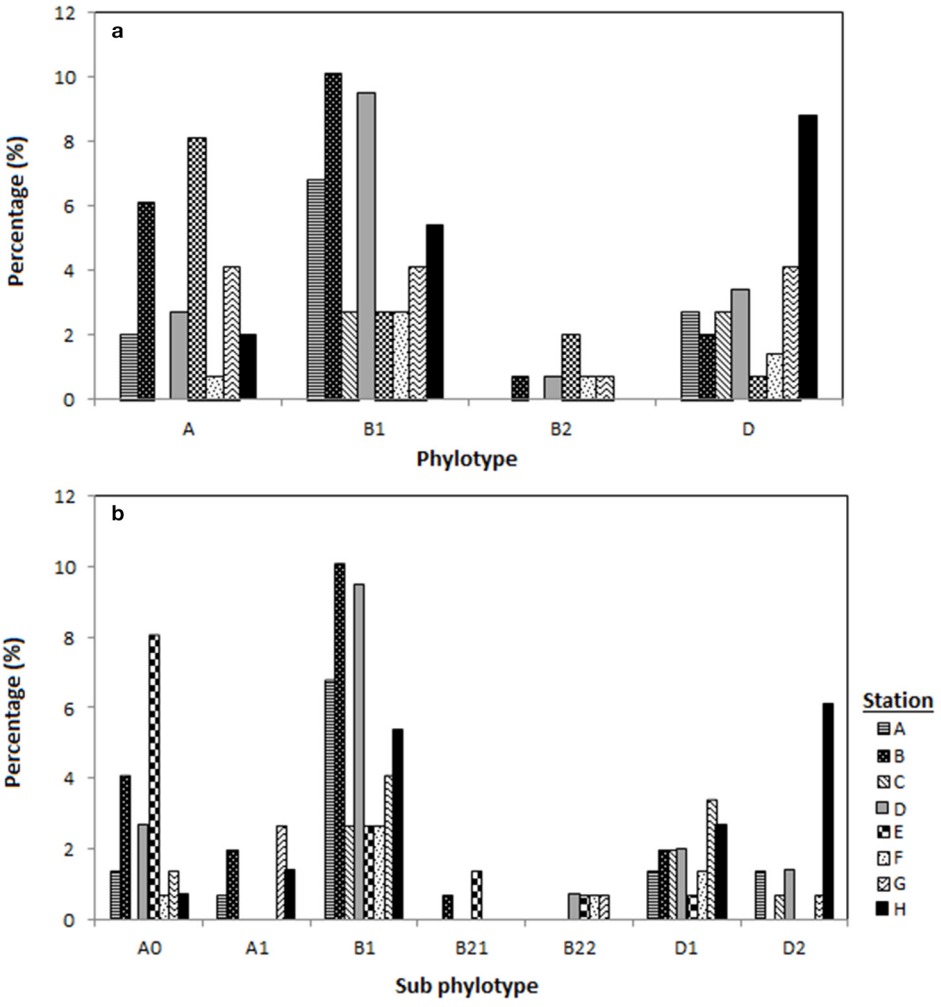


FIGURE 2 | Distribution of the *E. coli* phylogenetic groups in eight different stations located in Sepetang River (A), Sangga Besar River (B–E) and Selinsing River (G and H) and coastal waters (F), (a) distribution of *E. coli* according to the respective phylogenetic groups (A, B1, B2, D) and (b) according to phylogroup subtyping (A0, A1, B1, $B2_1$, $B2_2$, D1, D2).

phylogenetic group A and could be detected in almost all stations, while genotype A1 was only present in the upstream of Sangga Besar River (station A and B) and Selinsing River (station G and H). Genotype $B2_3$ was only detected from station D, E, F, and G which are all located downstream of a fishing village (**Figure 2b**).

None of the 148 isolates was EHEC, EPEC, ETEC, DAEC, or EIEC. However, two isolates originated from station D located upstream of Sangga Besar River and station G located upstream of Selinsing River were identified to be EAEC.

Antibiotic Resistance among the *E. coli* Isolates

Among the 148 *E. coli* isolates from Matang mangrove estuaries, 128 (87%) were non-susceptible to at least one of 15 antibiotics tested (**Table 2**). Only 20 (14%) *E. coli* isolates were sensitive to all the 15 tested antibiotics. The antibiotic resistance percentages were: N (74%), S (31%), K (19%), CN (3%), AMC (11%), AMP (33%), CRO (1%), EFT (3%), ENR (22%), CIP (10%), OA (18%), NA (22%), TE (28%), C (16%), and SXT (19%) (**Table 1** and Supplementary Table 2).

In comparisons among stations, Principle Component Analysis (PCA) showed that high antibiotic resistance among the *E. coli* isolates was detected at the stations (B and D) near to the fishing village (positive direction of PCA1, **Figure 3**). Most *E. coli* isolated from these stations were phylogenetic group B1 and carried class 1 integron. Moreover, high antibiotic resistances especially to aminoglycosides and beta-lactams were also detected in *E. coli* isolates from station F located on the cockle mud flat. On the other hand, low antibiotic resistance of *E. coli* isolates was detected at the stations with less anthropogenic influence (station H) and close to the fish cages (station E) (**Table 1** and **Figure 3**). Overall, *E. coli* isolates from Matang mangrove estuaries demonstrated high prevalence of multi-drug resistance (MDR). One third of the tested *E. coli* isolates (34%) were found to be non-susceptible to three or more antibiotics (3R); and about one in every five isolates (20%) were non-susceptible to six or more antibiotics (6R) tested in this study (**Table 1** and Supplementary Table 2). Further, four out of 148 isolates (3%) were non-susceptible to all of the 15 antibiotics examined in this work (**Table 1** and Supplementary Table 2). Fifty out of 148 (34%) *E. coli* samples isolated from Matang mangrove estuaries demonstrated resistance to at least three antibiotic classes and 10% were non-susceptible to all the seven antibiotic classes tested (**Table 1** and Supplementary Table 2).

The analysis of the phylogenetic groups with respect to their antibiotic resistance indicated that all groups showed some level of resistance to antibiotics. However, *E. coli* isolates in A and B1 phylogenetic groups (positive direction of PCA1) generally shows increasing resistance to most antibiotics as compared to the B2 and D phylogenetic groups (negative direction of PCA1). The phylogenetic group A and B1 demonstrated higher non-susceptibility level toward beta-lactams, (fluoro) quinolones, tetracycline, phenicols, and sulfamethoxazole/trimethoprim. Additionally, multi-drug resistance phenotype was more frequently identified in groups A and B1 (**Table 1** and Supplementary Table 2). Chi-square independence test found only significant association between phylogroups with enrofloxacin (ENR; $\chi^2 = 12.27$; $df = 3$; $p = 0.007$), oxolinic acid (OA; $\chi^2 = 11.71$; $df = 3$; $p = 0.008$) and nalidixic acid (NA; $\chi^2 = 12.04$; $df = 3$; $p = 0.001$), which are all (fluoro) quinolones.

TABLE 2 | Intergron and gene cassette detection in Matang estuary.

Characteristic	Isolates no	None integras gene	IntI1	IntI2	IntI1 & IntI2	Gene cassette	Contents of gene cassette
STATIONS							
A	17	16 (94)	1 (6)	1 (6)	1 (6)	0 (0)	
B	28	15 (54)	13 (46)	2 (7)	2 (7)	2 (7)	aadA22; dfrA17
C	8	7 (88)	1 (13)	0 (0)	0 (0)	0 (0)	
D	24	17 (71)	7 (29)	1 (13)	1 (13)	2 (8)	aadA1; dfrA5
E	20	20 (100)	0 (0)	0 (0)	0 (0)	0 (0)	
F	8	5 (63)	3 (38)	0 (0)	0 (0)	0 (0)	
G	19	15 (79)	4 (21)	0 (0)	0 (0)	2 (11)	dfrA17; dfrA5
H	24	22 (92)	2 (8)	0 (0)	0 (0)	1 (4)	dfrA1+aadA1
PHYLOGROUPES							
A	38	29 (76)	9 (24)	3 (8)	3 (8)	1 (3)	dfrA1+aadA1
B1	65	50 (77)	15 (23)	1 (2)	1 (2)	2 (3)	dfrA5; aadA22
B2	7	6 (86)	1 (14)	0 (0)	0 (0)	0 (0)	
D	38	32 (84)	6 (16)	0 (0)	0 (0)	4 (11)	dfrA5; aadA1; dfrA17; dfrA17
SEASONAL							
Dry	56	53 (95)	3 (5)	0 (0)	0 (0)	0 (0)	
Wet	92	64 (70)	28 (31)	4 (4)	4 (4)	7 (8)	aadA22; dfrA17; aadA1; dfrA5;dfrA17; dfrA5; dfrA1+aadA1
Total	148	117 (79)	31 (21)	4 (3)	4 (3)	7 (5)	

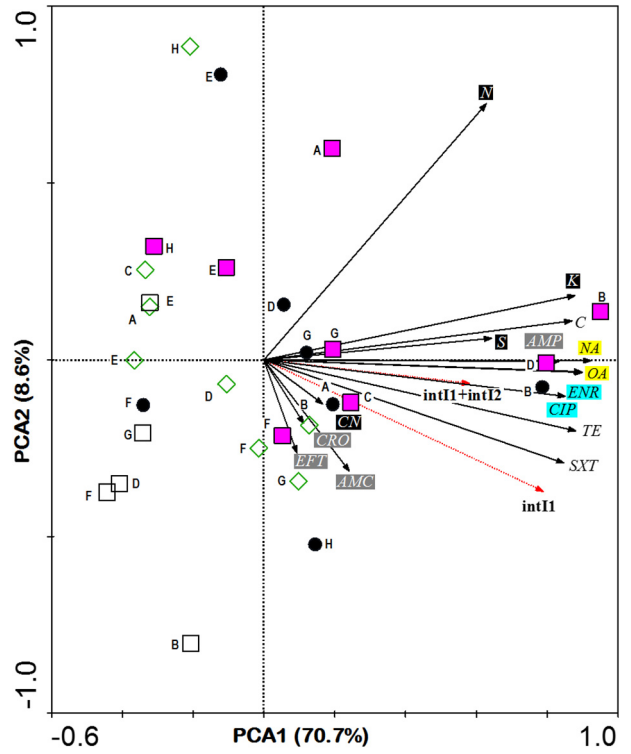


FIGURE 3 | PCA ordination biplot showing the distribution of *E. coli* phylogenetic groups by sampling stations with respect to their resistance to various antibiotics and the integras genes they carried. The first (PCA1) and second (PCA2) principal components respectively explained 70.7 and 8.6% of the total variance of the explanatory variables. Phylogroups are denoted by various symbols: black circle A, pink square B1, empty square B2 and empty diamond D. Bold letter beside symbol denotes sampling stations located in Sepetang River (A), Sangga Besar River (B–E), Selinsing River (G and H) and adjacent coastal water (F). Black solid arrows indicate isolates resistant to 15 antibiotics: Class I (black box), CN, aminoglycosidase: gentamicin; K, kanamycin; S, streptomycin; N, neomycin; Class II (gray box); AMC, β-lactams: amoxicillin/clavulanic acid; AMP, ampicillin; CRO, ceftriaxone; EFT, ceftiofur; Class III (blue box); ENR, fluoroquinolone: enrofloxacin; CIP, ciprofloxacin; Class IV (yellow box); NA, quinolones: nalidixic acid; OA, oxolinic acid; Class V; TE, tetracycline: tetracycline; Class VI; C, phenicols: chloramphenicol and Class VII; SXT, sulfamethoxazole/trimethoprim. Red dotted arrows indicate *E. coli* integron gene (s).

Prevalence of the Class 1, 2, and 3 integrons

All 148 *E. coli* isolates were screened for class I, II and III integrons. Class I and II integrons were detected in 31 (21%) and 4 (3%) isolates, respectively (**Table 2**). Class III integron was absent in the *E. coli* isolates in Matang mangrove estuaries. Four isolates (3%) harbored both class I and II integrons. Of the 31 *intI*1-positive isolates, only 7 (23%) harbored gene cassettes; while none of the *intI*2 positive isolates carried any gene cassette. The size of the gene cassette in class 1 integron ranged from 700 to 1200 bp. Only one out of 148 isolates was found to carry more than one gene cassette, and this particular isolate was obtained from downstream of the Selinsing River (station H). The DNA sequences of all class I integrons have been deposited in GenBank under accession nos. KR952338-KR952345.

Five types of the resistance gene cassettes were detected in this study (**Table 2**). Three types of *dfr*A (*dfr*A 1, *dfr*A 5, and *dfr*A 17) were identified, which encoded the dihydrofolate reductase enzyme, mediating resistance to trimethoprim. Additionally, two types of the *aad*A (*aad*A 1 and *aad*A 22) were detected in class 1 integron that confers resistance to spectinomycin and streptomycin. The *aad*A and *dfr*A genes can be present alone or in combination with each other and other resistance genes as only one cassette combination (*dfr*A1 + *aad*A1) was recovered in this study (**Table 2**). All *E. coli* isolates that carried the *aad*A and *dfr*A genes were resistant to aminoglycosides and SXT, respectively (**Table 2**).

Half of the *intI* positive *E.coli* belonged to group B1 ($n = 15$, 48%) followed by nine isolates (29%) in group A and six isolates (19%) in group D. Only one isolate in group B2 was detected as *intI*1 positive. This integron gene was closely associated with most of the antibiotic resistance. In the PCA biplot, the gradients of increasing number of *IntI* positive isolates were in the same direction as the gradients of antibiotic resistance (**Figure 3**). Resistance to all antibiotics (except N, AMC, CN, and CRO), i.e., 73.3% of the 15 tested antibiotics, was significantly correlated to *intI*1-positive isolates ($p < 0.001$), with correlation (R_n) values that ranged from 0.50 (S) to 0.81 (SXT) (Supplementary Table 3). Of the eleven antibiotic resistant isolates that were *intI*1-positive, seven were also significantly correlated to *intI*2-positive isolates ($p < 0.03$), with R_n values that ranged from 0.49 (TE) to 0.73 (C). Among four *intI*2 positive isolates, three and one isolates belonged to phylogenetic group A and B1, respectively. Moreover, four out of seven isolates (57%) harbored gene cassette class 1 belonged to phylogenetic group D (**Table 2**).

Genetic Diversity of *E. coli*

All the confirmed 148 *E. coli* isolates that were recovered in October 2011 and May 2012 were subjected to REP-PCR. REP-PCR was carried out to determine the diversity of *E. coli* using UPGMA (unweighted pair-group method using arithmetic averages). REP-PCR subtyped the 148 *E. coli* isolates into 118 different REP profiles comprising of 200 to 2000 bp DNA fragments. At 80% similarity, the coefficient of similarity (*F*-value) ranged from 0.69 to 1.0 and the 148 isolates were clustered into seven groups (Supplementary Table 4). Cluster I and V included 76 and 27 *E. coli* isolates from different stations, respectively. Another 45 *E. coli* isolates were grouped into five clusters with each comprising of two to 18 isolates. Most of resistant isolates including MDR phenotypes were grouped into cluster, I, V, and III of the REP-PCR profile (Supplementary Table 4).

Discussion

Multi-Drug Resistant (MDR) *E. coli* in Matang Mangrove Estuaries

In the present study, the Matang mangrove estuaries harbor a high prevalence of antibiotic resistant *E. coli* in surface waters and sediments., the results indicated that four in every five *E. coli* isolates from Matang estuaries were non-susceptible to one or more antibiotics tested (1R); and one out of four 1R Isolates

was non-susceptible to six or more antibiotics tested (**Table 1**). About one-third of the *E. coli* isolated from Matang estuaries were MDR and this figure is relatively higher compared to Tagus estuary in Portugal, in which 19% of the *E. coli* isolates were MDR (Pereira et al., 2013). However, the antibiotic resistant level was lower compared to the 88% found in Dongjiang River, China (Su et al., 2012). Laroche et al. (2009) also reported a relatively high level of MDR *E. coli* (39%) in Seine River, France. The high level of MDR *E. coli* observed in all of these studies from different parts of the world, including the current work highlighted the potential risk of dissemination of antibiotic resistance traits via the aquatic system.

PCA analysis showed that the prevalence of antibiotic resistance in *E. coli* was related to the sampling stations, in which antibiotic resistant *E. coli* was more frequently isolated from the upstream of both Sangga Besar and Selinsing Rivers. Station B was one of the stations with high numbers of MDR *E. coli* isolates recovered. This is likely due to its proximity to Kuala Sepetang fishing village where there is open sewage disposal and poor sanitation (Ghaderpour et al., 2014). Majority of *E. coli* isolates that demonstrated 100% non-susceptibility to all 15 antibiotics tested were isolated from station B. Also, *E. coli* isolates that showed non-susceptibility toward aminoglycosides and beta-lactams were more frequently isolated from stations located downstream of Kuala Sepetang or between the latter and Sangga Besar fishing village at the river mouth (**Table 1**). The findings suggested anthropogenic sources as the major contributor to the presence of antibiotic resistant *E. coli* in Matang mangrove estuaries. As the results show, beta-lactams resistant *E. coli* (i.e., non-susceptible to amocixillin-clavulanic acid, ampicillin, ceftriazone, or ceftiofur) was least frequently isolated from station H, but more frequently isolated from station F (75%) and station B (57%) located downstream of the villages. The highest antibiotic resistance prevalence was detected for neomycin (74%), ampicillin (33%), streptomycin (31%), and tetracycline (28%). The major antibiotic resistance found among *E. coli* isolates was in agreement with previous studies in aquatic environments (Hamelin et al., 2007; Laroche et al., 2009; Pereira et al., 2013). This resistance may reflect the abuse of antibiotics in human and veterinary medicine and aquaculture farms in the study area. The direct discharge of untreated sewage from the villages into the estuarine system, active fish and shrimp farming, as well as the presence of upstream swine farms are the main contributing factors to the high prevalence of MDR *E. coli* in Matang estuaries. Aquaculture could be a significant contributor to antibiotic resistance since the use of antibiotics, pesticides, and other chemotherapeutic agents in feed additives and treatment baths is as yet unregulated in Malaysia (Majusha et al., 2005; Ibrahim et al., 2010). Sulfonamides, tetracyclines, oxytetracycline, ampicillin, chloramphenicol, and quinoline are common antibiotics used in aquaculture farms in Perak as well as in the country (Mohamed et al., 2000; Keh, 2003). Nevertheless, reservoir sources of *E.coli* may include shorebirds and other feral animals such as monkeys, flying foxes and bats, as well as domestic animals, especially dogs (Frenzel and Couvillion, 2002).

It is interesting to note that even though the *E. coli* count at station F was low as reported by Ghaderpour et al. (2014), which resulted in lower isolation rate, a high level of antibiotic resistance was found in *E. coli* isolates from station F (**Table 1** and **Figure 2**). A high bacteria count is expected at the river mouth due to bacterial contamination from the upstream, but due to the fact that *E. coli* is sensitive to high salinity (Gao et al., 2013), at station F, most of the *E. coli* would have been inactivated except antibiotic resistant strains that are also tolerant to high salinity. On the other hand, low antibiotic resistance was found in station H, downstream of Selinsing River, despite the high count of *E. coli* reported previously (Ghaderpour et al., 2014). Since station H is far away from obvious anthropogenic sources, its high count of *E. coli* could originate from wildlife. It has been shown in other studies that the prevalence of antibiotic resistance was low in wild animals compared to human and agricultural sources (Sayah et al., 2005; Edge and Hill, 2007).

Distribution of Class I and II Integrons among *E. coli* from Matang Mangrove Estuaries

Integron is an important factor in developing MDR resistance phenotypes among *E. coli* and other bacterial species since they are able to transfer different resistance genes simultaneously (Martinez, 2009). In the present study, 21% of *E. coli* isolates were integron positive. It was higher than those values reported in previous studies, 11% in France and Portugal (Laroche et al., 2009; Pereira et al., 2013), and 15% in Czech Republic (Dolejská et al., 2009). The frequency of the class 2 integron (3%) among *E. coli* isolates in Matang estuaries concurred with earlier studies in estuary (Laroche et al., 2009), in animal and human *E. coli* isolates (Kang et al., 2005; Skurnik et al., 2005; Cocchi et al., 2007). No class 3 integron was found in this study since integron class 3 is rare even among the human and animal *E. coli.* This finding is consistent with other reports (Laroche et al., 2009; Su et al., 2012; Pereira et al., 2013).

Class Integron 1 can be considered as a marker for antibiotic resistance even without the presence of the gene cassette as they are usually located on the transposons or plasmids that carry other resistance genes (Leverstein-van Hall et al., 2003; Mokracka et al., 2011). This might explain why 90% of the integron class 1 positive isolates were found as multi-drug resistance (**Tables 1**, **2**).

Among the 31 integron positive *E. coli* isolates, only seven (22%) were detected carrying gene cassettes. All the gene cassettes were detected in class 1 integron positive *E. coli* isolates. Five different gene cassettes were found in this study (**Table 2**), and these have been previously reported among clinical *E. coli* (Lim et al., 2009; Ho et al., 2012; Su et al., 2012; Koczura et al., 2013). It is interesting to note that the gene cassettes found in class 1 integron among *E. coli* isolates in Matang estuaries were also reported in *E. coli* isolates from swine (Lapierre et al., 2010), poultry (Soufi et al., 2009), and humans (Lim et al., 2009; Ho et al., 2012).

Predominant Phylogenetic Groups in Matang Mangrove Estuaries

The *E. coli* phylogenetic group B1 was the most prevalent (44%) in Matang mangrove estuaries, followed by phylogenetic groups A and D. Phylogenetic groups A and B1 comprised more than

half (70%) of the total *E. coli* isolated from Matang mangrove estuaries. MLST genotyping has grouped phylogenetic groups A and B1 together, implying groups A and B1 to be sister groups (Gordon et al., 2008). Commensal *E. coli* was found to be predominantly in phylogenetic groups A and B1; whilst extraintestinal *E. coli* strains usually belong to groups B2 and D (Bingen et al., 1998; Picard et al., 1999; Johnson and Stell, 2000). Other studies have also reported that groups A and B1 were usually less pathogenic compared to groups B2 and D (Bingen et al., 1998; Picard et al., 1999; Johnson et al., 2001). Strains of A and B1 groups were recovered more frequently from aquatic environments than group B2 and D strains (Figueira et al., 2011). Walk et al. (2007) demonstrated that the majority of the *E. coli* strains that are able to persist in the environment belong to the B1 phylogenetic group. The findings from the present study are in agreement with previous studies in that A and B1 groups are predominant in the aquatic system, although phylogenetic group B1 comprised the most frequently isolated group in the present study. However, the distribution of these common phylogenetic groups did not hold true at station H, where phylogenetic group D was predomiant. Although, many studies have demonstrated groups B2 and D are usually more virulent (Bingen et al., 1998; Picard et al., 1999; Johnson et al., 2001), *E. coli* O157:H7 and other EHEC are in group D (Bidet et al., 2005), where cattle and birds (Escobar-Paìramo et al., 2006) are the main reservoirs. The findings obtained from the present study are realistic since station H was less influenced by human activity compared to the other stations where human activity was the main factor affecting the quality of the aquatic environment. Station H was least impacted by human activity where wild animals are thought to be the main source of *E. coli* at station H. However, due to the fact that pathogenic strains usually belong to group D, fecal contamination by feral animals can present a potential risk of pathogenic *E. coli*. Therefore, the identification of this kind of fecal contamination is necessary for appropriate management practices and remediation strategies to improve sustainable water resources.

Low prevalence of phylogenetic group B2 was found in our study which is in agreement with previous studies (Pereira et al., 2013). These isolates were detected in stations that were influenced more by human activities. It may show that this group is more associated with anthropogenic contamination as has been previously reported (Carlos et al., 2010). However, the percentage of *E. coli* in each group differs according to the sites of infection, geographical location, and level of antibiotic resistance (Duriez et al., 2001; Bukh et al., 2009). Carlos et al. (2010) reported that genotype $B2_3$ was found only in human feces and could be a good indicator for human fecal contamination in water. In the present study, genotype $B2_3$ was only detected from stations D, E, F, and G which were all located downstream of the fishing villages.

Many studies have reported the association of *E. coli* phylogenetic groups to their virulence, antibiotic resistance, and antibiotic resistant genes (Skurnik et al., 2005; Rijavec et al., 2006; Bukh et al., 2009; Garcia-Aljaro et al., 2009; Mataseje et al., 2009; Pereira et al., 2013; Mosquito et al., 2015). However, variable observations were reported from different studies. For instance, Mosquito et al. (2015) and Bukh et al. (2009) both reported that strains belonging to phylogenetic group D presented significantly higher percentages of multidrug resistance than the rest of the groups; while Skurnik et al. (2005), Rijavec et al. (2006), Garcia-Aljaro et al. (2009), Mataseje et al. (2009) and Pereira et al. (2013) had all found antibiotic resistance to be lower in *E. coli* phylogenetic group D and B2. In this study, we observed a lower percentage of antibiotic resistance in phylogenetic groups B2 (0%) and D (8%) as compared to group A (29%) and B1 (23%) (**Table 1**). However, the variation in observations from different studies could be due to the different origin of *E. coli.* Both studies (Bukh et al., 2009; Mosquito et al., 2015) reported an association of multidrug resistance with *E. coli* phylogenetic group D was of clinical origins but not environmental. Therefore, in our study, the higher percentage of multidrug resistance observed in group A and B1 was plausible. However, both *E. coli* phylogenetic group B2 and D demonstrated higher percentage of non-susceptibility to aminoglycosides, but not quinolones/fluoroquinolones. On the other hand, group A and B1 appear to be associated with resistance toward aminoglycosides and (fluoro) quinolones (**Table 1** and **Figure 3**). The low non-susceptibility percentage in group D is out of our expectation as most studies have reported on its high resistance toward (fluoro) quinolones (Sabaté et al., 2008; Bukh et al., 2009; Mosquito et al., 2015). Probably, a study into the origins of *E. coli* phylogenetic group D isolated in this study will yield an explanation for this variation.

Isolation of EAEC Pathotype from Matang Mangrove Estuaries

Our present finding also indicated that two isolates (1%) were pathogenic and identified as EAEC. Both EAEC isolates (SD4 and WGO3) belonged to phylogenetic group D and they were grouped in different clusters (Supplementary Table 4). Low percentage of the pathotypes in aquatic systems was also reported in a previous study (Hamelin et al., 2007). EAEC is known as an emergent pathogen causing diarrhea in developing countries and gastroenteritis outbreaks in industrialized countries, especially among children (Keskimäki et al., 2000; Villaseca et al., 2005). In Japan in 1993, a massive outbreak due to EAEC infection occurred among children (Keskimäki et al., 2000).

The source of EAEC remains unknown. The isolated EAEC were from stations D and G, and likely came from municipal wastewater as a potential source of pathogenic *E. coli*. The contaminated water may contaminate aquaculture products, while it is conceivable that their consumption could cause foodborne illness in humans. Water and food have been reported as the possible sources of EAEC infection in developing counties (Villaseca et al., 2005). This risk could become more significant if such bacteria are resistant to antibiotics.

Both EAEC isolates, SD4 and WGO3, contains class 1 integron with gene cassettes *aadA1* (1000 bp) and *dfrA5* (700 bp), respectively. The same gene cassettes with same length have been reported among clinical isolates of *E. coli* in the stools of infected persons in Malaysia (Lim et al., 2009; Ho et al., 2012). In addition, both EAEC isolates were MDR but were sensitive to fluoroquinolones. Fluoroquinolones has been found to be negatively associated with the presence of the

virulence factors among the clinical *E. coli* strain (Johnson et al., 2003; Kawamura-Sato et al., 2010), but has not been observed among isolates from animals (Johnson et al., 2003; Kawamura-Sato et al., 2010). This may indicate that *E. coli* population in Matang estuaries is contributed by different sources of contamination.

Genetic Diversity Using REP-PCR of *E. coli* isolates from Matang Mangrove Estuaries

REP-PCR is known as a fast and discriminatory technique to characterize the genomic diversity of *E. coli* isolates (Rademaker et al., 2000). Although the present results indicate a high diversity of *E. coli* isolates, the cluster analysis showed that the 148 isolates could be grouped into seven clusters at 80% similarity.

Overall, 76 (51%) and 27 (18%) *E. coli* isolates from eight different sampling sites were grouped into cluster I and V, respectively. The close similarity between some of the *E. coli* isolates over large spatial scale reflects both the hydrodynamics and interconnected waterways that characterize the Matang estuarine system. However, the diverse and variable nature of the *E. coli* population could be due to their survival rate, growth and inputs from different sources in the aquatic environment and, which differ according to the strain characteristic (Anderson et al., 2006; Kon et al., 2007, 2009). This explains why eight *E. coli* isolates in station F were grouped into four different clusters in the present study, while diversity was less in station C ($n = 8$) with two clusters, and station D ($n = 24$) with three clusters.

Although Selinsing River (stations G and H) was less used for aquaculture production compared to Sangga Besar River, the diversity of *E. coli* isolates was slightly higher. Besides the prolonged persistence and adaptation of *E. coli* in the water, heterogeneity among isolates could be due to erosion of the river banks and the presence of more wildlife that act as vectors of *E. coli* transmission (Solo-Gabriele et al., 2000; Kon et al., 2009). The high diversity was also found at stations A, B, and E as these stations were located next to human settlements and fish cage farms (E). However, there is no a single source that contributes to *E.coli* diversity and multiple sources are more likely (Kon et al., 2009).

Several studies reported different sources of *E. coli* isolates such as bird droppings (Edge and Hill, 2005), wildlife (Somarelli et al., 2007), and humans (Ram et al., 2004). However, it was not the main aim of this study to trace the sources of *E. coli* in the estuarine system. Perhaps, future work should compare the REP-PCR of *E. coli* isolates with those from humans and known animals in order to determine the sources of *E. coli* isolates in the Matang area.

Conclusion

Our study shows a high prevalence of the MDR (34%) among *E. coli* isolated from Matang estuaries. Phylogenetic group B1 has high resistance to antibiotics, thus playing a major role in the transmission of antimicrobial resistance via the aquatic environment. In particular, a high level of antibiotic resistance was observed at the upstream of the estuaries, which could be attributable to the adjacent human settlement and fish farms. Resistance to aminoglycosides, beta-lactams and tetracycline were most frequently detected in this study. Presence of integrons (21%) is another interesting result reflecting the contaminated MMFR estuaries that could act as a reservoir or source of MDR *E. coli* for the transmission of antibiotic resistance to the environment.

Acknowledgments

This research was financially supported by University of Malaya High Impact Research Grant UM.C/625/1/HIR/MOHE/SC/20 (UM.S/P/HIR/ MOHE/24) and IOES HiCoE Research Grant (IOES-2014E) from Ministry of Education Malaysia. Six strains (2060-004, E2348/69, JM221, E9034A, C1845, EC-12) were included as positive control for *E. coli* pathotyping, a gift from Oscar G. Gomez-Duarte, International Enteric Vaccines Research Program [IEVRP], University of Iowa Children's Hospital, Iowa City, IA.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00977

References

Anderson, M. A., Whitlock, J. E., and Harwood, V. J. (2006). Diversity and distribution of *Escherichia coli* genotypes and antibiotic resistance phenotypes in feces of humans, cattle, and horses. *Appl. Environ. Microbiol.* 27, 6914–6922. doi: 10.1128/AEM.01029-06

Ariffin, R., and Nik Mustafa, N. M. S. (eds.). (2013). *A Working Plan for the Matang Mangrove Forest Reserve, Perak.* 6th Revision. Ipoh: Perak State Forestry Department.

Bergthorsson, U., and Ochman, H. (1998). Distribution of chromosome length variation in natural isolates of *Escherichia coli*. *Mol. Biol. Evol.* 15, 6–16. doi: 10.1093/oxfordjournals.molbev.a025847

Bidet, P., Mariani-Kurkdjian, P., Grimont, F., Brahimi, N., Courroux, C., Grimont, P., et al. (2005). Characterization of *Escherichia coli* O157: H7 isolates causing haemolytic uraemic syndrome in France. *J. Med. Microbiol.* 54, 71–75. doi: 10.1099/jmm.0.45841-0

Bingen, E., Picard, B., Brahimi, N., Mathy, S., Desjardins, P., Elion, J., et al. (1998). Phylogenetic analysis of *Escherichia coli* strains causing neonatal meningitis suggests horizontal genetransfer from a predominant pool of highly virulent B2 group strain. *J. Infect. Dis.* 177, 642–650. doi: 10.1086/514217

Bukh, A. S., Schønheyder, H. C., Emmersen, J. M. G., Søgaard, M., Bastholm, S., and Roslev, P. (2009). *Escherichia coli* phylogenetic groups are associated with site of infection and level of antibiotic resistance in community-acquired bacteraemia: a 10 year population-based study in Denmark. *J. Antimicrob. Chemother.* 64, 163–168. doi: 10.1093/jac/dkp156

Burridge, L., Judith, S. W., Felipe, C., Jaime, P., and Katherine, B. (2010). Chemical use in salmon aquaculture: a review of current practices and possible environmental effects. *Aquaculture* 306, 7–23. doi: 10.1016/j.aquaculture.2010.05.020

Cambray, G., Guerout, A., and Mazel, D. (2010). Integrons. *Annu. Rev. Genet.* 44, 141–166. doi: 10.1146/annurev-genet-102209-163504

Carattoli, A. (2003). Plasmid-mediated antimicrobial resistance in *Salmonella enterica*. *Curr. Issues. Mol. Biol.* 5, 113–122. Available online at: http://www.horizonpress.com/cimb/v/v5/10.pdf

Carlos, C., Pires, M. M., Stoppe, N. C., Hachich, E. M, Sato, M. I., Gomes, T. A. T., et al. (2010). *Escherichia coli* phylogenetic group determination and its application in the identification of the major animal source of fecal contamination. *BMC Microbiol*. 10:161. doi: 10.1186/1471-2180-10-161

Chong, V. C., Lee, P. K. Y., and Lau, C. M. (2010). Diversity, extinction risk and conservation of Malaysian fishes. *J. Fish. Biol*. 76, 2009–2066. doi: 10.1111/j.1095-8649.2010.02685.x

Clermont, O., Bonacorsi, S., and Bingen, E. (2000). Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl. Environ. Microbiol*. 66, 4555–4558. doi: 10.1128/AEM.66.10.4555-4558.2000

CLSI. (2012). *Performance Standards for Antimicrobial Susceptibility Testing. CLSI Document M100-S22*.Wayne, PA: Clinical and Laboratory Standards Institute.

Cocchi, S., Grasselli, E., Gutacker, M., Benagli, C., Convert, M., and Piffaretti, J. C. (2007). Distribution and characterization of integrons in *Escherichia coli* strains of animal and human origin. *FEMS. Immunol. Med. Microbiol*. 50, 126–132. doi: 10.1111/j.1574-695X.2007.00242.x

Dolejská, M., Bierosová, B., Kohoutová, L., Literak, I., and Cízek, A. (2009). Antibiotic resistant *Salmonella* and *Escherichia coli* isolates with integrons and extended spectrum beta-lactamases in surface water and sympatric black-headed gulls. *J. Appl. Microbiol*. 106, 1941–1950. doi: 10.1111/j.1365-2672.2009.04155.x

Duriez, P., Clermont, O., Bonacorsi, S., Bingen, E., Chaventre, A., Elion, J., et al. (2001). Commensal *Escherichia coli* isolates are phylogenetically distributed among geographically distincthuman populations. *Microbiology* 147, 1671–1676. doi: 10.1099/00221287-147-6-1671

Edge, T. A., and Hill, S. (2005). Occurrence of antibiotic resistance in *Escherichia coli* from surface waters and fecal pollution sources near Hamilton, Ontario. *Can. J. Microbiol* 51, 501–505. doi: 10.1139/w05-028

Edge, T. A., and Hill, S. (2007). Multiple lines of evidence to identify the source of fecal pollution at a fresh water beach in Hamilton Harbour, Lake Ontario.*Water Res*. 41, 3585–3594. doi: 10.1016/j.watres.2007.05.012

Escobar-Páramo, P., Clermont, O., Blanc-Potard, A. B., Bui, H., Le Bouguénec, C., and Denamur, E. (2004). A specific genetic back ground is required for acquisition and expression of virulence factors in *Escherichia coli*. *Mol. Biol. Evol*. 21, 1085–1094. doi: 10.1093/molbev/msh118

Escobar-Pairamo, P., Le Menac'h, A., Le Gall, T., Amorin, C.,Gouriou, S., Picard, B. et al. (2006). Identification of forces shaping the commensal *Escherichia coli* genetic structure by comparing animal and human isolates. *Environ. Microbiol*. 8, 1975–1984. doi: 10.1111/j.1462-2920.2006.01077.x

Figueira, V., Serra, E., and Manaia, C. M. (2011). Differential patterns of antimicrobial resistance in population subsets of Escherichia coli isolated from waste and surface waters. *Sci. Total. Environ.* 409, 1017–1023. doi: 10.1016/j.scitotenv.2010.12.011

Frenzel, S. A., and Couvillion, C. S. (2002). Fecal-indicator bacteria in streams along a gradient ofresidential development. *J. Am. Water. Resource. Ass*. 38, 265–273. doi: 10.1111/j.1752-1688.2002.tb01550.x

Galvin, S., Boyle, F., Hickey, P., Vellinga, A., Morris, D., and Cormican, M. (2010). Enumeration and characterization of antimicrobial-resistant *Escherichia coli* bacteria in effluent from municipal, hospital, and secondary treatment facility sources. *Appl. Environ. Microbiol*. 76, 4772–4779. doi: 10.1128/AEM.02898-09

Gao, G., Falconer, R. A., and Lin, B. (2013). Modelling importance of sediment effects on fate and transport of enterococci in the Severn Estuary, UK. *Mar. Pollut. Bull*. 67, 45–54. doi: 10.1016/j.marpolbul.2012.12.002

Garcia-Aljaro, C., Moreno, E., Andreu, A., Prats, G., and Blanch, A. R. (2009). Phylogroups, virulence determinants and antimicrobial resistance in stx2 gene-carrying *Escherichia coli* isolated from aquatic environments. *Res. Microbiol*. 160, 585–591. doi: 10.1016/j.resmic.2009.08.004

Ghaderpour, A., KhairulNazrin, M. N., Chew, L. L., Chong, V. C., Thong, K. L., and Chai, L. C. (2014). Detection of multiple potentially pathogenic bacteria in Matang mangrove estuaries, Malaysia. *Mar. Pollut. Bull*. 8, 324–330. doi: 10.1016/j.marpolbul.2014.04.029

Gómez-Duarte, O. G., Bai, J., and Newel, E. (2009). Detection of *E. coli, Salmonella* spp., *Shigella* spp., *Yersinia enterocolitica*, *Vibrio cholerae*, and *Campylobacter* spp. enteropathogens by Three-reaction Multiplex PCR. *Diagn. Microbiol. Infect. Dis*. 63, 1–9. doi: 10.1016/j.diagmicrobio.2008.09.006

Gordon, D. M. (2004). "The influence of ecological factors on the distribution and genetic structure of *Escherichia coli*," in *Escherichia coli* and *Salmonella Typhimurium: Cellular and Molecular Biology*, ed F. Neidhardt (Washington, DC: American Society for Microbiology).

Gordon, D. M., Clermont, O., Tolley, H., and Denamur, E. (2008). Assigning *Escherichia coli* strains to phylogenetic groups: multi-locus sequence typing versus the PCR triplex method. *Environ. Microbiol.* 10, 2484–2496. doi: 10.1111/j.1462-2920.2008.01669.x

Gordon, D. M., and Cowling, A. (2003). The distribution and genetic structure of *Escherichia coli* in Australian vertebrates: host and geographic effects. *Microbiology* 149, 3575–3586. doi: 10.1099/mic.0.26486-0

Hall, R. M., and Collis, C. M. (1995). Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. *Mol. Microbiol*. 15, 593–600. doi: 10.1111/j.1365-2958.1995.tb02368.x

Hamelin, K., Bruant, G., El-Shaarawi, A., Hill, S., Edge, T. A., Fairbrother, J., et al. (2007). Occurrence of virulence and antimicrobial resistance genes in *Escherichia coli* isolates from different aquatic ecosystems within the St. Clair River and Detroit river areas. *Appl. Environ. Microbiol*. 73, 477–484. doi: 10.1128/AEM.01445-06

Ho, W. S., Balan, G., Puthucheary, S., Kong, B. H., Lim, K. T., Tan, L. K., et al. (2012). Prevalence and characterization of multidrug-resistant and extended-spectrum beta-lactamase-producing *Escherichia coli* from pediatric wards of a Malaysian hospital. *Microb. Drug. Resist*. 18, 408–416. doi: 10.1089/mdr.2011.0222

Ibrahim, A. B., Mohd khan, A., Ayob, M. Y., and Norrakiah, A. S. (2010). Pesticide and antibiotic residues in freshwater aquaculture fish: Chemical risk assessment from farm to table. *Asian J. Food Agro Indus.* 3, 328–334.

Jaime, R., Carmen, G. F., and Paola, N. (2012). "Antibiotics in aquaculture use, abuse and alternatives," in *Health and Environment in Aquaculture,* ed E. Carvalho, ISBN: 978-953-51-0497-1 (InTech). Available online at: http://www.intechopen.com/books/health-and-environment-in-aquaculture/antibiotics-in-aquaculture-use-abuse-and-alternatives

Johnson, J. R., Delavari, P., Kuskowski, M., and Stell, A. L. (2001). Phylogenetic distribution of extraintestinal virulence-associated traits in *Escherichia coli*. *J. Infect. Dis*. 183, 78–88. doi: 10.1086/317656

Johnson, J. R., Kuskowski, M. A., Owens, K., Gajewski, A., and Winokur, P. L. (2003). Phylogenetic origin and virulence genotype inrelation to resistance to fluoroquinolones and/or extended spectrum cephalosporins and cephamycins among *Escherichia coli* isolates from animals and humans. *J. Infect. Dis*. 188, 759–768. doi: 10.1086/377455

Johnson, J. R., and Stell, A. L. (2000). Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. *J. Infect. Dis*. 181, 261–272. doi: 10.1086/315217

Kang, H. Y., Jeong, Y. S., Oh, J. Y., Tae, S. H., Choi, C. H., Moon, D. C., et al. (2005). Characterization of antimicrobial resistance and class 1 integrons found in *Escherichia coli* isolates from humans and animals in Korea. *J. Antimicrob. Chemother*. 55, 639–644. doi: 10.1093/jac/dki076

Kawamura-Sato, K., Yoshida, R., Shibayama, K., and Ohta, M. (2010). Virulence genes, quinolone and fluoroquinolone resistance, and phylogenetic background of uropathogenic *Escherichia coli* strains isolated in Japan. *Jpn. J. Infect. Dis.* 63, 113–115. Available online at: http://www.nih.go.jp/niid/JJID/63/113.pdf

Keh, C. L. (2003). *Survey of the Use of Drugs and Chemicals in Aquaculture in Malaysia*. M.Sc. thesis, University of Malaya. 71.

Keskimäki, M., Mattila, L., Peltola, H., and Siitonen, A. (2000). Prevalence of diarrheagenic *Escherichia coli* in Finns with or without diarrhea during a round-the-world trip. *J. Clin. Microbiol.* 38, 4425–4429. Available online at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC87616/

Koczura, R., Mokracka, J., Barczak, A., Krysiak, N., and Kaznowski, A. (2013). Association between the presence of class 1 integrons, virulence genes, and phylogenetic groups of *Escherichia coli* isolates from river water. *Microb. Ecol.* 65, 84–90. doi: 10.1007/s00248-012-0101-3

Kon, T., Weir, S. C., Howell, T. E., Lee, H., and Trevors, T. (2009). Repetitive element (REP)-polymerase chain reaction (PCR) analysis of *Escherichia coli* isolates from recreational waters of southeastern Lake Huron, *Can. J. Microbiol.* 55, 269–276. doi: 10.1139/W08-123

Kon, T., Weir, S. C., Howell, E. T., Lee, H., and Trevor, J. T. (2007). Genetic Relatedness of *Escherichia coli* isolates in interstitial water from Lake Huron

(Canada) Beach. *Environ. Microbiol.* 73, 1961–1967. doi: 10.1128/AEM.02437-06

Lapierre, L., Martín, B. S., Araya-Jordán, C., and Borie, C. (2010). Comparison of integron linked antibiotic resistance genes in strains of *Salmonella* spp. isolated from swine in Chile in 2005 and 2008. *Can. J. Microbiol.* 56, 515–521. doi: 10.1139/W10-033

Laroche, E., Pawlak, B., Berthe, T., Skurnik, D., and Petit, F. (2009). Occurrence of antibiotic resistance andclass 1, 2 and 3 integrons in *Escherichia coli* isolated from a densely populated estuary (Seine, France). *FEMS. Microbiol. Ecol.* 68, 118–130. doi: 10.1111/j.1574-6941.2009.00655.x

Leverstein-van Hall, M. A., Blok, H. E. M., Rogier, A., Donders, T., Paauw, A., Fluit, A. C., et al. (2003). Multidrug resistance among Enterobacteriaceae is strongly associated with the presence of integronsand is independent of species or isolate origin. *J. Infect. Dis.* 187, 251–259. doi: 10.1086/345880

Lim, H. F., and Mohamad Parit, M. (2001). *Sustainable Development of the Matang Mangroves and Its Impacts on Local Socio-economic Livelihood*. Intensification of Research in Priority Areas Report, Government of Malaysia.

Lim, K. T., Yasin, R., Yeo, C. C., Puthucheary, S., and Thong, K. L. (2009). Characterization of multidrug resistant ESBL-producing *Escherichia coli* isolates from hospitals in Malaysia. *J. Biomed. Biotechnol.* 2009:165637. doi: 10.1155/2009/165637

Majusha, S., Sarita, G. B., Elyas, K. K., and Chandrasekaran, M. (2005). Multiple antibiotic resistances of vibrio isolates from coastal and brackish water areas. *Am. J. Biochem. Biotechnol.* 1, 201–206. doi: 10.3844/ajbbsp.2005.201.206

Martinez, J. L. (2009). Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environ. Pollut.* 157, 2893–2902. doi: 10.1016/j.envpol.2009.05.051

Mataseje, L. F., Neumann, N., Crago, B., Baudry, P., Zhanel, G. G., Louie, M., et al. (2009). ARO water study group characterization of cefoxitin-resistant *Escherichia coli* isolates fromrecreational beaches and private drinking water in Canada between 2004 and 2006. *Antimicrob. Agents. Ch.* 53, 3126–3130. doi: 10.1128/AAC.01353-08

Maynard, C., Bekal, S., Sanschagrin, F., Levesque, R. C., Brousseau, R., Masson, L., et al. (2004). Heterogeneity among virulence and antimicrobial resistance gene profiles of extraintestinal *Escherichia coli* isolates of animal and human origin. *J. Clin. Microbiol.* 42, 5444–5452. doi: 10.1128/JCM.42.12.5444-5452.2004

Mohamed, S., Nagaraj, G., Chua, F. H. C., and Wang, Y. G. (2000). "The use of chemicals in aquaculture in Malaysia and Singapore," in *Use of Chemicals in Aquaculture in Asia: Proceedings of the Meeting on the Use of Chemicals in Aquaculture in Asia 20-22 May 1996, Tigbauan, Iloilo, Philippines*, eds J. R. Arthur, C. R. Lavilla-Pitogo, and R. P. Subasinghe (Tigbauan: Aquaculture Department, Southeast Asian Fisheries Development Center), 127–140. Available online at: http://hdl.handle.net/10862/601

Mokracka, J., Koczura, R., Pawłowski, K., and Kaznowski, A. (2011). Resistance patterns and integron cassette arrays of *Enterobacter cloacae* complex strains of human origin. *J. Med. Microbiol.* 60, 737–743. doi: 10.1099/jmm.0.027979-0

Mosquito, S., Pons, M. J., Riveros, M., Ruis, J., and Ochoa, T. J. (2015). Diarrheagenic *Escherichia coli* phylogroups are associated with antibiotic resistance and duration of diarrheal episode. *Sci. World J.* 2015:610403. doi: 10.1155/2015/610403

Pereira, A., Santos, A., Tacão, M., Alves, A., Henriques, I., and Correia, A. (2013). Genetic diversity and antimicrobial resistance of *Escherichia coli* from Tagus estuary (Portugal). *Sci. Total. Environ.* 461, 65–71. doi: 10.1016/j.scitotenv.2013.04.067

Picard, B., Garcia, J. S., Gouriou, S., Duriez, P., Brahimi, N., Bingen, E., et al. (1999). The link between phylogeny and virulence in *Escherichia coli* extraintestinal infection. Infect. *Immun.* 67, 546–553.

Pruden, A., Pei, R., Storteboom, H., and Carlson, K. H. (2006). Antibiotic resistance genesas emerging contaminants: studies in northern Colorado. *Environ. Sci. Technol.* 40, 7445–7450. doi: 10.1021/es060413l

Rademaker, J. L. W., Hoste, B., Louws, F. J., Kersters, K., Swings, J., Vauterin, L., et al. (2000). Comparison of AFLPand REP-PCR genomic fingerprinting with DNA-DNA homologystudies: Xanthomonas as a model system. *Int. J. Sys. Evol. Micr.* 50, 665–677. doi: 10.1099/00207713-50-2-665

Ram, J. L., Ritchie, R. P., Fang, J., Gonzales, F. S., and Selegean, J. P. (2004). Sequence based source tracking of *E. coli* based on genetic diversity of beta glucuronidase. *J. Environ. Q.* 33, 1024–1032. doi: 10.2134/jeq2004.1024

Recchia, G. D., and Hall, R. M. (1995). Gene cassettes: a new class of mobile element. *Microbiol-UK* 141, 3015–3027. doi: 10.1099/13500872-141-12-3015

Rijavec, M., Starcic-Erjavec, M., Ambrozic-Avgustin, J., Reissbrodt, R., Fruth, A., Krizan-Hergouth, V., et al. (2006). High prevalence of multidrug resistance and random distribution of mobile genetic elements among uropathogenic *Escherichia coli* (UPEC) of the four major phylogenetic groups. *Curr. Microbiol.* 53, 158–162. doi: 10.1007/s00284-005-0501-4

Sabaté, M., Prats, G., Moreno, E., Ballest,é, E., Blanch, A. R., and Andreu, A. (2008). Virulence and antimicrobial resistance profiles among *Escherichia coli* strains isolated from human and animal wastewater. *Res. Microbiol.* 159, 288–293. doi: 10.1016/j.resmic.2008.02.001

Sayah, R. S., Kaneene, J. B., Johnson, Y., and Miller, R. (2005). Patterns ofantimicrobial resistance observed in *Escherichia coli* isolates obtained from domestic- and wild-animal fecal samples, human septage, and surface water. *Appl. Environ. Microbiol.* 71, 1394–1404. doi: 10.1128/AEM.71.3.1394-1404.2005

Skurnik, D., Le Menac'h, A., Zurakowski, D., Mazel, D., Courvalin, P., Denamur, E., et al. (2005). Integron associated antibiotic resistance and phylogenetic grouping of *Escherichia coli* isolates from healthy subjects free of recent antibiotic exposure. *Antimicrob. Agents. Ch.* 49, 3062–3065. doi: 10.1128/AAC.49.7.3062-3065.2005

Solo-Gabriele, H. M., Wolfert, M. A., Desmarais, T. R., and Palmer, C. J. (2000). Sources of *Escherichia coli* in a coastal subtropical environment. *Appl. Environ. Microbiol.* 66, 230–237. doi: 10.1128/AEM.66.1.230-237.2000

Somarelli, J. A., Makarewicz, J. C., Sia, R., and Simon, R. (2007). Wildlife identified as major source of *E. coli* in agriculturally dominated watersheds by BOX- AIR-derived genetic fingerprints. *J. Environ. Manage.* 82, 60–65. doi: 10.1016/j.jenvman.2005.12.013

Soufi, L., Abbassi, M. S., Saenz, Y., Vinue, L., Somalo, S., Zarazaga, M., et al. (2009). Torres C. Prevalence and diversity of integrons and associated resistance genes in *Escherichia coli* isolates from poultry meat in Tunisia. *Foodborne. Pathoge. Dis.* 6, 1067–1073. doi: 10.1089/fpd.2009.0284

Su, H. C., Ying, G. G., Tao, R., Zhang, Q. R., Zhao, L. J., and Liu, S. Y. (2012). Class 1 and 2 integrons, sul resistance genes and antibiotic resistance in *Escherichia coli* isolated from Dongjiang River, South China. *Environ. Pollut.* 169, 42–49. doi: 10.1016/j.envpol.2012.05.007

Ter Braak, C. J. F., and Smilauer, P. (2002). *CANOCO Reference Manual and CanoDraw for Windows User's Guide: Software for Canonical Community Ordination (Version 4.5)*. Ithaca, NY: Microcomputer Power. 500.

Vignaroli, C., Luna, G. M., Rinaldi, C., Di Cesare, A., Danovaroand, R., and Biavasco, F. (2012). New sequence types and multidrug resistance among pathogenic escherichia coli isolates from coastal marine sediments. *Appl. Environ. Microbiol.* 78, 3916–3922. doi: 10.1128/AEM.07820-11

Villaseca, J. M., Hernández, U., Sainz-Espuñes, T. R., Rosario, C., and Eslava, C. (2005). Enteroaggregative *Escherichia coli* an emergent pathogen with different virulence properties. *Rev. Latinoam. Microbiol.* 47, 140–159. Available online at: http://new.medigraphic.com/cgi-bin/resumen.cgi?IDREVISTA=23&IDARTICULO=5182&IDPUBLICACION=642

Walk, S. T., Alm, E. W., Calhoun, L. M., Mladonicky, J. M., and Whittam, T. S. (2007). Genetic diversity and population structure of *Escherichia coli* isolated from freshwater beaches. *Environ. Microbiol.* 9, 2274–2288. doi: 10.1111/j.1462-2920.2007.01341.x

Yu, X. K., and Thong, K. L. (2009). Multiplex PCR for simultaneous detection of virulence genes in *Escherichia coli*. *Malays. J. Med. Sci.* 28, 1–14. Available online at: http://eprints.um.edu.my/5657/1/Multiplex_PCR_for_simultaneous_detection_of_virulence_Genes_in_Escherichia_coli.pdf

Zar, J. H. (2010). *Biostatistical Analysis.* 5th Edn. Upper Saddle River, NJ: Pearson Education Inc.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 20 August 2014
doi: 10.3389/fmicb.2014.00426

Seawater is a reservoir of multi-resistant *Escherichia coli*, including strains hosting plasmid-mediated quinolones resistance and extended-spectrum beta-lactamases genes

Marta S. Alves, Anabela Pereira, Susana M. Araújo, Bruno B. Castro, António C. M. Correia and Isabel Henriques*

Department of Biology and CESAM, University of Aveiro, Aveiro, Portugal

Edited by:
Satoru Suzuki, Ehime University, Japan

Reviewed by:
Daniela Ceccarelli, University of Maryland, USA
Etinosa Igbinosa, University of Benin, Nigeria
Masaru Usui, Rakuno Gakuen, Japan

****Correspondence:***
Isabel Henriques, Department of Biology and CESAM, University of Aveiro, Campus Universitário de Santiago, Aveiro, 3810-193 Aveiro, Portugal
e-mail: ihenriques@ua.pt

The aim of this study was to examine antibiotic resistance (AR) dissemination in coastal water, considering the contribution of different sources of fecal contamination. Samples were collected in Berlenga, an uninhabited island classified as Natural Reserve and visited by tourists for aquatic recreational activities. To achieve our aim, AR in *Escherichia coli* isolates from coastal water was compared to AR in isolates from two sources of fecal contamination: human-derived sewage and seagull feces. Isolation of *E. coli* was done on Chromocult agar. Based on genetic typing 414 strains were established. Distribution of *E. coli* phylogenetic groups was similar among isolates of all sources. Resistances to streptomycin, tetracycline, cephalothin, and amoxicillin were the most frequent. Higher rates of AR were found among seawater and feces isolates, except for last-line antibiotics used in human medicine. Multi-resistance rates in isolates from sewage and seagull feces (29 and 32%) were lower than in isolates from seawater (39%). Seawater AR profiles were similar to those from seagull feces and differed significantly from sewage AR profiles. Nucleotide sequences matching resistance genes bla_{TEM}, *sul1*, *sul2*, *tet*(A), and *tet*(B), were present in isolates of all sources. Genes conferring resistance to 3rd generation cephalosporins were detected in seawater ($bla_{\text{CTX-M-1}}$ and $bla_{\text{SHV-12}}$) and seagull feces ($bla_{\text{CMY-2}}$). Plasmid-mediated determinants of resistance to quinolones were found: *qnrS1* in all sources and *qnrB19* in seawater and seagull feces. Our results show that seawater is a relevant reservoir of AR and that seagulls are an efficient vehicle to spread human-associated bacteria and resistance genes. The *E. coli* resistome recaptured from Berlenga coastal water was mainly modulated by seagulls-derived fecal pollution. The repertoire of resistance genes covers antibiotics critically important for humans, a potential risk for human health.

Keywords: *Escherichia coli*, antibiotic resistance, fecal pollution, water quality, Microbial source tracking

INTRODUCTION

Fecal contamination in aquatic environments contributes to the spread of human pathogens along with the dissemination of antibiotic-resistant bacteria. Overexposure to antibiotics is leading to increasing levels of resistance in the human (and other animals) commensal microbiota (Austin et al., 1999). The resistomes of fecal bacteria, once in environmental settings, contribute with antibiotic resistance (AR) genes to non-resistant indigenous microorganisms (Aminov, 2011; Tacão et al., 2014). In aquatic systems the cycle may include subsequent transmission of AR to human-associated bacteria (Devirgiliis et al., 2011; Figueira et al., 2011). At the same time, enrichment in AR bacteria is promoted by the presence of antimicrobials or other contaminants in the environment. This facts reinforce the need to identify the sources of antibiotic-resistant bacteria in aquatic environments of human usage (Rosewarne et al., 2010; Gomez-Alvarez et al., 2012).

Escherichia coli is a natural member of the intestinal microbiota of human and other homeothermic animals and is a fecal indicator bacteria of election (see review by Harwood et al., 2014). Multi-resistant strains are found in environments such as coastal waters. Although *E. coli* is considered harmless and commensal, several pathotypes have been recognized as significant human pathogens, comprising strains implicated in gastro-intestinal, urinary, or respiratory infections (Kaper et al., 2004). *E. coli* strains can be assigned to four main phylogenetic groups: A, B1, B2 and D (Clermont et al., 2000). Extraintestinal pathogenic bacteria usually belong to groups B2 and D, precisely those that include strains with a larger diversity of virulence factors. The commensal strains belong to groups A and B1 (Picard et al., 1999; Johnson et al., 2001).

In recent years, *E. coli* has been recognized as a major player in the dissemination of AR (Henriques et al., 2006a; Zhao and Dang, 2012). Resistance genotypes so far characterized include

genes for resistance to last-line antibiotics such as 3rd generation cephalosporins and fluoroquinolones (Alouache et al., 2012; Wellington et al., 2013; Tacão et al., 2014). To some extent, similar resistance phenotypes and genotypes are shared by strains exposed to similar environmental pressures (Wicki et al., 2011). Considering this fact, some animal hosts may favor the preferential development of certain genotypes.

Seagulls readily utilize food sources of human origin, especially garbage, which may contribute to the high levels of AR associated to their microbiota (Bonnedahl et al., 2009; Poirel et al., 2012). Multi-resistant phenotypes are found in *E. coli* strains isolated from seagull droppings in different geographic areas (Gionechetti et al., 2008; Bonnedahl et al., 2009; Dolejska et al., 2009; Poirel et al., 2012). Genes encoding ESBLs (extended spectrum beta-lactamases), such as $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-15}}$ (Bonnedahl et al., 2009; Dolejska et al., 2009; Simões et al., 2010), and resistance to quinolones (like the plasmid-mediated *qnrS*) (Literak et al., 2010; Vredenburg et al., 2014) integrate the repertoire of seagull's resistome.

Freshwater environments are well-recognized reactors for the dissemination and evolution of AR (Baquero et al., 2008; Figueira et al., 2011; Tacão et al., 2012) but processes occurring in seawater are less understood. The presence of AR strains of *E. coli* in coastal water represents a health issue in areas that are used for recreational activities.

Berlenga is an uninhabited island, recently added to the World Network of Biosphere Reserves due to its ecological relevance. Episodes of fecal pollution have been recorded in its coastal water (Araújo et al., 2014) with seagulls confirmed as the main source of fecal contamination. Human-derived sewage (from visiting tourists) was also suggested as a secondary source (Araújo et al., 2014).

Because it is a well-delimited ecosystem, the island constitutes a privileged location to study pathways implicated in dissemination of AR in natural environments. Resistance to 3rd generation cephalosporins or fluoroquinolones, used as front-line drugs for treatment of Gram-negative infections (Cantón and Coque, 2006; Cattoir et al., 2007), deserved special attention.

In this study the focus was put in coastal water and in the specific contribution of the two previously identified sources of fecal pollution. To accomplish our aims, *E. coli* strains were characterized at the phenotypic level with respect to their resistances profiles; at the genotypic level, specific genes were used as indicators for the resistance to last-line use antibiotics. Phenotypic and genotypic data from coastal water strains was compared with data obtained from the strains of each of the two sources of fecal contamination. Main contributions to the water resistome are hypothesized and discussed.

MATERIALS AND METHODS

STUDY SITE AND SAMPLE COLLECTION

Berlenga Island is the main island of the Berlengas archipelago, located in the Portuguese continental shelf, 5.7 miles northwest of Cape Carvoeiro. The island has a great diversity of habitats, marine species, and avifauna, including a large population of the yellow-legged gull (*Larus* [*cachinnans*] *michahellis*). Recently, Berlengas was added to the UNESCO'S World Biosphere Reserve. Although uninhabited, many tourists visit the island in the summer mainly for recreational activities such as swimming and diving (Araújo et al., 2014). Human-derived sewage is collected on a settlement tank and discharged near the coastline of the island.

Samples of seagull feces, human-derived sewage and seawater from the Berlenga beach were collected as described in Araújo et al. (2014). Sampling was performed every 2 weeks between May and September 2011. Briefly, samples of seagull feces, human-derived sewage and seawater were collected in each sampling date and consisted in: (i) 5 composite samples of seagull feces scattered on the beach; (ii) 250 mL of raw human-derived sewage taken from the effluent of the sanitary infrastructures; and (iii) 2 L of seawater collected about 30 cm below the surface. Samples were collected in sterile containers and kept on ice until processing.

E. COLI STRAINS

A total of 939 *E. coli* isolates were retrieved in Chromocult Coliform Agar plates (Merck, Germany) from seagull feces ($n = 427$), sewage ($n = 170$) and seawater ($n = 342$) as described by Araújo et al. (2014). Genetic diversity of the *E. coli* isolates was inspected by BOX-PCR with primers and conditions previously described (Araújo et al., 2014). Fingerprinting analysis allowed the selection of 414 non-clonal isolates, which were included in the present study. From those, 179 isolates were from seagull feces, 69 isolates were from sewage and 166 were from seawater.

DETERMINATION OF *E. COLI* PHYLOGENETIC GROUPS

The triplex PCR developed by Clermont et al. (2000), with primers for genes *chuA* and *yjaA* and for the DNA fragment TSPE4-C2, was used to determine the phylogenetic groups of the *E. coli* strains. Template DNA was obtained by suspending 2 bacterial colonies in 100 μL of sterile water. For each PCR reaction, 1 μL of this suspension was used as template. Appropriate positive and negative controls were included in the assay.

ANTIBIOTIC SUSCEPTIBILITY TESTING

The *E. coli* isolates were tested for susceptibility to 16 antibiotics by disk diffusion method on Mueller-Hinton Agar (Oxoid, Basingstoke, UK) according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2012). The following disks (Oxoid, UK) were used: ampicillin (10 μg), amoxicillin (10 μg), amoxicillin/clavulanic acid (20/10 μg), piperacillin (100 μg), piperacillin/tazobactam (100/10 μg), cephalothin (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), gentamicin (10 μg), streptomycin (10 μg), imipenem (10 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), tetracycline (30 μg), chloramphenicol (30 μg) and trimethoprim/sulfamethoxazole (1.25/23.75 μg). *E. coli* ATCC 25922 was used as quality control. Isolates were classified as sensitive, intermediate or resistant according to the CLSI recommendations after 24 h incubation at 37°C. To determine the percentage of resistant strains to each antibiotic and to define resistance phenotypes, intermediate and resistant isolates were subsequently grouped in a same resistant class.

ANTIBIOTIC RESISTANCE GENE DETECTION

Escherichia coli strains displaying resistance and intermediate resistance phenotypes were screened by PCR to detect genes conferring resistance to β-lactams (bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{IMP}, bla_{VIM}, bla_{KPC}, bla_{OXA-48}, bla_{GES}, $bla_{AmpC-like}$), tetracycline [*tet*(A), *tet*(B)], quinolones (*qnrA*, *qnrB*, *qnrS*) and sulfonamides (*sul1*, *sul2*). The genes tested were chosen among the most prevalent in clinical and environmental *E. coli* isolates. The PCR reactions were performed in a MyCycler Thermal cycler (Bio-Rad, USA). Primers and PCR conditions are presented in **Table 1** and Table S1 (Supporting Information). The reaction mixtures (25 μL total volume) consisted of 6.25 μL NZYTaq 2x Green Master Mix (2.5 mM $MgCl_2$; 200 μM dNTPs; 0.2 U/μL DNA polymerase) (NZYtech, Portugal), 16.25 μL of ultrapure water, 0.75 μL of each primer (reverse and forward), and 1 μL of cell suspension prepared as described above. The presence of $bla_{AmpC-like}$ genes was inspected using a multiplex PCR as described by Dallenne et al. (2010). Negative and positive controls were included in each PCR experiment. The negative control differed from other reaction mixtures by substituting the cell suspension for the same volume of dH_2O; positive control strains are indicated in **Table 1**. PCR products were analyzed by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. All amplicons obtained using primers for bla_{SHV}, bla_{CTX-M}, $bla_{AmpC-like}$, *qnrA*, *qnrB*, and *qnrS* and PCR products obtained using primers for bla_{TEM} from 3rd generation cephalosporins-resistant isolates were sequenced (accession numbers KM094197 to KM094211). For this PCR products were purified with DNA Clean & Concentrator (Zymo Research, USA) following manufacturer's instructions, and used as template in the sequencing reactions. Online similarity searches were performed with the BLAST software at the National Center for Biotechnology Information website.

The genomic contexts of both genes were determined by PCR using previously described primers and conditions (Saladin et al., 2002; Eckert et al., 2006; Tacão et al., 2012). Primers targeted either $bla_{CTX-M-1}$ or bla_{CMY-2} and genes previously found in the vicinity of these resistance genes (i.e., IS*ECP1* for $bla_{CTX-M-1}$ and bla_{CMY-2}; *ORF477* for $bla_{CTX-M-1}$) (Saladin et al., 2002; Eckert et al., 2006; Tacão et al., 2012). PCR fragments were sequenced as described above.

Table 1 | PCR primers and conditions for AR genes amplification.

Target	Primer sequence (5′–3′)	Amplicon size (bp)	Program[a]	Annealing temperature (°C)	References	Control strains
bla_{OXA-48}	OXA_F:TTGGTGGCATCGATTATCGG OXA_R: GAGCACTTCTTTTGTGATGGC	744	E	55	Poirel et al., 2004	*S. xiamenensis* IR34
bla_{TEM}	TEM_F: AAAGATGCTGAAGATCA TEM_R: TTTGGTATGGCTTCATTC	425	B	44	Speldooren et al., 1998	*K. pneumoniae* 6T
bla_{SHV}	SHV_F: GCGAAAGCCAGCTGTCGGGC SHV_R: GATTGGCGGCGCTGTTATCGC	304	B	62	Henriques et al., 2006a	*K. pneumoniae* 2s
bla_{CTX-M}	CTX_F: SCVATGTGCAGYACCAGTAA CTX_R: GCTGCCGGTYTTATCVCC	652	A	55	Lu et al., 2010	*K. pneumoniae* Kp40
bla_{IMP}	IMP_F: GAATAGAGTGGCTTAATTGTC IMP_R: GGTTTAAYAAAACAACCACC	232	B	55	Henriques et al., 2006b	*K. pneumoniae* KP99c196
bla_{VIM}	VIM_F: GATGGTGTTTGGTCGCATATCG VIM_R: GCCACGTTCCCCGCAGACG	475	B	58	Henriques et al., 2006a	*P. aeruginosa* NTU-39/00
bla_{KPC}	KPC_F: CATTCAAGGGCTTTCTTGCTGC KPC_R: ACGACGGCATAGTCATTT	538	B	55	Dallenne et al., 2010	*K. oxytoca* Ko25
bla_{GES}	GES_F: AGTCGGCTAGACCGGAAAG GES_R: TTTGTCCGTGCTCAGGAT	399	D	57	Dallenne et al., 2010	*K. pneumoniae* 22K9
tet(A)	tetA_F: GCTACATCCTGCCTTC tetA_R: GCATAGATCGGAAGAG	211	C	53	Nawaz et al., 2006	*E. coli* M.I.10.2
tet(B)	tetB_F: TCATTGCCGACCTCAG tetB_R: CCAACCATCACCATCC	391	C	53	Nawaz et al., 2006	*E. coli* M.I.10.1
*qnr*A	qnrA_F: TTCTCACGCCAGGATTTG qnrA_R: CCATCCAGATCGGCAAA	521	C	53	Guillard et al., 2011	*K. pneumoniae* Kp 51
*qnr*B	qnrB_F: GGMATHGAAATTCGCCACTG qnrB_R: TTYGCBGYYCGCCAGTCG	261	C	53	Cattoir et al., 2007; Guillard et al., 2011	*K. pneumonia* Kp1
*qnr*S	qnrS_F: GCAAGTTCATTGAACAGGGT qnrS_R: TCTAAACCGTCGAGTTCGGCG	428	C	54	Cattoir et al., 2007	*K. oxytoca* Ko25
sul1	sul1_F: CTGAACGATATCCAAGGATTYCC sul1_R: AAAAATCCCACGGRTC	239	C	50	Heuer and Smalla, 2007	*A. media*
sul2	sul2_F: GCGCTCAAGGCAGATGGCAT sul2_R: GCGTTTGATACCGGCACCCG	293	C	60	Henriques et al., 2006a	*E. coli* A237

[a] *A, B, C, D, and E represent different PCR programs (see Table S1 for details).*

STATISTICAL ANALYSIS

AR phenotypes for each isolate were converted into a numeric code for each antibiotic: 0 signifying susceptibility, 1 representing intermediate resistance and 2 representing resistance. Principal Component Analysis (PCA, using a covariance matrix model) was used to explore AR patterns, reducing the multidimensional data matrix to a bidimensional biplot fit for interpretation, as performed in suchlike studies (Parveen et al., 1997; Su et al., 2012; Pereira et al., 2013). One-way analyses of variance (ANOVA) were performed on the PCA sample scores (principal components 1 and 2—PC1 and PC2) to assess significant differences ($p \leq 0.05$) in the AR profiles of *E. coli* isolates among sampling sources or phylogenetic groups. When applicable, *post-hoc* Tukey tests followed ANOVA tests. ANOVAs and PCA were conducted using Minitab and CANOCO for Windows version 4.5 (Scientia Ltd., UK), respectively.

RESULTS

PHYLOGENETIC DIVERSITY OF *E. COLI* ISOLATES

The set of 414 *E. coli* isolates were assigned to the main phylogenetic groups: A, B1, B2, and D (**Figure 1**). Overall, group A was the most prevalent (45% of the total number of isolates) followed by group B1 (31%), group D (21%) and group B2 (3%). Considering the different sampling sources (e.g., seawater, seagull feces and human-derived sewage) distribution among phylogenetic groups was rather similar, except for group B2 isolates that were only detected in sewage (10% of the total number of isolates retrieved from this source) and in seagull feces (4%).

ANTIBIOTIC RESISTANCE PROFILES

The susceptibility patterns of the isolates to 16 antibiotics are shown in **Figure 2**. Resistance (or intermediate resistance) was detected to all antibiotics tested and 94% of the isolates were resistant to at least one of the antibiotics. The most prevalent AR was toward streptomycin (83–100%), followed by tetracycline and cephalothin in seawater (48 and 42% respectively), and tetracycline and amoxicillin in the fecal sources (35 and 34% in seagull feces and 23% for both antibiotics in sewage). Isolates were more susceptible to imipenem, 3rd generation cephalosporins, gentamicin and the combination piperacillin/tazobactam.

For the majority of the antibiotics tested (e.g., penicillins, cephalothin, tetracycline), resistance rates were higher in isolates from seawater and seagull feces (**Figure 2**). Resistance to imipenem, 3rd generation cephalosporins and ciprofloxacin was most common in sewage. Multi-resistance levels (i.e., resistance to antibiotics of at least three different classes) were higher among isolates recovered from seawater (39%), followed by isolates from seagull feces (32%) and sewage (29%).

PCA analysis confirmed a clear AR gradient, with less resistant isolates (isolates resistant to a lower number of antibiotics) consistently displaying lower PC1 scores (i.e., being located at the left of the biplot) and more resistant isolates displaying higher PC1 scores (i.e., being located at the right of the biplot) (**Figure 3**). Although resistant and multi-resistant isolates were found in all sampling sources, significant differences in PC1 scores were found among sources [ANOVA: $F_{(2,\ 411)} = 3.7$, $p = 0.025$]. This was mostly due to a significantly higher PC1 score in seawater isolates comparatively to sewage isolates (Tukey test, $p < 0.05$). No significant differences in terms of the AR profile were found across phylogenetic groups, either for PC1 [ANOVA: $F_{(2,\ 397)} = 1.59$, $p = 0.206$] or PC2 [ANOVA: $F_{(2,\ 397)} = 0.43$, $p = 0.649$]; group B2 was excluded from this analysis due to low representativeness.

A total of 126 different resistance (or intermediate resistance) phenotypes were observed but most ($n = 86$) were represented by only one isolate. Resistance to streptomycin was the most common phenotype, identified in 90 isolates, followed by resistance to streptomycin and tetracycline (30 isolates) and streptomycin and cephalothin (27 isolates). Several multi-resistance phenotypes were detected. Among these, 10 phenotypes included 8 antibiotics and 9 phenotypes included 9 antibiotics. Twenty-four phenotypes (**Table 2**) were common to seawater and to at least one of the putative pollution sources (i.e., sewage and seagull feces). From these, 45% ($n = 11$) were common to seawater and

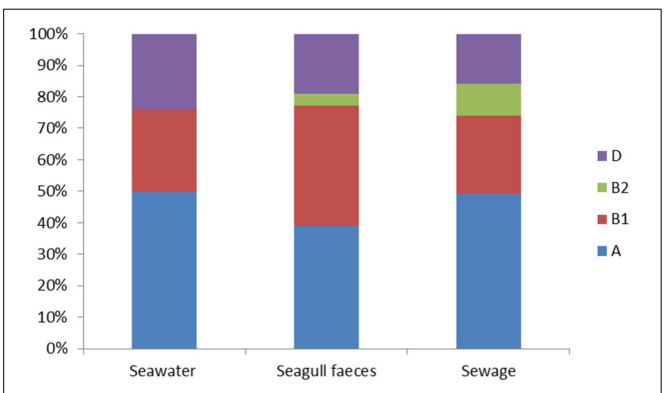


FIGURE 1 | Distribution of the *E. coli* phylogenetic groups (A, B1, B2 and D) among seawater ($n = 166$), seagull feces ($n = 179$) and human-derived sewage ($n = 69$).

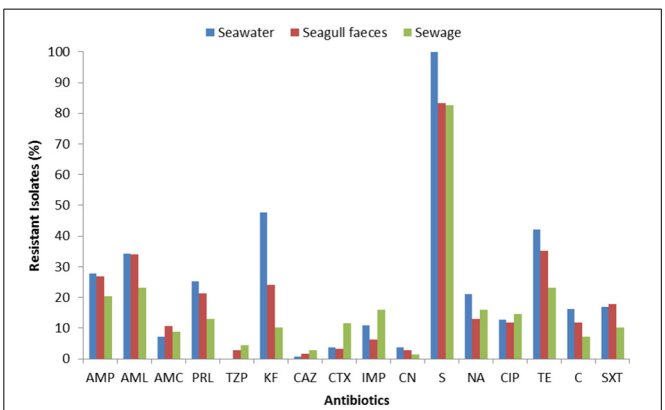


FIGURE 2 | Total resistance frequencies to 16 antibiotics of *E. coli* strains isolated from seawater ($n = 166$), seagull feces ($n = 179$) and human-derived sewage ($n = 69$). AMP, ampicillin; AML, amoxicillin; AMC, amoxicillin/clavulanic acid; PRL, piperacillin; TZP, piperacillin/tazobactam; KF, cephalothin; CAZ, ceftazidime; CTX, cefotaxime; IMP, imipenem; CN, gentamicin; S, streptomycin; NA, nalidixic acid; CIP, ciprofloxacin; TE, tetracycline; C, chloramphenicol; SXT, sulfamethoxazole/trimethoprim.

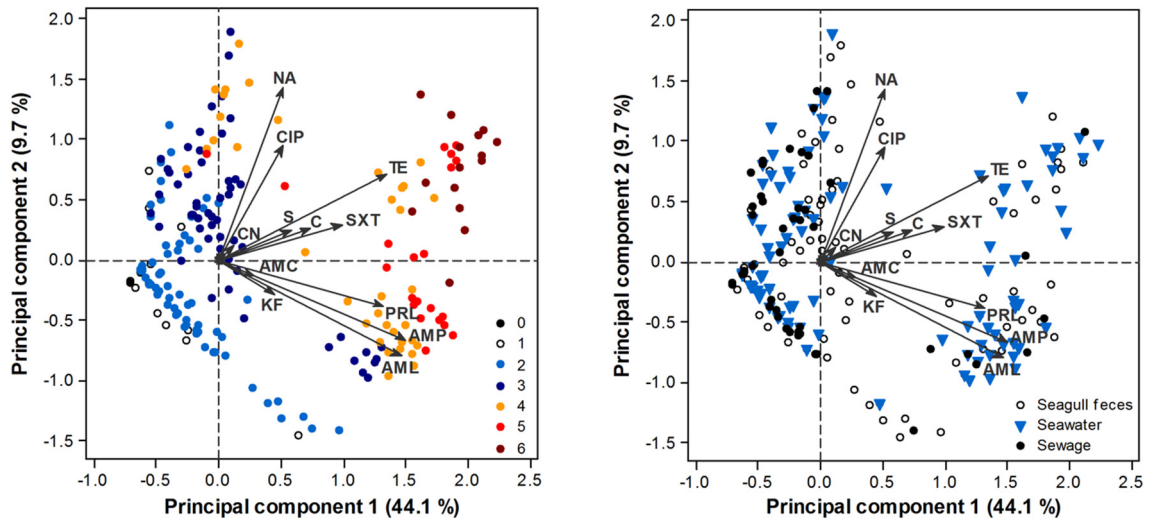


FIGURE 3 | PCA biplot of *E. coli* isolate scores in terms of their AR profile. Arrows represent antibiotics: AMP, ampicillin; AML, amoxicillin; AMC, amoxicillin/clavulanic acid; PRL, piperacillin; TZP, piperacillin/tazobactam; KF, cephalothin; CAZ, ceftazidime; CTX, cefotaxime; IMP, imipenem; CN, gentamicin; S, streptomycin; NA, nalidixic acid; CIP, ciprofloxacin; TE, tetracycline; C, chloramphenicol; SXT, sulfamethoxazole/trimethoprim. TZP, CAZ, CTX, IMP have little influence in the ordination, thus respective arrows are not perceptible. Scores are depicted according to the number of antibiotic classes that isolates are resistant to (0–6; **left panel**) or source of origin (**right panel**).

seagull feces but were absent from human-derived sewage, while only 8% ($n = 2$) of the phenotypes were exclusively detected in both seawater and sewage.

DETECTION OF ANTIBIOTIC RESISTANCE GENES

The resistance genes elected for this study were detected in 157 isolates out of 390 displaying a resistance phenotype (**Table 3**). The most commonly detected genes were bla_{TEM}, *sul1*, *sul2*, *tet*(A), and *tet*(B), which were prevalent in 6 out of 8 sampling moments (data not shown). The bla_{TEM} gene ($bla_{\text{TEM-1}}$ according to sequence analysis of representative isolates) was more prevalent in isolates from seawater (69% of 68 isolates resistant to penicillins) than in isolates from seagull feces (38% of) or sewage (24%). Both *tet*(A) and *sul2* were commonly detected in seawater (69 and 64%, respectively) and seagull feces (70 and 60%), but were less frequent in sewage (31 and 43%). Eighty six isolates displaying resistance to quinolones were tested for the presence of *qnr* determinants. Among these, *qnrS* (identified as *qnrS1* by sequence analysis) was the most prevalent, being more frequent in seagull feces (17% of 30 resistant isolates). This gene was detected in 5 out of 8 sampling dates. Gene *qnrB* (*qnrB19* according to sequence analysis) was detected in two sampling dates in isolates from seawater and seagull feces and *qnrA* was not detected. Gene $bla_{\text{SHV-12}}$ was detected in one isolate from seawater. Genes $bla_{\text{CTX-M}}$ and $bla_{\text{AmpC-like}}$ were detected in one isolate each, from seawater and seagull feces respectively. Sequencing analysis revealed 100% identity with $bla_{\text{CTX-M-1}}$ and $bla_{\text{CMY-2}}$. The genomic contexts of both genes were determined and results revealed genomic contexts identical to the ones previously described in clinical isolates (Kang et al., 2006; Tacão et al., 2012): IS*ECP1* was detected upstream of both genes and *ORF477* was identified downstream $bla_{\text{CTX-M-1}}$. The genes encoding resistance to carbapenems (i.e., $bla_{\text{OXA-48}}$, bla_{IMP}, bla_{VIM}, bla_{GES} and bla_{KPC}) were not detected in any of the *E. coli* isolates studied.

A total of 27 different AR genotypes were observed. Among these, 7 included only 1 resistance gene, 7 included 2 genes and 8 included 3 genes. Four genotypes included 4 genes: bla_{TEM}-*tet*(A)-*sul1*-*sul2*, detected in 4 isolates from seawater and 5 isolates from seagull feces; bla_{TEM}-*tet*(B)-*sul1*-*sul2*, detected in 2 isolates from seawater and 2 isolates from sewage; bla_{TEM}-$bla_{\text{SHV-12}}$-*tet*(A)-*sul2* and bla_{TEM}-$bla_{\text{CTX-M-1}}$-*tet*(A)-*qnrS1*, each detected in 1 seawater isolate. The genotype bla_{TEM}-*tet*(B)-*qnrS1*-*sul1*-*sul2* was detected in 1 isolate from seagull feces. Fourteen genotypes were shared between seawater and at least 1 of the putative pollution sources (**Table 4**). From these, 50% ($n = 7$) were shared between seawater and seagull feces, exclusively. Only 1 genotype was common to seawater and sewage, and absent from seagull feces.

DISCUSSION

The threats for human health represented by fecal pollution in aquatic environments depend on whether the source is human or non-human (Field and Samadpour, 2007). Dissemination of AR is certainly a risk factor. The particular characteristics of the Berlenga Island allowed evaluation of the contribution given by human-derived sewage and seagull feces, to the AR patterns found in *E. coli* strains that can be grown and isolated from coastal water samples.

High prevalence of resistance to streptomycin, tetracycline, cephalothin, and amoxicillin was detected in all sources. Results are in agreement with previous studies that demonstrated a wide dissemination of resistance to these antibiotics among *E. coli* isolates from Portuguese clinical settings (Freitas et al., 2014),

Table 2 | AR phenotypes detected in seawater and in at least one of the putative pollution sources (i.e., seagull feces and sewage).

Phenotypes	Number of isolates			
	Seawater	Seagull feces	Sewage	Total (% of the total number of isolates)
S	31	39	20	90 (21.7)
S, TE	14	13	3	30 (7.2)
KF, S	22	5	0	27 (6.5)
AML, KF, S	6	4	1	11 (2.7)
IPM, S	4	4	0	8 (1.9)
S, NA	4	1	1	6 (1.4)
AMP, AML, PRL, KF, S, TE	2	3	1	6 (1.4)
AMP, AML, PRL, KF, S, TE, STX	2	4	0	6 (1.4)
AML, S	1	3	1	5 (1.2)
AMP, S	1	0	3	4 (1.0)
S, NA, TE	2	1	1	4 (1.0)
AMP, AML, PRL, S, TE	2	1	1	4 (1.0)
AMC, S	1	2	0	3 (0.7)
S, TE, STX	2	1	0	3 (0.7)
AML, KF, S, TE	2	1	0	3 (0.7)
AMP, AML, KF, S	1	2	0	3 (0.7)
AMP, AML, PRL, S	1	2	0	3 (0.7)
AMP, AML, PRL, S, TE, STX	2	1	0	3 (0.7)
AMP, AML, AMC, PRL, KF, S, TE, C, STX	1	1	1	3 (0.7)
AMP, AML, PRL, KF, S, NA, CIP, TE, C, STX	2	1	0	3 (0.7)
AMP, AML, AMC, KF, S	0	2	0	2 (0.5)
KF, S, TE	1	1	0	2 (0.5)
AMP, AML, PRL, KF, S, TE, C, STX	1	1	0	2 (0.5)
AMP, AML, AMC, PRL, IPM, S, TE, C, STX	1	0	1	2 (0.5)

The number of isolates from each sampling source displaying each phenotype is presented.

AMP, ampicillin; AML, amoxicillin; AMC, amoxicillin/clavulanic acid; PRL, piperacillin; KF, cephalothin; IPM, imipenem; S, streptomycin; NA, nalidixic acid; CIP, ciprofloxacin; TE, tetracycline; C, chloramphenicol; STX, thrimethoprim/sulfamethoxazole.

Table 3 | Prevalence of resistance genes detected in the *E. coli* strains according to the isolation source.

Antibiotics	Genes	Seawater		Seagull feces		Sewage	
		Resistant isolates	Positive PCR results	Resistant isolates	Positive PCR results	Resistant isolates	Positive PCR results
3rd generation cephalosporins and/or penicillins	bla_{TEM}	68[a]	47 (69%)	82[a]	31 (38%)	33[a]	8 (24%)
	bla_{SHV-12}		1 (2%)		0		0
	$bla_{CTX-M-1}$	6[b]	1 (17%)	8[b]	0	8[b]	0
	bla_{CMY-2}		0		1 (13%)		0
Quinolones	*qnrB19*	39	1 (3%)	30	2 (7%)	17	0
	qnrS1		2 (5%)		5 (17%)		1 (6%)
Tetracycline	*tet*(A)	70	48 (69%)	63	44 (70%)	16	5 (31%)
	tet(B)		22 (31%)		15 (24%)		6 (38%)
Sulfonamide + trimethoprim	*sul1*	28	8 (29%)	32	8 (25%)	7	2 (29%)
	sul2		18 (64%)		19 (60%)		3 (43%)

[a] *bla_{TEM} and bla_{SHV} were inspected in all penicillin-resistant isolates (n = 183), 22 of which were also resistant to 3rd generation cephalosporins.*

[b] *bla_{CTX-M} and bla_{CMY} were inspected in isolates displaying resistance both to penicillins and 3rd generation cephalosporins (n = 22).*

Table 4 | AR genotypes detected in seawater and in at least one of the putative pollution sources (i.e., seagull feces and sewage).

Genotype	Seawater	Seagull feces	Sewage	Total (%)
tet(A)	21	20	3	44 (11.3)
bla_{TEM}, *tet*(A)	13	6	2	21 (5.4)
tet(B)	7	8	2	17 (4.4)
bla_{TEM}, *tet*(B)	7	3	2	12 (3.1)
bla_{TEM}, *tet*(A), *sul2*	4	5	0	9 (2.3)
bla_{TEM}, *tet*(A), *sul1*, *sul2*	4	5	0	9 (2.3)
bla_{TEM}	3	4	1	8 (2.1)
bla_{TEM}, *tet*(B), *sul2*	4	1	0	5 (1.3)
sul2	1	3	0	4 (1.0)
bla_{TEM}, *tet*(B), *sul1*, *sul2*	2	0	2	4 (1.0)
tet(A), *sul2*	1	2	0	3 (0.8)
bla_{TEM}, *sul2*	1	1	1	3 (0.8)
bla_{TEM}, *tet*(A), *qnrS1*	1	2	0	3 (0.8)
bla_{TEM}, *tet*(A), *tet*(B)	1	1	0	2 (0.5)

The number of isolates from each sampling source displaying each phenotype is presented.

animals (Radhouani et al., 2009) and aquatic systems (Pereira et al., 2013). For antibiotics mainly used in human medicine such as 3rd generation cephalosporins, ciprofloxacin and imipenem, levels of resistance were low. An uncommon phenotype combining resistance to imipenem and streptomycin and susceptibility to penicillins was detected in eight strains, six of which were isolated from sewage. Genes encoding carbapenemases were not detected in these strains, an indication that non-enzymatic mechanisms, such as the expression of efflux pumps or porin loss (Papp-Wallace et al., 2011), may contribute to the phenotype. Concerning 3rd generation cephalosporins, the resistance phenotype was explained only in three out of twenty-two resistant isolates. Novel or uncommon resistance genes may be present in the genomes of the remaining strains. On the other hand, mutations in the promoter and attenuator sequences of the chromosomal *ampC* gene may also contribute to reduced susceptibility to these antibiotics. These mutations have been described in *E. coli* isolates from different sources, including aquatic systems (Mataseje et al., 2009).

Levels of AR in coastal water isolates were expected to be low, considering the moderate human impact and the limits to survival imposed to *E. coli* by water salinity. However, experimental data revealed resistance levels similar to those previously reported for polluted freshwater (Ham et al., 2012; Pereira et al., 2013) or wastewater (Figueira et al., 2011). Multi-resistance levels were higher in coastal water (39%) than in the two sources of fecal contamination (32% for seagull feces and 29% found in human sewage).

Genes frequently hosted by clinical isolates and sharing similar genomic contexts (Saladin et al., 2002; Eckert et al., 2006), were also hosted by seawater isolates. Examples are ESBL-encoding genes such as $bla_{CTX\text{-}M\text{-}1}$ and $bla_{SHV\text{-}12}$ or plasmid-mediated quinolone resistance genes (e.g., *qnrB19*). It is to point that dissemination of these genes or related ones is a matter of serious concern in a scenario of lack of innovation in what concerns antimicrobial therapies. In fact, 3rd generation cephalosporins and fluoroquinolones are front-line therapeutics for the treatment of Gram-negative infections (Cantón and Coque, 2006; Cattoir et al., 2007).

AR features of seawater *E. coli* strains are mainly dependent on their animal source (Wicki et al., 2011). At the same time, *E. coli* strains are able to adapt to non-human environments, a process that implies changes in their genetic background (van Elsas et al., 2011). Adaptation to the more stressful saline conditions of coastal waters may favor strains with genotypes that are more efficient to deal with other stresses.

The results strongly suggest that the *E. coli* resistome found in coastal water was mainly impacted by fecal pollution from seagulls. The AR patterns found on seagull feces and seawater shared abundant common elements, while differing significantly from the resistance patterns found on sewage. Differences were not attributable to a different distribution in terms of phylogenetic groups. Based on these observations it is possible to hypothesize that the enteric environment of seagulls positively selects *E. coli* strains more prone to survive in marine environments. The same data allows proposing resistome analysis as a microbial source-tracking tool (see Harwood et al., 2014). Such approach has been previously tested, using almost exclusively data resulting from the analysis of resistance phenotypes (Parveen et al., 1997; Field and Samadpour, 2007; Carroll et al., 2009). Data on resistance genotypes add valuable information for the identification of the contamination source. The resistance gene pool in the analyzed sources was quite stable along the period of the study, except for the sporadic detection of infrequent genes (data not shown). Our recommendation is toward the use of genotypic data only in combination with other data. Our data further confirmed the conclusion of Araújo et al. (2014) establishing seagulls as the main source of fecal pollution on Berlenga Island.

This study highlights the risks associated to seagull-derived pollution. This is a critical aspect since wild life-derived pollution is frequently neglected due to the assumption that host-specificity of pathogens (Field and Samadpour, 2007) is a preventive barrier, that makes difficult if not impossible, the establishment on a new host. Indeed, the contribution of seagull fecal bacteria to AR dissemination constitutes an undeniable risk to humans, especially because of the close contact between these birds and human waste. Previous studies have confirmed seagulls as a reservoir of AR (Gionechetti et al., 2008; Bonnedahl et al., 2009; Dolejska et al., 2009). In our study, besides the high level of multi-resistance in seagull feces, we retrieved isolates carrying genes conferring resistance to antibiotics that are critically important for human medicine. For example, the prevalence of isolates carrying the *qnrS1* gene was higher in seagull feces than in sewage. This plasmid-encoded gene was previously identified in isolates from seagull feces (Literak et al., 2010) and is frequently found in human pathogens (Cattoir et al., 2007). Likewise, we detected the presence of *qnrB19* in seawater and seagull feces, a gene which was previously detected in animal and human isolates (Dolejska et al., 2013). Moreover, the plasmid-encoded gene $bla_{CMY\text{-}2}$, conferring resistance to 3rd generation cephalosporins, was detected in seagull feces and in a genomic context identical to the one found in

clinical isolates (Verdet et al., 2009). Poirel et al. (2012) reported a high proportion of CMY-2-positive strains among resistant *E. coli* isolated from seagull feces in Miami Beach. So, data obtained in different contexts indicate seagulls as important vectors of antibiotic resistant bacteria.

A previous study in the same Island (Radhouani et al., 2009), showed that all isolates were susceptible to carbapenems and 3rd generation cephalosporins. These antibiotics are mainly used in human medicine (Cantón and Coque, 2006; Papp-Wallace et al., 2011) and resistance to them is known to be still limited in unpolluted or moderately polluted environments (Henriques et al., 2012; Tacão et al., 2012). As reported here, resistance to these antibiotics was most common in human-derived sewage, but its presence on seagull feces isolates suggests an undesirable progress in terms of resistance dissemination. Seagulls based on the Island fly to urban areas on the coast for feeding. Like suggested for other geographic regions (Bonnedahl et al., 2009), human activities may considerably influence the AR patterns associated to seagulls in remote areas, such as Berlenga. In fact, it is a matter of concern that levels of resistance to 3rd generation cephalosporins have been consistently increasing in isolates from clinical settings in Portugal (ECDC, 2013). For example, gene $bla_{\text{CMY-2}}$, which was here detected in one isolate from seagull feces, has been detected in *E. coli* from clinical samples in Portuguese health care settings (Freitas et al., 2014).

CONCLUSIONS

This study confirms seawater as a relevant reservoir of *E. coli* strains adapted to marine environments and carrying AR determinants that are common to clinical strains. It is evidenced that the seawater *E. coli* resistome in Berlenga is mainly modulated by seagulls-derived pollution. This fact confirms seagulls as a main spread vehicle of AR bacteria to coastal water. Occurrence of high levels of multi-resistance and of clinically relevant genes underlies the need to carefully consider the human health risks subjacent to seagull-derived pollution. The assessment of these risks is essential for an effective management of coastal water used for recreational activities. Lastly, we believe that this study—along with correlated data from previous studies—has succeeded in showing that AR could be used as a tool to aid in the identification of the fecal sources of contamination.

ACKNOWLEDGMENTS

The Portuguese Foundation for Science and Technology (FCT) supported this study through projects PTDC/AAC-AMB/109155/2008 and FCOMP-01-0124-FEDER-008640. FCT also financed the fellowships of A Pereira (SFRH/BPD/26685/2006). I Henriques was supported by the project MARES (Sustainable Use of Marine Resources; ref. SCT_2011_02_033_4904) funded by National Strategic reference Framework. Bruno B. Castro was supported by the Programme Ciência 2008, co-funded by the Human Potential Operational Programme (National Strategic Reference Framework 2007–2013) and European Social Fund (EU). This work was supported by European Funds through COMPETE and by National Funds through the Portuguese Science Foundation (FCT) within project PEst-C/MAR/LA0017/2013.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00426/abstract

REFERENCES

Alouache, S., Kada, M., Messai, Y., Estepa, V., Torres, C., and Bakour, R. (2012). Antibiotic resistance and extended-spectrum beta-lactamases in isolated bacteria from seawater of Algiers beaches (Algeria). *Microb. Environ.* 27, 80–86. doi: 10.1264/jsme2.ME11266

Aminov, R. I. (2011). Horizontal gene exchange in environmental microbiota. *Front. Microbiol.* 2:158. doi: 10.3389/fmicb.2011.00158

Araújo, S., Henriques, I. S., Leandro, S. M., Alves, A., Pereira, A., and Correia, A. (2014). Gulls identified as major source of faecal pollution in coastal waters: a microbial source tracking study. *Sci. Total Environ.* 470–471, 84–91. doi: 10.1016/j.scitotenv.2013.09.075

Austin, D. J., Kristinsson, K. G., and Anderson, R. M. (1999). The relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance. *Proc. Natl. Acad. Sci. U.S.A.* 96, 1152–1156. doi: 10.1073/pnas.96.3.1152

Baquero, F., Martínez, J. L., and Cantón, R. (2008). Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol.* 19, 260–265. doi: 10.1016/j.copbio.2008.05.006

Bonnedahl, J., Drobni, M., Gauthier-Clerc, M., Hernandez, J., Granholm, S., Kayser, Y., et al. (2009). Dissemination of *Escherichia coli* with CTX-M type ESBL between humans and yellow-legged gulls in the south of France. *PLoS ONE* 4:e5958. doi: 10.1371/journal.pone.0005958

Cantón, R., and Coque, T. M. (2006). The CTX-M beta-lactamase pandemic. *Curr. Opin. Microbiol.* 9, 466–475. doi: 10.1016/j.mib.2006.08.011

Carroll, S. P., Dawes, L., Hargreaves, M., and Goonetilleke, A. (2009). Faecal pollution source identifcation in an urbanizing catchment using antibiotic resistance profling, discriminant analysis and partial least squares regression. *Water Res.* 43, 1237–1246. doi: 10.1016/j.watres.2008.12.017

Cattoir, V., Poirel, L., and Nordmann, P. (2007). Plasmid-mediated quinolone resistance determinant QnrB4 identified in France in an *Enterobacter cloacae* clinical isolate coexpressing a QnrS1 determinant. *Antimicrob. Agents Chemother.* 51, 2652–2653. doi: 10.1128/AAC.01616-06

Clermont, O., Bonacorsi, S., and Bingen, E. (2000). Rapid and simple determination of *Escherichia coli* phylogenetic group. *Appl. Environ. Microbiol.* 66, 4555–4558. doi: 10.1128/AEM.66.10.4555-4558.2000

CLSI (2012). *Performance Standard for Antimicrobial Susceptibility Testing e Document Approved Standard M100-S22*. Wayne, PA: CLSI.

Dallenne, C., Da Costa, A., Decré, D., Favier, C., and Arlet, G. (2010). Development of a set of multiplex PCR assays for the detection of genes encoding important beta-lactamases in *Enterobacteriaceae*. *J. Antimicrob. Chemother.* 65, 490–495. doi: 10.1093/jac/dkp498

Devirgiliis, C., Barile, S., and Perozzi, G. (2011). Antibiotic resistance determinants in the interplay between food and gut microbiota. *Genes Nutr.* 6, 275–284. doi: 10.1007/s12263-011-0226-x

Dolejska, M., Bierosova, B., Kohoutova, L., Literak, I., and Cizek, A. (2009). Antibiotic-resistant *Salmonella* and *Escherichia coli* isolates with integrons and extended-spectrum beta-lactamases in surface water and sympatric black-headed gulls. *J. Appl. Microbiol.* 106, 1941–1950. doi: 10.1111/j.1365-2672.2009.04155.x

Dolejska, M., Villa, L., Hasman, H., Hansen, L., and Carattoli, A. (2013). Characterization of IncN plasmids carrying blaCTX-M-1 and qnr genes in *Escherichia coli* and Salmonella from animals, the environment and humans. *J. Antimicrob. Chemother.* 68, 333–339. doi: 10.1093/jac/dks387

ECDC. (2013). *European Centre for Disease Prevention and Control. Antimicrobial resistance surveillance in Europe 2012. Annual report of the European Antimicrobial Resistence Surveillance Network (EARS-Net)*. Available online at http://ecdc.europa.eu/ (Accessed 07 July, 2014).

Eckert, C., Gautier, V., and Alert, G. (2006). DNA sequence analysis of the genetic environment of various *bla*CTX-M genes. *J. Antimicrob. Chemother.* 57, 14–23. doi: 10.1093/jac/dki398

Field, K. G., and Samadpour, M. (2007). Faecal source tracking, the indicator paradigm, and managing water quality. *Water Res.* 41, 3517–3538. doi: 10.1016/j.watres.2007.06.056

Figueira, V., Serra, E., and Manaia, C. M. (2011). Differential patterns of antimicrobial resistance in population subsets of *Escherichia coli* isolated from waste- and surface waters. *Sci. Total Environ.* 409, 1017–1023. doi: 10.1016/j.scitotenv.2010.12.011

Freitas, F., Machado, E., Ribeiro, T. G., Novais, Â., Peixe, L. (2014). Long-term dissemination of acquired AmpC β-lactamases among *Klebsiella* spp. and *Escherichia coli* in Portuguese clinical settings. *Eur. J. Clin. Microbiol. Infect. Dis.* 33, 551–558. doi: 10.1007/s10096-013-1983-9

Gionechetti, F., Zucca, P., Gombac, F., Monti-Bragadin, C., Lagatolla, C., Tonin, E., et al. (2008). Characterization of antimicrobial resistance and class 1 integrons in *Enterobacteriaceae* isolated from Mediterranean herring gulls (*Larus cachinnans*). *Microb. Drug Resist.* 14, 93–99. doi: 10.1089/mdr.2008.0803

Gomez-Alvarez, V., Revetta, R. P., and Santo Domingo, J. W. (2012). Metagenomic analysis of drinking water receiving different disinfection treatments. *Appl. Environ. Microbiol.* 78, 6095–6102. doi: 10.1128/AEM.01018-12

Guillard, T., Moret, H., Brasmea, L., Carliera, A., Vernet-Garniera, V., Cambau, E., et al. (2011). Rapid detection of *qnr* and *qepA* plasmid mediated quinolone resistance genes using real-time PCR. *Diagn. Microbiol. Infect. Dis.* 70, 253–259. doi: 10.1016/j.diagmicrobio.2011.01.004

Ham, Y. S., Kobori, H., Kang, J. H., Matsuzaki, T., Iino, M., and Nomura, H. (2012). Distribution of antibiotic resistance in urban watershed in Japan. *Environ. Pollut.* 162, 98–103. doi: 10.1016/j.envpol.2011.11.002

Harwood, V. J., Staley, C., Badgley, B. D., Borges, K., and Korajkic, A. (2014). Microbial source tracking markers for detection of faecal contamination in environmental waters: relationships between pathogens and human health outcomes. *FEMS Microbiol. Rev.* 38, 1–40. doi: 10.1111/1574-6976.12031

Henriques, I., Araújo, S., Azevedo, J. S., Alves, M. S., Chouchani, C., Pereira, A., et al. (2012). Prevalence and diversity of carbapenem-resistant bacteria in untreated drinking water in Portugal. *Microb. Drug Resist.* 18, 531–537. doi: 10.1089/mdr.2012.0029

Henriques, I., Fonseca, F., Alves, A., Saavedra, M. J., and Correia, A. (2006a). Occurrence and diversity of integrons and beta-lactamase genes among ampicillin-resistant isolates from estuarine waters. *Res. Microbiol.* 157, 938–947. doi: 10.1016/j.resmic.2006.09.003

Henriques, I., Moura, A., Alves, A., Saavedra, M. J., and Correia, A. (2006b). Analysing diversity among beta-lactamase encoding genes in aquatic environments. *FEMS Microbiol. Ecol.* 56, 418–429. doi: 10.1111/j.1574-6941.2006.00073.x

Heuer, H., and Smalla, K. (2007). Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. *Environ. Microbiol.* 9, 657–666. doi: 10.1111/j.1462-2920.2006.01185.x

Johnson, J. R., Delavari, P., Kuskowski, M., and Stell, A. L. (2001). Phylogenetic distribution of extraintestinal virulence-associated traits in *Escherichia coli*. *J. Infect. Dis.* 183, 78–88. doi: 10.1086/317656

Kang, M. S., Besser, T. E., and Call, D. R. (2006). Variability in the region downstream of the blaCMY-2 beta-lactamase gene in *Escherichia coli* and Salmonella enterica plasmids. *Antimicrob. Agents Chemother*. 50, 1590–1593. doi: 10.1128/AAC.50.4.1590-1593.2006

Kaper, J. B., Nataro, J. P., and Mobley, H. L. (2004). Pathogenic *Escherichia coli*. *Nat. Rev. Microbiol.* 2, 123–140. doi: 10.1038/nrmicro818

Literak, I., Dolejska, M., Janoszowska, D., Hrusakova, J., Meissner, W., Rzyska, H., et al. (2010). Antibiotic-resistant *Escherichia coli* bacteria, including strains with genes encoding the extended-spectrum beta-lactamase and QnrS, in waterbirds on the Baltic Sea Coast of Poland. *Appl. Environ. Microbiol.* 76, 8126–8134. doi: 10.1128/AEM.01446-10

Lu, S., Zhang, Y., Geng, S., Li, T., Ye, Z., Zhang, D., et al. (2010). High diversity of extended-spectrum beta- lactamase-producing bacteria in an urban river sediment habitat. *Appl. Environ. Microbiol.* 76, 5972–5976. doi: 10.1128/AEM.00711-10

Mataseje, L. F., Neumann, N., Crago, B., Baudry, P., Zhanel, G. G., Louie, M., et al. (2009). Characterization of cefoxitin-resistant *Escherichia coli* isolates from recreational beaches and private drinking water in Canada between 2004 and 2006. *Antimicrob. Agents Chemother.* 53, 3126–3130. doi: 10.1128/AAC.01353-08

Nawaz, M., Sung, K., Khan, S. A., Khan, A. A., and Steel, R. (2006). Biochemical and molecular characterization of tetracycline-resistant *Aeromonas veronii* isolates from catfish. *Appl. Environ. Microbiol.* 72, 6461–6466. doi: 10.1128/AEM.00271-06

Papp-Wallace, K. M., Endimiani, A., Taracila, M. A., and Bonomo, R. A. (2011). Carbapenems: past, present, and future. *Antimicrob. Agents Chemother.* 55, 4943–4960. doi: 10.1128/AAC.00296-11

Parveen, S., Murphree, R. L., Edmiston, L., Kaspar, C. W., Portier, K. M., and Tamplin, M. L. (1997). Association of multiple-antibiotic-resistance profiles with point and nonpoint sources of *Escherichia coli* in Apalachicola Bay. *Appl. Environ. Microbiol.* 63, 2607–2612. doi: 10.1128/AEM.00763-07

Pereira, A., Santos, A., Tacão, M., Alves, A., Henriques, I., and Correia, A. (2013). Genetic diversity and antimicrobial resistance of *Escherichia coli* from Tagus estuary (Portugal). *Sci. Total Environ.* 461–462, 65–71. doi: 10.1016/j.scitotenv.2013.04.067

Picard, B., Garcia, J. S., Gouriou, S., Duriez, P., Brahimi, N., Bingen, E., et al. (1999). The link between phylogeny and virulence in *Escherichia coli* extraintestinal infection. *Infect. Immu.* 67, 546–553.

Poirel, L., Héritier, C., Tolün, V., and Nordmann, P. (2004). Emergence of oxacillinase-mediated resistance to imipenem in *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother*. 48, 15–22. doi: 10.1128/AEM.00271-06

Poirel, L., Potron, A., de La Cuesta, C., Cleary, T., Nordmann, P., and Munoz-Price, L. S. (2012). Wild coastline birds as reservoirs of broad-spectrum-beta-lactamase-producing *Enterobacteriaceae* in Miami Beach, Florida. *Antimicrob. Agents Chemother.* 56, 2756–2758. doi: 10.1128/AAC.05982-11

Radhouani, H., Poeta, P., Igrejas, G., Gonçalves, A., Vinu,é, L., and Torres, C. (2009). Antimicrobial resistance and phylogenetic groups in isolates of *Escherichia coli* from seagulls at the Berlengas nature reserve. *Vet. Rec.* 165, 138–142. doi: 10.1136/vr.165.5.138

Rosewarne, C. P., Pettigrove, V., Stokes, H. W., and Parsons, Y. M. (2010). Class 1 integrons in benthic bacterial communities: abundance, association with Tn402-like transposition modules and evidence for co-selection with heavy-metal resistance. *FEMS Microbiol. Ecol.* 72, 35–46. doi: 10.1111/j.1574-6941.2009.00823.x

Saladin, M., Cao, V. T., Lambert, T., Donay, J. L., Herrmann, J. L., Ould-Hocine, Z., et al. (2002). Diversity of CTX-M beta-lactamases and their promoter regions from *Enterobacteriaceae* isolated in three Parisian hospitals. *FEMS Microbiol. Lett.* 209, 161-168. doi: 10.1111/j.1574-6968.2002.tb11126.x

Simões, R. R., Poirel, L., Da Costa, P. M., and Nordmann, P. (2010). Seagulls and beaches as reservoirs for multidrug-resistant *Escherichia coli*. *Emerg. Infect. Dis.* 6, 110–112. doi: 10.3201/eid1601.090896

Speldooren, V., Heym, B., Labia, R., and Nicolas-Chanoine, M. H. (1998). Discriminatory detection of inhibitor-resistant beta-lactamases in *Escherichia coli* by single-strand conformation polymorphism-PCR. *Antimicrob. Agents Chemother.* 42, 879–884.

Su, H. C., Ying, G. G., Tao, R., Zhang, R. Q., Zhao, J. L., and Liu, Y. S. (2012). Class 1 and 2 integrons, *sul* resistance genes and antibiotic resistance in *Escherichia coli* isolated from Dongjiang River, South China. *Environ. Pollut.* 169, 42–49. doi: 10.1016/j.envpol.2012.05.007

Tacão, M., Correia, A., and Henriques, I. (2012). Resistance to broad-spectrum antibiotics in aquatic systems: anthropogenic activities modulate the dissemination of *bla*CTX-M-like genes. *Appl. Environ. Microbiol.* 78, 4134–4140. doi: 10.1128/AEM.00359-12

Tacão, M., Moura, A., Correia, A., and Henriques, I. (2014). Co-resistance to different classes of antibiotics among ESBL-producers from aquatic systems. *Water Res.* 48, 100–107. doi: 10.1016/j.watres.2013.09.021

van Elsas, J. D., Semenov, A. V., Costa, R., and Trevors, J. T. (2011). Survival of *Escherichia coli* in the environment: fundamental and public health aspects. *ISME J.* 5, 173–183. doi: 10.1038/ismej.2010.80

Verdet, C., Gautier, V., Chachaty, E., Ronco, E., Hidri, N., Decré, D., et al. (2009). Genetic context of plasmid-carried $bla_{\text{CMY-2-like}}$ genes in *Enterobacteriaceae*. *Antimicrob. Agents Chemother.* 53, 4002–4006. doi: 10.1128/AAC.00753-08

Vredenburg, J., Varela, A. R., Hasan, B., Bertilsson, S., Olsen, B., Narciso-da-Rocha, C., et al. (2014). Quinolone-resistant *Escherichia coli* isolated from birds of prey in Portugal are genetically distinct from those isolated from water environments and gulls in Portugal, Spain and Sweden. *Environ. Microbiol.* 16, 995–1004. doi: 10.1111/1462-2920.12231

Wellington, E. M. H., Boxall, A. B. A., Cross, P., Feil, E. J., Gaze, W. H., Hawkey, P. M., et al. (2013). The role of natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. *Lancet Infect. Dis.* 13, 155–165. doi: 10.1016/S1473-3099(12)70317-1

Wicki, M., Karabulut, F., Auckenthaler, A., Felleisen, R., Tanner, M., and Baumgartner, A. (2011). Identification of faecal input sites in spring water by selection and genotyping of multiresistant *Escherichia coli*. *Appl. Environ. Microbiol.* 77, 8427–8433. doi: 10.1128/AEM.05651-11

Zhao, J. Y., and Dang, H. (2012). Coastal seawater bacteria harbor a large reservoir of plasmid mediated quinolone resistance determinants in Jiaozhou Bay, China. *Microb. Ecol.* 64, 187–199. doi: 10.1007/s00248-012-0008-z

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 May 2014; accepted: 29 July 2014; published online: 20 August 2014.

Citation: Alves MS, Pereira A, Araújo SM, Castro BB, Correia ACM and Henriques I (2014) Seawater is a reservoir of multi-resistant Escherichia coli, including strains hosting plasmid-mediated quinolones resistance and extended-spectrum beta-lactamases genes. Front. Microbiol. ***5****:426. doi: 10.3389/fmicb.2014.00426*

This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 04 August 2015
doi: 10.3389/fmicb.2015.00796

Sulfonamide and tetracycline resistance genes in total- and culturable-bacterial assemblages in South African aquatic environments

Satoru Suzuki[1], Mitsuko Ogo[1], Tatsuya Koike[2], Hideshige Takada[2] and Brent Newman[3]*

[1] Center for Marine Environmental Studies, Ehime University, Matsuyama, Japan, [2] Tokyo University of Agriculture and Technology, Fuchu, Japan, [3] Coastal Systems Research Group, Natural Resources and the Environment, The Council for Scientific and Industrial Research, Durban, South Africa

Antibiotic resistant bacteria are ubiquitous in the natural environment. The introduction of effluent derived antibiotic resistance genes (ARGs) into aquatic environments is of concern in the spreading of genetic risk. This study showed the prevalence of sulfonamide and tetracycline resistance genes, *sul1*, *sul2*, *sul3,* and *tet*(M), in the total bacterial assemblage and colony forming bacterial assemblage in river and estuarine water and sewage treatment plants (STP) in South Africa. There was no correlation between antibiotic concentrations and ARGs, suggesting the targeted ARGs are spread in a wide area without connection to selection pressure. Among *sul* genes, *sul1* and *sul2* were major genes in the total (over 10^{-2} copies/16S) and colony forming bacteria assemblages ($\sim 10^{-1}$ copies/16S). In urban waters, the *sul3* gene was mostly not detectable in total and culturable assemblages, suggesting *sul3* is not abundant. *tet*(M) was found in natural assemblages with 10^{-3} copies/16S level in STP, but was not detected in colony forming bacteria, suggesting the non-culturable (yet-to-be cultured) bacterial community in urban surface waters and STP effluent possess the *tet*(M) gene. Sulfamethoxazole (SMX) resistant (SMX^r) and oxytetracycline (OTC) resistant (OTC^r) bacterial communities in urban waters possessed not only *sul1* and *sul2* but also *sul3* and *tet*(M) genes. These genes are widely distributed in SMX^r and OTC^r bacteria. In conclusion, urban river and estuarine water and STP effluent in the Durban area were highly contaminated with ARGs, and the yet-to-be cultured bacterial community may act as a non-visible ARG reservoir in certain situations.

Keywords: antibiotic resistance, *sul*, *tet*(M), yet-to-be cultured, South Africa, sewage treatment plant

OPEN ACCESS

Edited by:
David W. Graham, Newcastle University, UK

Reviewed by:
Andrew C. Singer, Natural Environment Research Council-Centre for Ecology and Hydrology, UK
Joakim Larsson, University of Gothenburg, Sweden

****Correspondence:***
Satoru Suzuki, Center for Marine Environmental Studies, Ehime University, Matsuyama, Ehime 790-8577, Japan
ssuzuki@ehime-u.ac.jp

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *30 April 2015*
Accepted: *22 July 2015*
Published: *04 August 2015*

Citation:
Suzuki S, Ogo M, Koike T, Takada H and Newman B (2015) Sulfonamide and tetracycline resistance genes in total- and culturable-bacterial assemblages in South African aquatic environments. Front. Microbiol. 6:796. doi: 10.3389/fmicb.2015.00796

Introduction

Antibiotic resistance genes (ARGs) are found not only in the clinical but also the natural environment, which can eventually produce antibiotic resistant bacteria (ARB). Antibiotics and ARB are released to the environment from hospitals, livestock facilities, and sewage treatment plants (STP) (Pruden et al., 2013). Although antibiotics are decomposed and diluted in the aquatic environment water, even at low concentrations they may act as signaling molecules in microbes (Fajardo and Martinez, 2008). Selection of ARG mutation by very low concentrations of antibiotics

is reported (Gullberg et al., 2011). It is, therefore, critical to understand the fate of released antibiotics, ARB and ARGs in the environment, and whether residual ARGs in the environment pose a risk to humans. The aim of this study was to assess the status of antibiotics and ARGs in anthropogenically impacted surface waters in one area of South Africa.

The status of antibiotic use and STP operation differs between countries. Consequently, the status of antibiotic contamination and presence of ARBs and ARGs in aquatic ecosystems must be established on a case by case basis. In previous monitoring we showed the status of antibiotic contamination (Shimizu et al., 2013) and ARGs (Suzuki et al., 2013) in numerous Asian countries. In many tropical Asian countries an integrated system of agriculture is followed, which includes animal husbandry, aquaculture, and crop farming (Suzuki and Hoa, 2012). In this system the major antibiotic used for animals is sulfonamides. Tetracyclines are also used in aquaculture. STPs receive wastewater and excreta from humans and livestock facilities, which intimates the mixing of waters containing various antibiotics, ARB and ARGs. The main purpose of conventional STPs is to prevent the spread of infectious diseases and reduce solid and nutrient loads from excreta entering surface waters, not to decompose pharmaceuticals and genes. Although advanced disinfection technologies can greatly reduce the danger of waterborne diseases (United States Environmental Protection and Agency, 2004), antibiotics and ARGs are not completely decomposed in the STP process and are released into the environment (Rizzo et al., 2013; Berkner et al., 2014).

The populations and economies of African countries are developing. Although South Africa has a relatively well developed economy by African standards, many STPs are not functioning efficiently and are overloaded and has been identified as a serious cause for concern (Snyman et al., 2006; Water Research and Commission, 2006). Furthermore, most South African cities are characterized by large informal settlements where sanitation facilities are poor and in some cases essentially non-existent, with pit latrines and mobile toilets usually the only form of sanitation. This might result in the introduction of antibiotics, ARB and ARGs into the aquatic environment. Omulo et al. (2015) reviewed many articles on ARB research from Eastern Africa, which mainly studied on human and animal bacteria. Environmental ARB needs to be studied further.

It is well known that the majority of bacteria in aquatic environments are non-culturable or yet-to-be cultured bacteria (Bloomfield et al., 1988; Amann et al., 1995; Takami et al., 2009). In recent monitoring in the Philippines we showed that the total bacterial community in seawater possessed minor sulfonamide resistance gene *sul3*, which was not detected in colony forming bacteria (Suzuki et al., 2013). This suggests that the abundant non-culturable or yet-to-be cultured bacteria in aquatic environments are a reservoir of ARGs, but these are not detectable by culture methods. The *sul3* gene was detected in human and non-human isolates of *Salmonella* in Portuguese waters, although *sul3* was a minor contributor compared to *sul1* and *sul2* genes (Antunes et al., 2005). In Denmark, *Escherichia coli* isolated from pork and pigs possessed *sul3*, but this gene was not found in human isolates (Hammerum et al., 2006). In Germany, *sul3* was not found in *E. coli* of human isolates, but was found in cattle, pig, and poultry isolates (Guerra et al., 2003). These studies suggest that *sul3* is spreading widely amongst animals but not amongst humans, possibly due to the use of sulfonamide for animal husbandry but not in humans in developed countries, and that *sul3* is transferred by a different gene cassette to *sul1* and *sul2* (Antunes et al., 2005). Although recent advances in metagenomics can detect total resistome, quantitative estimation of ARGs in the microbial community is not yet possible. An understanding of the reservoir of culturable- and non-culturable bacteria in the environment might thus be useful in assessing whether environmental ARGs are posing a risk.

The aim of this study was to assess the abundance of *sul1*, *sul2*, *sul3,* and *tet*(M) genes in total- and colony forming-bacterial assemblages in surface waters and STP effluent in the eThekwini area of South Africa. As far as we are aware, no information in this context is available for this area, or indeed for other areas in South Africa. Sulfonamides and tetracyclines have a long use as human and animal therapeutic agents and animal growth promoters. Sulfonamide resistance occurs mainly by mutation of the dihydropteroate synthase (DHPS) gene, although other mechanisms are known (Radstrom and Swedberg, 1988; Huang et al., 2004). As to tetracycline resistance, 45 *tet* genes are known at this time (Roberts et al., 2012). Among the *tet* genes, *tet*(M), a ribosomal protection protein gene is suspected of having the broadest host range (Roberts et al., 2012) and its origin is reported to be ancient (Kobayashi et al., 2007). Additionally, *tet*(M) shows high genetic diversity (Rizzotti et al., 2009) and wide distribution in the natural environment (D'Costa et al., 2011). Therefore, we focused on the *sul* genes and *tet*(M) as monitoring targets. We hypothesized that effluent from inefficient STPs or wastewater derived from poor sanitary conditions should contain high concentrations of ARGs from human bacteria. The comparison of ARGs using culture-dependent and independent methods should, therefore, provide an understanding on whether bacterial communities of natural or human origin are the major reservoir of ARGs in aquatic ecosystems.

Materials and Methods

Sampling of Water

Samples were collected with an ethanol rinsed stainless steel bucket between September 3 and 5 in 2012, in the eThekwini Metropolitan Municipality area in the province of KwaZulu-Natal, on the subtropical northeast cost of South Africa (**Figure 1**). Characteristics of the sampling sites are summarized in Supplementary Table S1. The municipality has a population size of about 3,400,000 (Statistics South Africa, http://www.statssa.gov.za/?page_id=1021&id=ethekwini-municipality). The city of Durban and a number of smaller towns fall in the municipal area. Rainfall in the eThekwini area is seasonal, falling predominantly in summer. Although it was not raining at the times that samples were collected, about 31 mm of rain was recorded at rain monitoring gage in Durban during the sampling

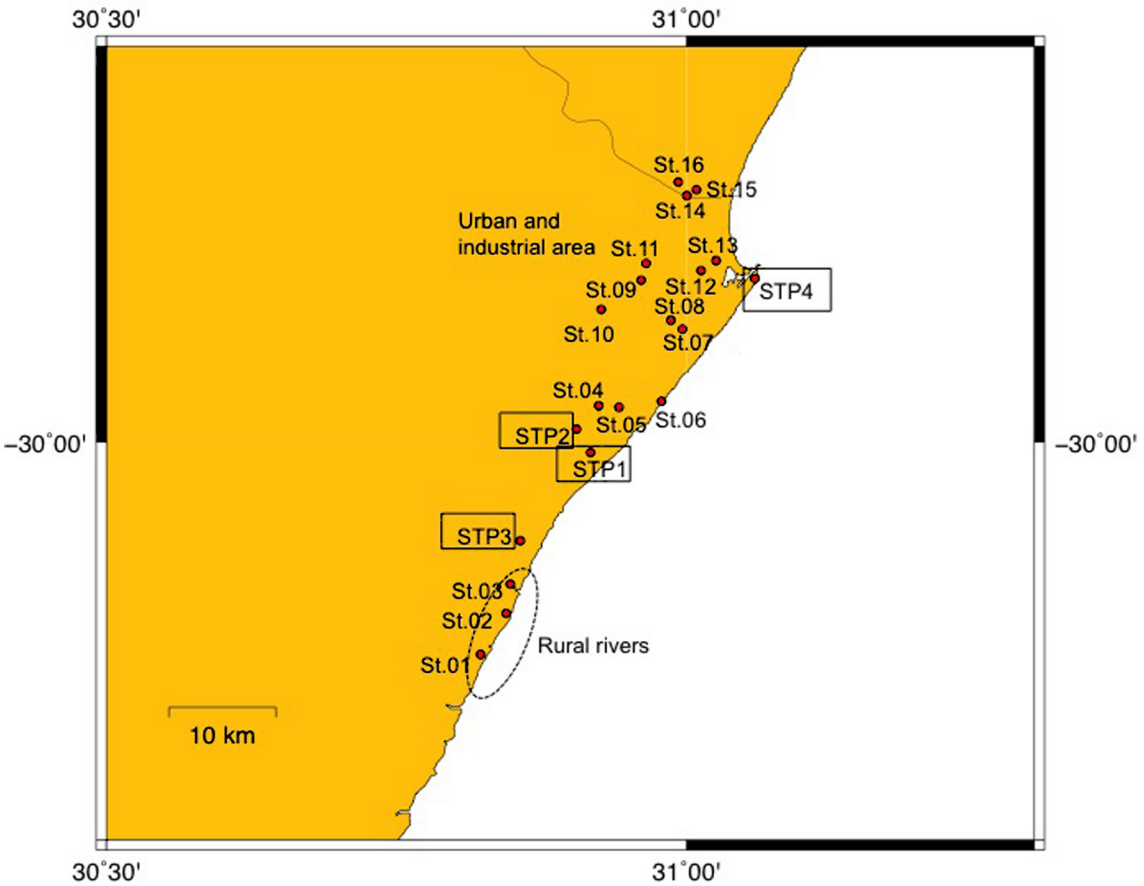


FIGURE 1 | Map of sampling sites. Sites 01–03 are rural river sites, Sites 04–16 are urban river sites, and STP1-4 are sewage treatment plants (STPs).

period. Because samples were collected at a single point in time we recognize this study does not provide an understanding on the temporal variability of ARBs and ARGs in surface waters and STP effluents in the study area.

Surface water samples were taken at three sites (Sites 01–03) in the estuarine parts of rivers situated in rural locations. Thirteen sites (Sites 04–16) were sampled in the riverine and estuarine parts of rivers with urbanized and industrialized catchments in the greater Durban area, and effluent was collected from four STPs (STP1–4). The water and effluent was filtered through 50 μm mesh plankton net to remove large debris, and stored on ice for a few hours until analysis. Further detail on the condition of surface waters at the river and estuarine sites is provided in Segura et al. (2015). Water samples indicate present status of contamination (Takasu et al., 2011).

Antibiotic Concentration

Sulfonamides and tetracyclines were analyzed using a liquid chromatograph (Accela, Thermo Scientific) equipped with a tandem mass spectrometer (LC-MS/MS; Quantum Access, Thermo Scientific) after extraction using a solid-phase cartridge (Oasis HLB resin, Waters). The analytical process was the same as that provided in Segura et al. (2015).

Bacterial Count

Total bacterial cell number was counted by DAPI staining according to Sato-Takabe et al. (2015). Total viable count and sulfamethoxazole resistant (SMX^r) and oxytetracycline resistant (OTC^r) bacterial numbers were enumerated on nutrient agar plates (LB plus 1.5% agar) incubated at 30°C for 24 h. To estimate SMX^r and OTC^r bacteria, 60 μg/mL of each drug was supplemented to the medium (Hoa et al., 2011). All plate counts were performed in duplicate.

Quantitative Analysis of Antibiotic Resistance Genes (ARGs)

The sulfonamide resistance genes, *sul1*, *sul2,* and *sul3*, and tetracycline resistance gene, *tet*(M), were quantified by quantitative PCR (qPCR) from total assemblage using total DNA trapped on 0.2 μm pore filter. For the culturable bacterial assemblage, all colonies on agar plates were mixed and used for qPCR. DNA extraction from the filter and mixtures of colonies was previously reported (Suzuki et al., 2013). DNA from filters and colonies were obtained from triplicate biological samples. qPCR was performed using a CFX 96 Real-Time system (BioRad, Laboratories, Hercules, CA, USA) to detect an increase of double-stranded DNA with an increase in fluorescence

according to Suzuki et al. (2013). PCR amplifications were performed in a 20 μl reaction volume containing 1 X Sso Fast EvaGreen Supermix (Bio-Rad), 500 nM of each primer and 1 μl of sample DNA. qPCR was performed using previously designed primers; bacterial 16S rRNA genes (Suzuki et al., 2000), *sul1* (Heuer and Smalla, 2007), *sul2* (Heuer et al., 2008), *sul3* (Pei et al., 2006), and *tet*(M) (Tamminen et al., 2011). Serial 1:10 dilutions of plasmids constructed from the pGEM-T Easy vector (Promega, Madison, WI, USA) and 16S rRNA gene from *E. coli* K12, *sul1* from plasmid R388, *sul2* from plasmid RSF1010, *sul3* from plasmid pUVP4401 (Heuer and Smalla, 2007), and *tet*(M) from pFD310 fragments (Smith et al., 1992) were used as standards for quantification. The qPCR program consisted of an initial denaturation of 30 s at 95°C and 40 cycles of 5 s at 95°C and 10 s at 50°C for 16S rRNA gene and 10 s at 51°C for *sul1* and *sul2* and 20 s at 60°C for *sul3,* and 20 s at 57°C for *tet*(M), respectively. Melting curves for the amplicons were measured by raising the temperature slowly from 60°C and 65°C to 95°C for 16S rRNA gene, *sul1*, *sul2, sul3, tet*(M), and *sul3,* respectively, while monitoring fluorescence. Each sample was measured in triplicate. The copy numbers of *sul1*, *sul2, sul3,* and *tet*(M) were normalized by dividing by the 16S rRNA gene copy number at the respective time points to take into account any temporal variation in bacterial cell numbers. Unit of the copy number is described as copies/16S in the text. The results were analyzed using a Big Dye terminator kit on a 3130 ABI Prism sequencer (Applied Biosystems, Foster City, CA, USA). PCR products were sequenced to confirm they were not non-specific products.

Results and Discussion

Drug Contamination

The distribution of antibiotic concentrations in surface waters and STP effluent showed that SMX was a major contaminant along with trimethoprim, which is a combination drug. The SMX concentrations were: rural surface waters - 48.2 ± 71.2 ng/L ($n = 3$), urban surface waters - 2561 ± 51.3 ng/L ($n = 13$), STP effluent - 3612 ± 1733.4 ng/L ($n = 4$). High SMX concentrations in urban surface waters and STP effluent indicate its frequent use in human chemotherapy. It is also reported that SMX is frequently used in African countries to control bacteria and protozoan infections in HIV patients (Zachariah et al., 2007). Recently report in Ghana, Mozambique, Kenya, and South Africa showed that the SMX is the highest concentration among selected 18 antibiotics in all countries (Segura et al., 2015). Data from STP in the present study showed high concentration compared to these. Tetracyclines were mostly not detectable in surface waters and STP effluent (maximum 18 ng/L, and mostly below detection limit). At one STP (STP4), however, 291 ng/L of OTC was detected, indicating real time use of the drug. The results suggest that SMX is used frequently in the Durban area. The concentration over 1000 ng/L was similar to a pig farm in Vietnam (Hoa et al., 2011; Shimizu et al., 2013), and double that of STP effluent in Michigan, U. S. (Gao et al., 2012b). Erythromycin (1194 ng/L) was also present in STP4 effluent, but was not particularly prevalent in surface water samples, suggesting the antibiotics originated from human medicines. The high contamination of surface waters and STP effluents by antibiotics suggests that ARGs in hospitals are also likely entering the environment (Pruden, 2014).

Bacterial Numbers

The counts of bacteria in different surface water and STP effluent samples are shown in **Table 1**, as enumerated by DAPI count (total number), plate count (colony forming number), and SMX^r and OTC^r bacterial counts. Total cell number was almost the same in the rural and urban surface waters, with 10^6 cells/ml, but an order of magnitude higher in STP effluents. The colony forming number was two orders of magnitude lower than the total cell number. The contribution of culturable bacteria to the total cell number was 1.0–1.5% in surface waters and 6.8% in STP effluents, a statistically significant difference ($p < 0.05$, *t*-test). The culturable bacterial contribution to the total cell number in freshwater is reported to be approximately 0.25% (Amann et al., 1995), indicating that the number of culturable bacteria was higher in surface waters and STP effluents in Durban. Dominance rate of viable number was higher in urban surface waters and STP effluent compared to rural surface waters, suggesting contamination of culturable bacteria is derived from human sources. Iweriebor et al. (2015) reported in South Africa that resistance rate of *Enterococcus* from hospital and STP effluents was 67–100%. The contribution of ARB in STP effluent was higher than in surface waters in our study (SMX^r, $p < 0.05$ and OTC^r, $p < 0.01$). Culturable bacteria in STP effluent should include enteric bacteria, which form colonies on agar plates with a contribution of 15% (Langendijk et al., 1995) compared to 0.1% in seawater (Amann et al., 1995; Fuhrman and Hagström, 2008). Abundances of SMX^r and OTC^r bacteria were not positively correlated to antibiotic concentrations. It is reported that drug concentrations and occurrence of ARB are not correlated to fluoroquinolones in environment (Takasu et al., 2011). Although the reason why sulfonamide- and tetracycline-resistance are frequently found in non-contaminated environments is not known, the heavy use of sulfonamides and tetracyclines in the 20th century could be one of the reasons for the selection of SMX^r - and OTC^r -genes in bacterial communities. Sediment stores *sul* and *tet* genes for a long time in non-contaminated areas (Tamminen et al., 2011; Muziasari et al., 2014), whereas water samples indicate present status. The abundance of ARB in surface waters suggests their continuous input into the environment. The ARGs for these drugs should be distributed in various environmental bacteria around the world. There are factors other than antibiotics, such as metals (Knapp et al., 2011), that may select for ARB and ARGs in natural bacterial assemblages.

The *sul* and *tet*(M) Genes in Total- and Culturable-Assemblages

Among *sul* genes, *sul1* and *sul2* were detected at a similar copy number in total assemblages in all categories of water (**Figure 2A**), and also in colony forming bacteria (**Figure 2B**). This indicates that *sul1* and *sul2* are ubiquitous in bacterial

TABLE 1 | Bacterial number in three categorized sites.

Site	Total cell count (cells/ml)	Colony count (CFU/ml) (% of total)	Sulfamethoxazole resistant (SMX^r; CFU/ml) (% of colony count)	Oxytetracycline resistant (OTC^r; CFU/ml) (% of colony count)
Rural river ($n = 3$)	$(1.1 \pm 0.47) \times 10^6$	$(1.1 \pm 1.4) \times 10^4$ (1.0%)	$(1.8 \pm 3.0) \times 10^3$(16.8%)	$(5.0 \pm 7.0) \times 10^2$(4.7%)
Urban and industrial river ($n = 13$)	$(2.7 \pm 2.7) \times 10^6$	$(4.1 \pm 4.8) \times 10^4$(1.5)	$(8.1 \pm 7.7) \times 10^3$ (20.0)	$(4.8 \pm 6.9) \times 10^3$(11.9)
Sewage treatment plant (STP) ($n = 4$)	$(1.0 \pm 1.0) \times 10^7$	$(7.0 \pm 11) \times 10^5$(6.8)	$(2.1 \pm 3.0) \times 10^5$ (30.4)	$(4.8 \pm 5.1) \times 10^4$(6.9)

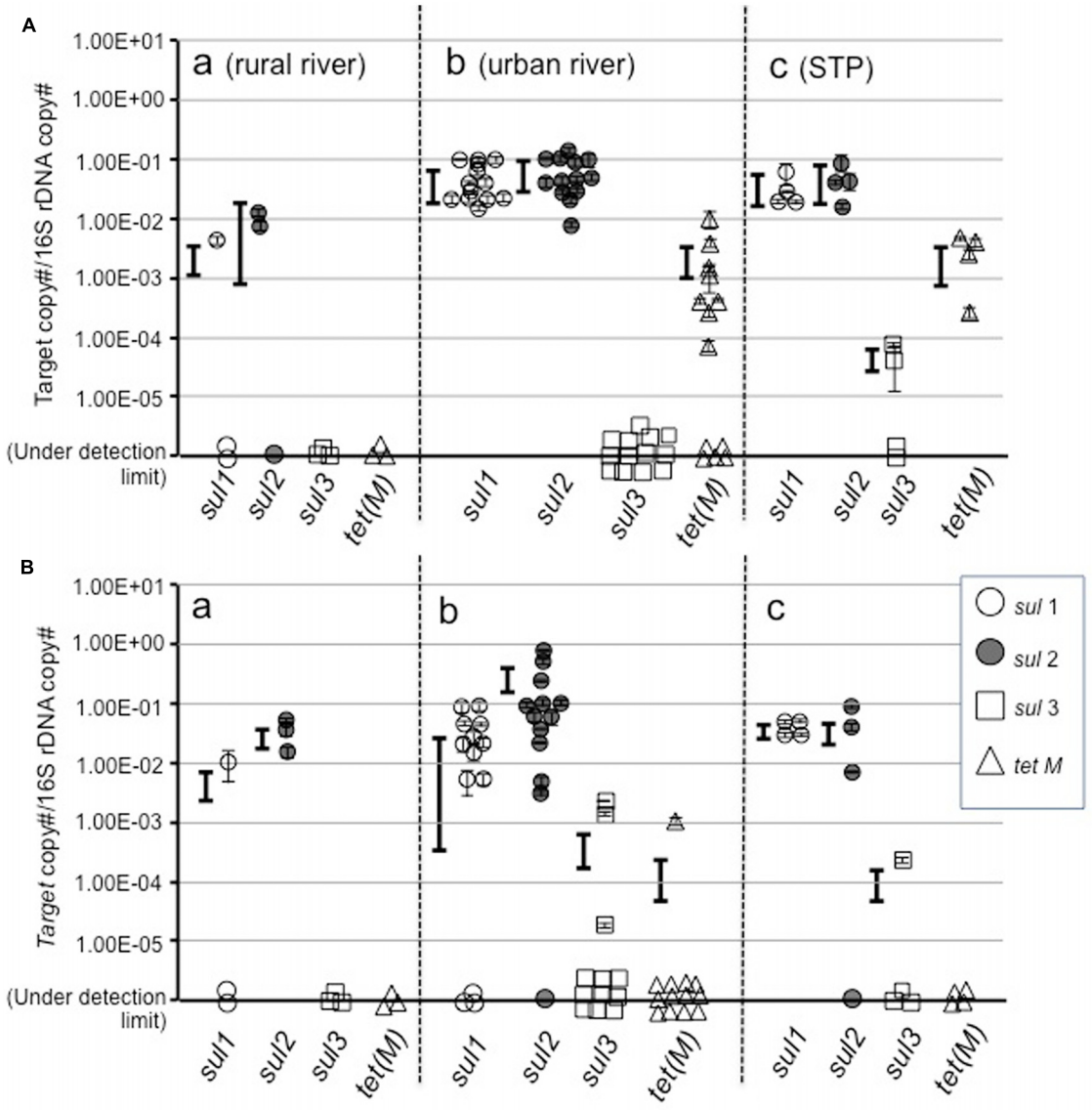


FIGURE 2 | Abundance of *sul1, sul2, sul3,* and *tet*(M) genes in total (A) and culturable bacterial assemblages (B).

communities, including yet-to-be cultured and culturable bacteria in aquatic environments in the Durban area. At most urban river and estuarine and STP sites, *sul1* and *sul2* were present at copy numbers of 10^{-2}–10^{-1}/16S. These values are higher than at rural sites in the Durban area, and in the Philippines (Suzuki et al., 2013) and in Finnish sediment (Muziasari et al., 2014), but are comparable to values reported for suspended solids in lagoon waters (McKinney et al., 2010).

Profiles for *sul3* and *tet*(M) were different from *sul1* and *sul2* between total assemblage and culturable bacteria. The *sul3* gene was not detected or was at a very low abundance in the total assemblage at most sites, although two sites showed

10^{-5}–10^{-4}/16S. In the case of the Philippines, *sul3* was not detected in natural assemblages or colony forming bacteria in freshwater lakes and rivers, whereas a high copy number was detected in seawater assemblages (Suzuki et al., 2013). The fact that *sul3* was not at a high copy number in culturable bacteria (**Figure 2B**) suggests this gene is not abundant in the Durban area. Gao et al. (2012a) reported similar results in freshwater.

In the case of *tet*(M), the total assemblage in urban surface waters and STP effluents possessed approximately 10^{-3}/16S, whereas culturable bacteria did not. This suggests the yet-to-be cultured community possesses *tet*(M). Since the yet-to-be cultured bacteria comprise the major component of the bacterial community, the gene pool of *tet*(M) in environment should be large. A risk assessment for ARGs amongst this silent majority is required.

The copy numbers of the targeted ARGs were measured in a pooled colony from SMX^r and OTC^r bacteria (**Figure 3**). The *sul1* and *sul2* were higher than 10^{-1}/16S at urban and STP sites, with *sul3* around 10^{-3}∼10^{-2}/16S (**Figure 3A**). The rural sites also showed high copies of *sul1* and *sul2*, but *sul3* was detected at only one site at a low concentration. This indicates that colony forming SMX^r bacteria possess *sul* genes, which were

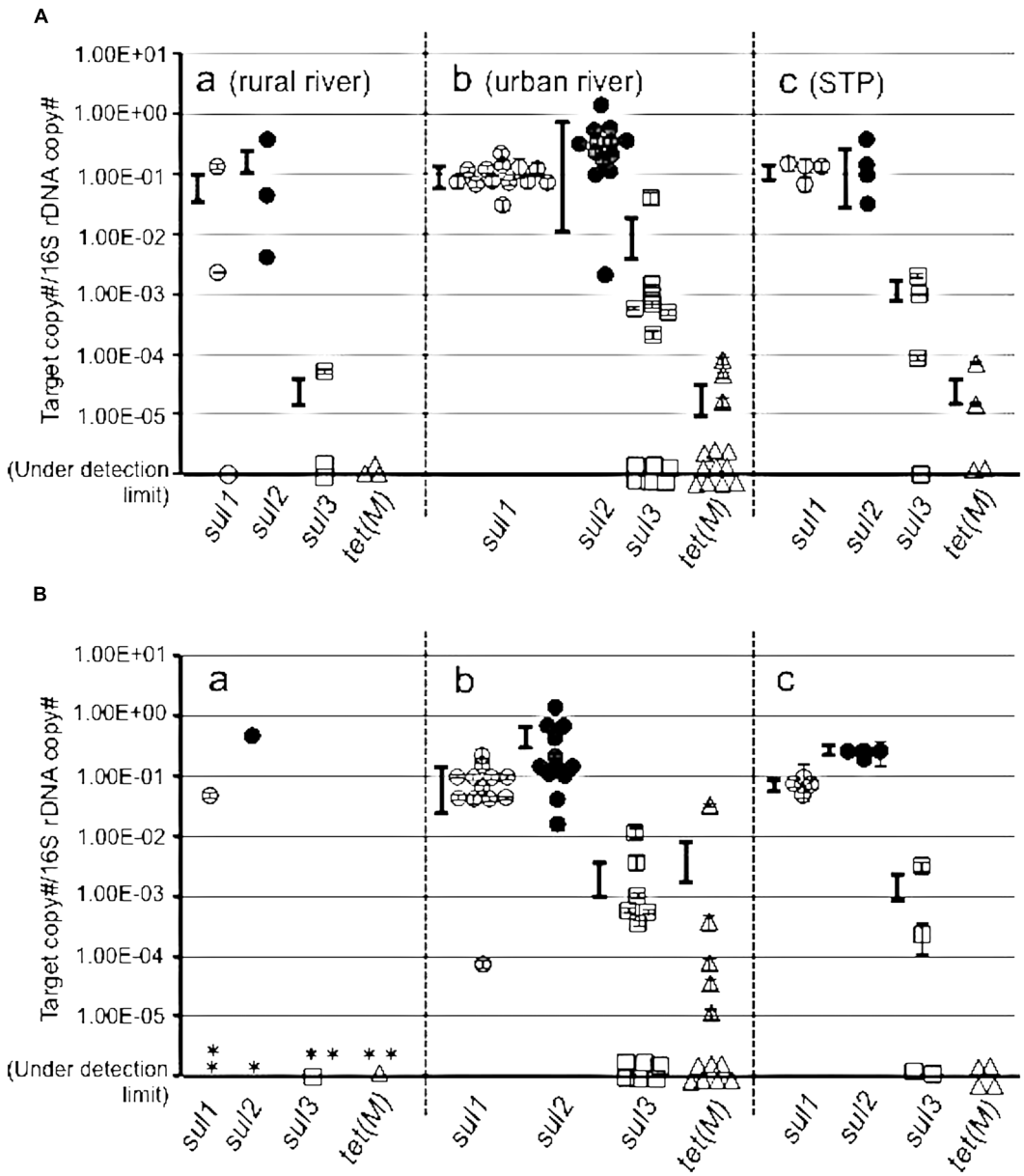


FIGURE 3 | Abundance of *sul1, sul2, sul3,* and *tet*(M) genes in sulfamethoxazole resistant (SMX^r) colonies mix (A) and in oxytetracycline resistant (OTC^r) colonies mix (B). (B) Asterisk shows sites where resistant isolate was not obtained. Symbols are the same to **Figure 2.**

selected on SMX-containing agar plate. The *sul* genes in the OTC^r assemblage also showed a high copy number of *sul* genes (**Figure 3B**). It is reported that *sul* and *tet* genes are sometimes coded on the same plasmid of aquatic bacteria (Kim et al., 2008; Nonaka et al., 2012), and SMX^r and OTC^r phenotypes are frequently linked (Hu et al., 2008). The present study supports the findings in terms of gene copy numbers in assemblages by cross checking with SMX^r and OTC^r bacteria. On the other hand, *tet*(M) copy number was less than 10^{-2} in SMX^r and OTC^r bacteria at most sites, suggesting two possibilities. One is that the selected bacteria by SMX and OTC possess other *tet* genes than *tet*(M), and is the other that *tet*(M) is abundant in total assemblages but not in culturable resistant bacteria.

Conclusion

Quantitative PCR and culture methods revealed that *sul* genes are conveyed by bacterial communities in urban surface waters and STP effluent in the Durban area of South Africa. Additionally, *sul3* was detected in the culturable bacteria assemblage. The yet-to-be cultured bacterial community may act as a non-visible reservoir of ARGs in certain situations.

Acknowledgments

We appreciate the assistance of Dr. T. Yokokawa and other members in the field and laboratory. The authors thank Grant-in-Aids from JSPS (90196816, 22241014, 22254001) for funding this study.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00796

References

Amann, R. I., Ludwig, W., and Schleifer, K.-H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* 59, 143–169.

Antunes, P., Machado, J., Sousa, J. C., and Peixe, L. (2005). Dissemination of sulfonamide resistance genes (sul1, sul2, and sul3) in *Portuguese Salmonella* enterica strains and relation with integrons. *Antimicrob. Agents Chemother.* 49, 836–839. doi: 10.1128/AAC.49.2.836-839.2005

Berkner, S., and Konradi, S. and Schönfeld, J. (2014). Antibiotic resistance and the environment—there and back again. *EMBO Rep.* 15, 740–744. doi: 10.15252/embr.201438978

Bloomfield, S. F., Gordon, S., Stewart, A. B., Dodd, C. E. R., Booth, I. R., and Power, E. G. M. (1988). The viable but non-culturable phenomenon explained? *Microbiology* 144, 1–3. doi: 10.1099/00221287-144-1-1

D'Costa, V. M., King, C. E., Kalan, L., Morar, M., Sung, W. W. L., Schwarz, C., et al. (2011). Antibiotic resistance is ancient. *Nature* 477, 458–461.

Fajardo, A., and Martinez, J. L. (2008). Antibiotics as signals that trigger specific bacterial responses. *Curr. Opin. Microbiol.* 11, 161–167. doi: 10.1016/j.mib.2008.02.006

Fuhrman, J. A., and Hagström, Å. (2008). "Bacterial and archaeal community structure and its patterns," in *Microbial Ecology of the Oceans*, 2nd Edn, ed. D. L. Kirchman (Hoboken, NJ: Wiley Sons), 45–90.

Gao, P., Mao, D., Luo, Y., Wang, L., Xu, B., and Xu, L. (2012a). Occurrence of sulfonamide and tetracycline-resistant bacteria and resistance genes in aquaculture environment. *Wat. Res.* 46, 2355–2364. doi: 10.1016/j.watres.2012.02.004

Gao, P., Munir, M., and Xagoraraki, I. (2012b). Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. *Sci. Total Environ.* 421–422, 173–183. doi: 10.1016/j.scitotenv.2012.01.061

Guerra, B., Junker, E., Schroeter, A., Malorny, B., Lehmann, S., and Helmuth, R. (2003). Phenotypic and genotypic characterization of antimicrobial resistance in German *Escherichia coli* isolates from cattle, swine and poultry. *J. Antimicrob. Chemother.* 52, 489–492. doi: 10.1093/jac/dkg362

Gullberg, E., Cao, S., Berg, O. G., Ilback, C., Sandegren, L., Hughes, D., et al. (2011). Selection of resistant bacteria at very low antibiotic. *PLoS Pathog.* 7:e1002158. doi: 10.1371/journal.ppat.1002158

Hammerum, A. M., Sandvang, D., Andersen, S. R., Seyfarth, A. M., Porsbo, L. J., Frimodt-Moller, N., et al. (2006). Detection of sul1, sul2 and sul3 in sulphonamide resistant *Escherichia coli* isolates obtained from healthy humans, pork and pigs in Denmark. *Int. J. Food Microbiol.* 106, 235–237. doi: 10.1016/j.ijfoodmicro.2005.06.023

Heuer, H., Focks, A., Lamshoft, M., and Smalla, K. (2008). Fate of sulfadiazine administered to pigs and its quantitative effect on the dynamics of bacterial resistance genes in manure and manured soil. *Soil Biol. Biochem.* 40, 1892–1900. doi: 10.1016/j.soilbio.2008.03.014

Heuer, H., and Smalla, K. (2007). Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. *Environ. Microbiol.* 9, 657–666. doi: 10.1111/j.1462-2920.2006.01185.x

Hoa, P. T. P., Managaki, S., Nakada, N., Takada, H., Shimizu, A., Anh, D. H., et al. (2011). Antibiotic contamination and occurrence of antibiotic-resistant bacteria in aquatic environments of northern Vietnam. *Sci. Total Environ.* 409, 2894–2901. doi: 10.1016/j.scitotenv.2011.04.030

Hu, J., Shi, J., Chang, H., Li, D., Yang, M., and Kamagata, Y. (2008). Phenotyping and genotyping of antibiotic-resistant *Escherichia coli* isolated from a natural river basin. *Environ. Sci. Technol.* 42, 3415–3420. doi: 10.1021/es7026746

Huang, L., Crothers, K., Atzori, C., Benfield, T., Miller, R., Rabodonirina, M., et al. (2004). Dihydropteroate synthase gene mutations in pneumocystis and sulfa resistance. *Emmerg. Infect. Dis.* 10, 1721–1728. doi: 10.3201/eid1010.030994

Iweriebor, B. C., Gaqavu, S., Obi, L. C., Nwodo, U. U., and Okoh, A. I. (2015). Antibiotic susceptibilities of *Enterococcus* species isolated from hospital and domestic wastewater effluents in Alice, Eastern Cape Province of South Africa. *Int. J. Environ. Res. Public Health* 12, 4231–4246. doi: 10.3390/ijerph120404231

Kim, M.-J., Hirono, I., Kurokawa, K., Maki, T., Hawke, J., Kondo, H., et al. (2008). Complete DNA sequence and analysis of the transferable multiple-drug resistance plasmids (R plasmids) from *Photobacterium damselae* subsp. *piscicida* isolates collected in Japan and the United States. *Antimicrob. Agents Chemother.* 52, 606–611. doi: 10.1128/AAC.01216-07

Knapp, C. W., McCluskey, S. M., Singh, B. K., Campbell, C. D., Hudson, G., and Graham, D. W. (2011). Antibiotic resistance gene abundances correlate with metal and geochemical conditions in archived Scottish soils. *PLoS ONE* 6:e27300. doi: 10.1371/journal.pone.0027300

Kobayashi, T., Nonaka, L., Maruyama, F., and Suzuki, S. (2007). Molecular evidence for the ancient origin of the ribosomal protection protein that mediates tetracycline resistance in bacteria. *J. Mol. Evol.* 65, 228–235. doi: 10.1007/s00239-007-9006-z

Langendijk, P. S., Schut, F., Jansen, G. J., Raangs, G. C., Kamphuis, G. R., Wilkinson, M. H. F., et al. (1995). Quantitative fluorescence in situ hybridization of *Bifidobacterium* spp. *with genus-specific* 16S rRNA-targeted probes and its application in fecal samples. *Appl. Environ. Microbiol.* 61, 3069–3075.

McKinney, C. W., Loftin, K. A., Meyer, M. T., Davis, J. G., and Pruden, A. (2010). tet and sul antibiotic resistance genes in livestock lagoons of various operation type, configuration, and antibiotic occurrence. *Environ. Sci. Technol.* 44, 6102–6109. doi: 10.1021/es9038165

Muziasari, W. A., Managaki, A., Pärnänen, K., Karkman, A., Lyra, C., Tamminen, M., et al. (2014). Sulphonamide and trimethoprim resistance genes persist in sediments at Baltic Sea aquaculture farms but are not detected in the surrounding environment. *PLoS ONE* 9:e92702. doi: 10.1371/journal.pone.0092702

Nonaka, L., Maruyama, F., Miyamoto, M., Miyakoshi, M., Kurokawa, K., and Masuda, M. (2012). Novel conjugative transferable multiple drug resistance plasmid pAQU1 from *Photobacterium damselae* subsp. *damselae* isolated from marine aquaculture environment. *Microbes Environ.* 27, 263–272. doi: 10.1264/jsme2.ME11338

Omulo, S., Thumbi, S. M., Njenga, M. K., and Call, D. R. (2015). A review of 40 years of enteric antimicrobial resistance research in Eastern Africa: what can be done better? *Antimicrob. Resist. Infect. Cont.* 4, 1–13. doi: 10.1186/s13756-014-0041-4

Pei, R., Kim, S.-C., Carlson, K. H., and Pruden, A. (2006). Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Wat. Res.* 40, 2427–2435. doi: 10.1016/j.watres.2006.04.017

Pruden, A. (2014). Balancing water sustainability and public health goals in the face of growing concerns about antibiotic resistance. *Environ. Sci. Technol.* 48, 5–14. doi: 10.1021/es403883p

Pruden, A., Joakim Larsson, D. G., Amézquita, A., Collignon, P., Brandt, K. K., Graham, D. W., et al. (2013). Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. *Environ. Health Perspect.* 121, 878–885. doi: 10.1289/ehp.1206446

Radstrom, P., and Swedberg, G. (1988). RSF1010 and a conjugative plasmid contain sulII, one of two known genes for plasmid-borne sulfonamide resistance dihydropteroate synthase. *Antimicrob. Agents Chemother.* 32, 1684–1692. doi: 10.1128/AAC.32.11.1684

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M. C., et al. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. *Sci. Total Environ.* 447, 345–360. doi: 10.1016/j.scitotenv.2013.01.032

Rizzotti, L., La Gioia, F., Dellaglio, F., and Torriani, S. (2009). Molecular diversity and transferability of the tetracycline resistance gene tet(M), carried on Tn916-1545 family transposons, in enterococci from a total food chain. *Antonie Van Leeuwenhoek* 96, 43–52. doi: 10.1007/s10482-009-9334-7

Roberts, M. C., Schwarz, S., and Aarts, H. J. M. (2012). Erratum: acquired antibiotic resistance genes: an overview. *Front. Microbiol* 3:384. doi: 10.3389/fmicb.2012.00384

Sato-Takabe, Y., Suzuki, S., Shishikura, R., Hamasaki, K., Tada, Y., Kataoka, T., et al. (2015). Spatial distribution and cell size of aerobic anoxygenic phototrophic bacteria in the Uwa Sea. *Japan. J. Oceanogr.* 71, 151–159. doi: 10.1007/s10872-014-0267-z

Segura, P. A., Takada, H., Correa, J. A., Saadi, K. E., Koike, T., Onwona-Agyeman, S., et al. (2015). Global occurrence of anti-infectives in contaminated surface waters: impact of income inequality between countries. *Environ. Int.* 80, 89–97. doi: 10.1016/j.envint.2015.04.001

Shimizu, A., Takada, H., Koike, T., Takeshita, T., Saha, M., Rinawati, et al. (2013). Ubiquitous occurrence of sulfonamides in tropical Asian waters. *Sci. Total Environ.* 45, 108–115. doi: 10.1016/j.scitotenv.2013.02.027

Smith, C. J., Rogers, M. B., and McKee, M. L. (1992). Heterologous gene expression in *Bacteroides fragilis*. *Plasmid* 27, 141–154. doi: 10.1016/0147-619X(92)90014-2

Snyman, H. G., Van Niekerk, A. M., and Rajasakran, N. (2006). "Sustainable wastewater treatment – what has gone wrong and how do we get back on track," in *Proceedings of the water Institute of South Africa (WISA) Conference, 21–25 May 2006*, Durban.

Suzuki, M. T., Taylor, L. T., and Delong, E. F. (2000). Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5-nuclease assays. *Appl. Environ. Microbiol.* 66, 4605–4614. doi: 10.1128/AEM.66.11.4605-4614.2000

Suzuki, S., and Hoa, P. T. P. (2012). Distribution of quinolone, sulfonamides, tetracyclines in aquatic environment and antibiotic resistance in Indochina. *Front. Microbiol.* 3:67. doi: 10.3389/fmicb.2012.00067

Suzuki, S., Ogo, M., Miller, T. W., Shimizu, A., Takada, H., and Sringan, M. A. T. (2013). Who possesses drug resistance genes in the aquatic environment? :sulfamethoxazole (SMX) resistance genes among the bacterial community in water environment of Metro-Manila, Philippines. *Front. Microbiol* 4:102. doi: 10.3389/fmicb.2013.00102

Takami, H., Hanada, S., Sekiguchi, Y., Tanaka, Y., and Kamagata, Y. (2009). Effect of gelling agent on colony formation in solid cultivation of microbial community in lake sediment. *Environ. Microbiol.* 11, 1827–1834. doi: 10.1111/j.1462-2920.2009.01907.x

Takasu, H., Suzuki, S., Reungsang, A., and Viet, P. H. (2011). Fluoroquinolone (FQ) Contamination does not correlate with occurrence of FQ-resistant bacteria in aquatic environments of Vietnam and Thailand. *Microbes Environ.* 26, 135–143. doi: 10.1264/jsme2.ME10204

Tamminen, M., Karkman, A., Lõhmus, A., Muziasari, W. I., Takasu, H., Wada, S., et al. (2011). Tetracycline resistance genes persist at aquaculture farms in the absence of selection pressure. *Environ. Sci. Technol.* 45, 386–391. doi: 10.1021/es102725n

United States Environmental Protection and Agency. (2004). *Primer for Municipal Wastewater Treatment Systems*. EPA 832-R-04-001. Washington DC: United States Environmental Protection and Agency, 25. Available at: http://www.epa.gov/npdes/pubs/primer.pdf

Water Research and Commission. (2006). *Wastewater Treatment in South Africa: From Crisis to Compliance*. WRC Report, Report No. 8001/8295/3/P. Pretoria: Water Research and Commission.

Zachariah, R., Harries, A. D., Luo, C., Bachman, G., and Graham, S. M. (2007). Scaling-up co-trimoxazole prophylaxis in HIV-exposed and HIV-infected children in high HIV-prevalence countries. *Lancet Inf. Dis.* 7, 686–693. doi: 10.1016/S1473-3099(07)70239-6

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 08 March 2016
doi: 10.3389/fmicb.2016.00263

Survival of Antibiotic Resistant Bacteria and Horizontal Gene Transfer Control Antibiotic Resistance Gene Content in Anaerobic Digesters

*Jennifer H. Miller, John T. Novak, William R. Knocke and Amy Pruden **

Virginia Tech, Charles E. Via Department of Civil and Environmental Engineering, Blacksburg, VA, USA

OPEN ACCESS

Edited by:
Charles W. Knapp, University of Strathclyde, UK

Reviewed by:
Jose Luis Balcazar, Catalan Institute for Water Research, Spain
Andrew C. Singer, NERC Centre for Ecology & Hydrology, UK

***Correspondence:**
Amy Pruden
apruden@vt.edu

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *26 December 2015*
Accepted: *17 February 2016*
Published: *08 March 2016*

Citation:
Miller JH, Novak JT, Knocke WR and Pruden A (2016) Survival of Antibiotic Resistant Bacteria and Horizontal Gene Transfer Control Antibiotic Resistance Gene Content in Anaerobic Digesters. Front. Microbiol. 7:263. doi: 10.3389/fmicb.2016.00263

Understanding fate of antibiotic resistant bacteria (ARB) vs. their antibiotic resistance genes (ARGs) during wastewater sludge treatment is critical in order to reduce the spread of antibiotic resistance through process optimization. Here, we spiked high concentrations of tetracycline-resistant bacteria, isolated from mesophilic (Iso M1-1—a *Pseudomonas* sp.) and thermophilic (Iso T10—a *Bacillus* sp.) anaerobic digested sludge, into batch digesters and monitored their fate by plate counts and quantitative polymerase chain reaction (QPCR) of their corresponding tetracycline ARGs. In batch studies, spiked ARB plate counts returned to baseline (thermophilic) or 1-log above baseline (mesophilic) while levels of the ARG present in the spiked isolate [*tet*(G)] remained high in mesophilic batch reactors. To compare results under semi-continuous flow conditions with natural influent variation, *tet*(O), *tet*(W), and *sul1* ARGs, along with the *intI1* integrase gene, were monitored over a 9-month period in the raw feed sludge and effluent sludge of lab-scale thermophilic and mesophilic anaerobic digesters. *sul1* and *intI1* in mesophilic and thermophilic digesters correlated positively (Spearman rho = 0.457–0.829, $P < 0.05$) with the raw feed sludge. There was no correlation in *tet*(O) or *tet*(W) ratios in raw sludge and mesophilic digested sludge or thermophilic digested sludge (Spearman rho = 0.130–0.486, $P = 0.075$–0.612). However, in the thermophilic digester, the *tet*(O) and *tet*(W) ratios remained consistently low over the entire monitoring period. We conclude that the influent sludge microbial composition can influence the ARG content of a digester, apparently as a result of differential survival or death of ARBs or horizontal gene transfer of genes between raw sludge ARBs and the digester microbial community. Notably, mesophilic digestion was more susceptible to ARG intrusion than thermophilic digestion, which may be attributed to a higher rate of ARB survival and/or horizontal gene transfer between raw sludge bacteria and the digester microbial community.

Keywords: mesophilic anaerobic digestion, thermophilic anaerobic digestion, antibiotic resistant bacteria, tetracycline, antibiotic resistance gene, horizontal gene transfer, biosolids

INTRODUCTION

Bacterial resistance to antibiotics is a worldwide problem resulting in untreatable infections, death, and escalating healthcare costs. A large body of research has focused on the connection between antibiotic abuse in human medicine and agricultural settings with clinical antibiotic resistance, but the spread of antibiotic resistance continues to be a problem. Thus, the landscape of the war against antibiotic resistance is expanding, with growing attention on environmental reservoirs, such as wastewater treatment plants (WWTPs). In particular, there is interest in the means by which the transfer of antibiotic resistance genes (ARGs) via environmental routes to clinical pathogens can be mitigated (Davison, 1999; Wellington et al., 2013). It is now understood that even non-pathogenic organisms, such as commensals, can be of concern and can serve as a source of ARGs to pathogenic bacteria of clinical significance (Poirel et al., 2002; Rossolini et al., 2008).

WWTPs are of interest not only because they receive wastewater laden with antibiotics, antibiotic resistant bacteria (ARBs), and the ARGs that they carry, but also because they hypothetically create a hot spot for horizontal transfer of ARGs (Dröge et al., 2000; Schlüter et al., 2007) and can elevate ARGs in impacted receiving environments (Storteboom et al., 2010; LaPara et al., 2011). The nutrient-rich, microbially-dense nature of WWTP biomass, in concert with the presence of antibiotics or other selection factors, has been linked with the elevation and persistence of ARGs in activated sludge (Stalder et al., 2013), wastewater effluent (da Silva et al., 2007; Zhang et al., 2009; Luczkiewicz et al., 2010), and land-applied biosolids (Munir and Xagoraraki, 2011; Munir et al., 2011). Early studies focused on tracking specific ARBs or ARGs in wastewater matrices, while mobile genetic elements (e.g., transposons, integrons, and plasmids) have been the subject of more recent attention given their essential role in facilitating transfer of ARGs from the environment to the clinic (Muniesa et al., 2011; Lupo et al., 2012; Moura et al., 2012; Stalder et al., 2012; Ashbolt et al., 2013; Rizzo et al., 2013; Gillings et al., 2014). The prevalence of plasmid-borne ARGs of clinical relevance in WWTPs (Szczepanowski et al., 2009) is of concern and calls for research to establish understanding of the mechanisms governing ARG persistence and proliferation and means by which to limit ARG propagation.

Sludge digestion is of particular interest as a potential means to mitigate the spread of antibiotic resistance, given that it controls the fate of the vast majority of WWTP biomass and ARGs (Munir et al., 2011). Prior studies have collectively measured tetracycline, sulfonamide, erythromycin, and Class 1 integrase genes, which are considered to be key markers of horizontal gene transfer, in mesophilic and thermophilic digestion (Ghosh et al., 2009; Diehl and LaPara, 2010; Ma et al., 2011; Miller et al., 2013). Thermophilic digestion reduced all ARGs to some extent (1–2 log), whereas mesophilic digestion had variable effects on gene levels. Ma et al. (2011) also reported an enhanced removal (2–3 log) of ARGs during thermal hydrolysis pretreatment. However, the concentration of ARGs increased, with the exception of *sul*1 and *tet*(G), in the subsequent mesophilic stage. Recent research has focused on metagenomics of ARGs and ARBs in anaerobic digestion (Resende et al., 2014; Yang et al., 2014; Zhang et al., 2015). Prior research suggests that the digester microbial community and the operating conditions play an important role in determining ARG composition and fate. However, the role of the raw sludge influent ARG composition has not previously been examined and has important implications in terms of relative importance of upstream mitigation strategies (e.g., limiting antibiotic use, separating waste streams) vs. digester operation (e.g., maintaining ideal digester microbial community by seeding or selection by temperature or other factors).

It is difficult to measure horizontal gene transfer events in complex environmental matrices (Luby et al., 2016). Rizzo et al. (2013) summarized that there are three types of retrospective evidence of horizontal gene transfer, including association of the ARG with a mobile genetic element, loss of co-location of the insertion site in the host and the acquired ARG, or the lack of similarity between the ARG phylogeny and the ARB phylogeny. Microcosm studies in sediment (Bonot and Merlin, 2010), activated sludge, biofilm, and anaerobic digestion (Merlin et al., 2011) used an increasing ratio of introduced plasmid DNA normalized to ARB chromosomal DNA over time to signify horizontal gene transfer between the introduced ARB and indigenous bacteria. In the present study, evidence of horizontal gene transfer is approached by tracking the fate of a donor ARB by plate culturing and tracking a particular ARG that it carries via quantitative polymerase chain reaction (QPCR) in experimental digesters spiked with the ARB.

The purpose of this study was to examine the effect of elevated influent ARGs originating from influent ARB hosts during mesophilic and thermophilic sludge digestion. A feature of the experimental approach was that the spiked tetracycline-resistant ARBs (designated Iso M1-1 and Iso T10) were isolated from the mesophilic and thermophilic sludge itself and thus presented the opportunity to observe the behavior of native ARBs and their ARGs when elevated in the digester environment. Iso M1-1 was isolated from mesophilic sludge and was identified as a *Pseudomonas* sp. carrying the *tet*(G) tetracycline ARG, which was monitored along with *tet*(W), an ARG not carried by the isolate and representative of the background. Iso T10 was isolated from thermophilic sludge and was identified as *Bacillus* sp. carrying *tet*(W) tetracycline ARG. Quantitative polymerase chain reaction (QPCR) of ARGs along with tetracycline-resistant plate counts provided insight into the fate of the isolate and evidence of horizontal gene transfer. To further examine the fates of ARGs under different digester operating conditions, *sul*1, *tet*(O), and *tet*(W), the *intI1* gene encoding the integrase enzyme of Class 1 integrons were monitored in influent raw sludge and digested sludge from lab-scale mesophilic and thermophilic digesters. Overall results of this study help support management options for reducing the spread of antibiotic resistance via sludge digestion.

Abbreviations: ARG, antibiotic resistance gene; DNA, deoxyribonucleic acid; MRSA, methicillin-resistant *Staphylococcus aureus;* PBS, phosphate buffered saline; QPCR, quantitative polymerase chain reaction; VBNC, viable but non-culturable; USEPA, United States Environmental Protection Agency.

MATERIALS AND METHODS

Batch Digestion of Tetracycline-Resistant Isolates

Culture Media

Tryptic soy agar (TSA, 40 g/L) was added to reverse osmosis water, dissolved by heating, autoclaved at 121°C for 20 min, and cooled to 55°C in a water bath prior to antibiotic amendment. Filter-sterilized solutions of tetracycline hydrochloride (Sigma Aldrich) and cycloheximide (Sigma Aldrich) were added to the agar at final concentrations of 16 and 200 mg/L, respectively. Agar was mixed at low speed on a stir plate prior to pouring Petri dishes (100 × 15 mm). These plates were used in the isolation of tetracycline-resistant microorganisms from sludge and in monitoring their fate in batch digestion experiments.

Isolation of Tetracycline-Resistant Bacteria

Tetracycline-amended (16 mg/L) TSA plates were inoculated with 100 μL of 1x, 10x, and 100x diluted mesophilic or thermophilic digested sludge and incubated at 37°C for 24 h. After 24 h, individual colonies were aseptically re-streaked on fresh tetracycline-amended TSA plates and incubated again for 24 h. This process was repeated at least 3 times for 5 mesophilic colonies and 13 thermophilic colonies. DNA extracts of all colonies were screened for *tet*(C), *tet*(G), *tet*(O), or *tet*(W) presence using QPCR because these genes have been associated with wastewater (Storteboom et al., 2010). However, *tet*(C) and *tet*(O) were not detected in any colonies. The mesophilic colony labeled Iso M1-1 was positive only for *tet*(G) and thermophilic colony labeled Iso T10 was positive only for *tet*(W). Consistent morphology and growth curves were obtained for Iso M1-1 and Iso T10 (6 and 10-h growth times to stationary phase, respectively). Freezer stocks of the isolates were prepared with cultures grown to density, concentrated by centrifugation, rinsed with phosphate buffered saline, re-concentrated, resuspended in tryptic soy broth with 20% glycerol, and stored at −80°C until use.

Cloning and sequencing of the Iso M1-1 16S rRNA gene indicated that the isolate was a *Pseudomonas* sp. (accession number KC211303.1, 99% of identity Phylum Proteobacteria, Class Gammaproteobacterium) in 20 of the 20 tested clones. The closest match to Iso T10 was *Bacillus* sp. (accession number KJ5464450.1, 99% of identity, Phylum Firmicutes) in 20 of the 20 tested clones. Cloning used 16S rRNA primers 8F and 1492R and sequencing returned ∼640 basepairs spanning the V1 and partial V2 regions. These results suggest that the isolation procedure was successful in isolating only one genus from each sludge.

Batch Digester Preparation

Six hundred mL flasks of tryptic soy broth were each spiked with 1 mL of Iso M1-1 or Iso T10 freezer stock and cultured to late log phase. Cells were harvested by centrifugation of individual flask contents at 10,000 rpm for 10 min and re-suspended and rinsed in phosphate buffered saline (PBS). The rinse step was repeated three times prior to final resuspension of pellets from two flasks per biological replicate in PBS (∼30 mL total per biological replicate). The absorbance at 600 nm was measured for a 10x dilution of the concentrated cell suspension. An absorbance of 1.2 approximated a cell density of 10^8 cells per mL (10^9 cells per mL in the undiluted suspension). Digested mesophilic or thermophilic sludge (4.5 mL) was aliquoted to sterile plastic disposable culture tubes (100 × 17 mm, Fisher Scientific) and spiked with the concentrated Iso M1-1 or Iso T10 pure culture, respectively. Additional triplicates were unamended and served as control blanks at time zero. Tubes were incubated in a water bath at the test temperature (i.e., 37or 53°C) over a range of time points up to 40 days in order to capture the death/decay curves. Triplicate sample tubes were removed at each time point and immersed in a water/ice bath to quench the temperature reaction. After quenching, plating on tetracycline-amended agar was done immediately and an aliquot of the sample was stored at −20°C for later DNA extraction of an ARG contained within the isolate and a control ARG (not contained within the isolate).

Cell Culturing/Plating

Aliquots (100 μL) of serial 10x dilutions of the original sample were plated immediately on two tetracycline-amended TSA plates and incubated at 37°C for 24 h prior to cell count. Prior to each dilution and plating, culture tubes were vortexed a moderately high speed for 30 s to disengage bacteria from sludge particles.

pH and TS/VS

Total and volatile solids (Method 2540-G) and pH (Method 4500) were analyzed at the start and end of the batch studies as specified in Standard Methods for the Examination of Water and Wastewater (APHA/AWWA/WEF, 1995).

Continuous Feed Digester Study

Continuous Feed Digester Setup

One 15 L, 12-day solids retention time (SRT) thermophilic (53°C) digester and one 10 L, 20-day SRT mesophilic (37°C) digester were operated as control digesters for a series of digestion studies, as reported elsewhere (Miller et al., 2013). In brief, high density polyethylene cone fermenters (Hobby Beverage Equipment Company, Temecula, CA) with nominal volumes of 6.5 gallons (24.6 L) were fed daily with a 70% primary sludge and 30% thickened waste activated sludge mixture from the Christiansburg, Virginia WWTP, which predominantly receives residential wastewater with minimal industrial contribution and no hospital contributions. The mesophilic and thermophilic digesters were originally seeded with digested (37°C) Christiansburg WWTP sludge and laboratory-scale thermophilic (48–57°C) digester material, respectively. Feed to both digesters was diluted with tap water to maintain a consistent influent total solids (TS) of 2.5% and reduce variations in operating parameters (e.g., pH, alkalinity, VFAs, volatile solids reduction). Raw sludge was collected every 4–6 weeks and stored at 4°C to minimize biological activity until fed into the digesters. TS, volatile solids (VS), and pH of the raw sludge were routinely measured over the 9-month study period. A peristaltic pump was used to mix digester contents by recycling headspace gas to the bottom of the cone digester. Evolved gases were collected in 25-L Tedlar bags (SKC,

Inc., 84, Pennsylvania). Both digesters were maintained in a 37°C constant temperature room with the thermophilic digester modified with electric heating tape with a temperature controller (Model No. BSAT 101-100, Thermolyne, Dubuque, Iowa) to further elevate the temperature. Miller et al. (2013) reports data associated with control digesters performance monitoring, including total and volatile solids, total alkalinity, pH, gas volume, headspace methane and carbon dioxide, and volatile fatty acids. DNA samples were collected then stored at −20°C for later DNA extraction from feed sludge (after refrigeration, directly prior to feeding), mesophilic control digester effluent, and thermophilic control digester effluent over the 9-month study period. ARGs [*tet*(O) and *tet*(W)] were selected for analysis because they are typically associated with wastewater sources (Storteboom et al., 2010) and they have been shown to have variable treatment efficiencies in anaerobic digestion (Diehl and LaPara, 2010; Ma et al., 2011; Miller et al., 2013). *intI1* and ARG *sul1* were selected for analysis because they are associated with Class 1 integrons (Mazel, 2006), which have been suggested as a relative indicator of antibiotic resistance (Gillings et al., 2014). In the present study, ARG data points that were used to generate previously published bar chart averages of ARG concentration (Miller et al., 2013) are combined with previously unpublished ARG data points and plotted to examine Spearman rho correlations between ARGs concentrations in the raw feed sludge and digester effluents. These correlations provide insight into the influence of the ARG content of raw sludge on the ARG content of digester effluent (biosolids).

Statistics

Sigmaplot 11.0 (2008) was used to calculate Spearman's rho correlation coefficients and *P*-values (Helsel and Hirsch, 2002) between ARG-16S ratios measured in the raw sludge feed and digester effluent in the continuous feed digester study. If the correlation coefficient was positive, the variables tend to increase together. If the correlation coefficient was negative, the values tend to decrease together. A $P < 0.05$ was considered significant.

Quantification of ARGs for Batch and Continuous Feed Studies

DNA was extracted from 175 μL of sludge samples using the MagMax Total Nucleic Acid Extraction Kit (Ambion, Life Technologies) according to manufacturer's protocol. Extracted DNA was diluted 50× to minimize inhibitory effects and stored at −20°C until analysis by QPCR for bacterial 16S rRNA genes, *tet*(G), *tet*(W), *tet*(O), *sul1*, and *intI1*. A 10 μL reaction mixture was comprised of 5.0 μ L SsoFast Evagreen Supermix (Bio-Rad, Hercules, CA), 0.8 μL of each 5 μM primer (**Table 1**), 2.4 μL molecular biology grade water, and 1 μL of DNA template. All samples were quantified in triplicate. Standards (serial dilutions of cloned genes ranging from 10^1 to 10^7 gene copies per μL) and a reagent blank (reaction mixture with molecular biology grade water substituted for DNA template) were included in triplicate with each QPCR well plate. Bio-rad CFX Manager 3.0 generated a standard curve of threshold cycle against log gene concentration. Successful standard curves were characterized by $R^2 > 0.98$ and amplification efficiency between 90 and 105% and were used to calculate ARG concentration in the diluted sample extract. ARG gene copy number concentration in sludge was calculated by adjusting the diluted sample extract ARG concentration by reduced volume subsequent to DNA extraction (0.3x), dilution of the extract to reduce QPCR inhibitor concentrations (50x), and unit conversion from μL to mL (1000x). The lowest standard on the QPCR curve (10 gene copy numbers (gcn) per μL) was equivalent to 1.5×10^5 gcn per mL digested sludge.

RESULTS

Fate of Tetracycline Resistant Isolate (Iso M1-1) during Mesophilic Digestion

Tetracycline-resistant CFU and *tet*(G) were monitored to provide insight into the fate of Iso M1-1 over a 40-day period after it was spiked to batch mesophilic digesters (**Figures 1A,B**). Because *tet*(W) was absent in Iso M1-1, it was monitored as a control ARG present in the background digester community. ARG numbers are presented both as gene copy numbers per volume of sludge (**Figure 1A**) and normalized to background 16S rRNA genes (**Figure 1B**), as an indicator of the relative proportion of bacteria carrying ARGs and a means to normalize minor variation in DNA extraction efficiency.

Tetracycline-resistant CFU were elevated by about 5-log above background (1.3×10^3 CFU/ml) following the initial spike and subsequently decreased over a 5-day period to $\sim 10^4$

TABLE 1 | Primer sequence and annealing temperature of the QPCR assays.

Target Gene	Primer	Primer sequence (5′–3′)	Annealing temperature	References
*sul*I	*sul*I–Fw	CGCACCGGAAACATCGCTGCAC	69.9	Pei et al., 2006
	*sul*I–Rv	TGAAGTTCCGCCGCAAGGCTCG	69.9	
tet(O)	*tet*(O)–Fw	ACGGARAGTTTATTGTATACC	50.3	Aminov et al., 2001
	tet(O)–Rv	TGGCGTATCTATAATGTTGAC		
tet(W)	*tet*(W)–Fw	GAGAGCCTGCTATATGCCAGC	60.0	Aminov et al., 2001
	tet(W)–Rv	GGGCGTATCCACAATGTTAAC		
intI1	HS463a	CTGGATTTCGATCACGGCACG	60.0	Hardwick et al., 2008
	HS464	ACATGCGTGTAAATCATCGTCG		
16S rRNA (bacterial)	1369F	CGGTGAATACGTTCYCGG	60.0	Suzuki et al., 2000
	1492R	GGWTACCTTGTTACGACTT		

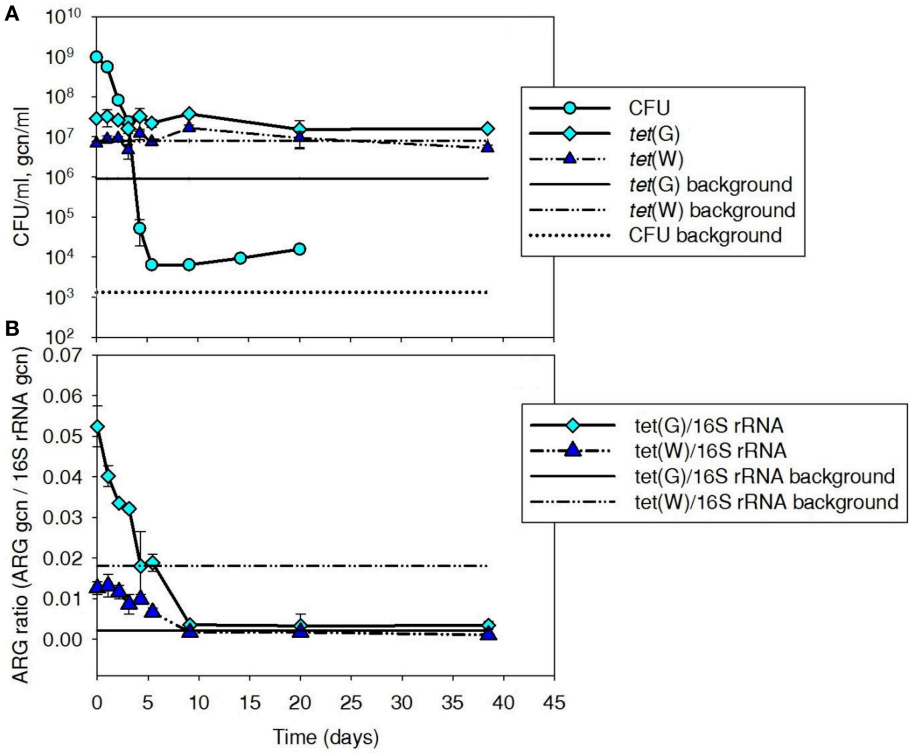


FIGURE 1 | Plate count (CFU/ml sludge) and (A) *tet*(G) and (B) normalized ARG ratios (ARG gcn/16S rRNA) for *tet*(G) associated with tetracycline resistant bacteria, including Iso M1-1, during mesophilic treatment at 37°C in digested sludge. *tet*(W) was included as a control gene because it was not associated with Iso M1-1, but was present in the background community. Background line represents the pre-spike concentration in digested sludge.

CFU/ml, where they remained ∼1-log above background for the remainder of the experiment. At these dilution levels, a few different cell morphologies were evident, but it was not possible to distinguish the Iso M1-1 from other background tetracycline-resistant bacteria.

Digester *tet*(G) content was elevated by about 1.5-log to 2.9×10^7 gcn/ml following the initial spike of Iso M1-1. *tet*(G) normalized to 16S rRNA ratio was 0.28 in the pure culture preparation just prior to spiking, suggesting that at least 28% of the Iso M1-1 population carried *tet*(G). The percentage is likely closer to 100% [i.e., all cells carried *tet*(G)], as *Pseudomonas* spp. are reported to contain a median number of 4 copies (range is between four and seven copies) of the 16S rRNA gene per cell (Stoddard et al., 2015). The *tet*(G) content of the background digester community was 9.1×10^5 gcn/ml (**Figure 1A**). Unlike CFUs, levels of *tet*(G) did not decrease with time after the initial spike of IsoM1-1 into the batch digesters, but remained ∼1–1.5-log above the background throughout the experiment. When normalized to 16S rRNA genes, *tet*(G) ratios displayed a decreasing trend and returned to background by day 10 (**Figure 1B**). This is because 16S rRNA genes increased over this same period, which is suggestive of bacterial growth.

In contrast to *tet*(G), *tet*(W) was not influenced by the initial spike of Iso M1-1 and remained at or near the background level throughout the experiment (**Figure 1A**). This was as expected, given that IsoM1-1 does not carry *tet*(W). The 16S rRNA gene-normalized *tet*(G) and *tet*(W) ratios showed similar trends (**Figure 1B**).

Fate of Tetracycline-Resistant Isolate (Iso T10) during Thermophilic Digestion

Tetracycline-resistant plate counts in initial spiked thermophilic digester microcosms were elevated to 4.0×10^8 CFU/ml (**Figure 2**), but decreased immediately and returned to slightly above background (7.3×10^2 CFU/ml) within 15 min and remained at $\sim 10^3$ CFU/ml over an additional 40-day monitoring period. Iso T10 contained *tet*(W), not *tet*(G) (as carried by Iso M1-1). The *tet*(W) content of the background digester community was high (ranged 4.0×10^6–1.2×10^7 gcn/ml over the 40-day experiment), resulting in only a slight increase in *tet*(W) as a result of the spiked Iso T10, which was within the QPCR analytical error range (data not shown).

Lab-Scale Semi-Continuous Feed Digester Study

ARG-16S ratios in the 1) feed and ARG-16S ratios in the 2a) thermophilic digester and 2b) mesophilic digester were compared using Spearman's rho correlation analysis. *intI1* ratios in the feed sludge were positively correlated to *intI1* ratios in both thermophilic and mesophilic digesters (**Figure 3**). *sul1* ratios in the feed sludge were also positively correlated to *sul1* ratios in both thermophilic and mesophilic digesters (**Figure 4**).

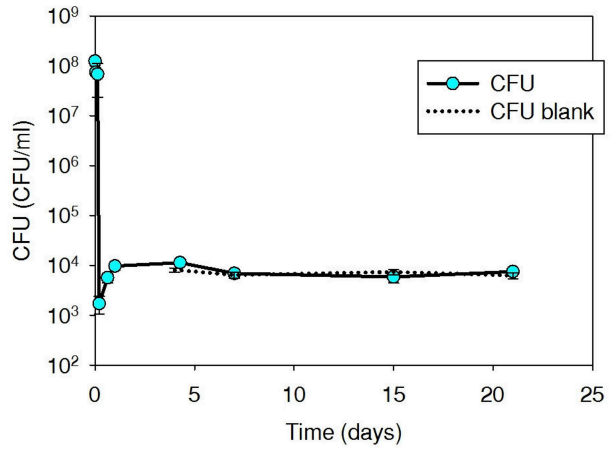


FIGURE 2 | Plate count (CFU/ml sludge) of tetracycline-resistant bacteria, including spiked Iso T10, during thermophilic treatment at 53°C in digested sludge.

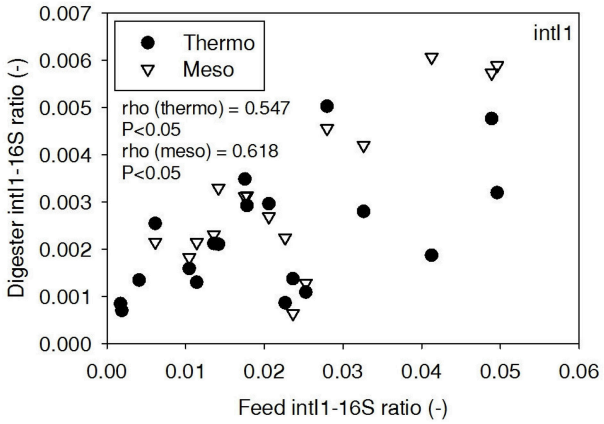


FIGURE 3 | Spearman rho correlation coefficients and *P*-values for *intI1* ARG ratios (normalized to 16S rRNA) in raw sludge feed compared to *intI1* ARG ratios in thermophilic and mesophilic digester effluent. A subset of this data (78% of data points) were previously published in Miller et al. (2013) as bar chart ARG concentration averages in a study of the effects of antibiotic concentration on ARGs in digestion or as ARG trends with time as a function of raw sludge storage temperature.

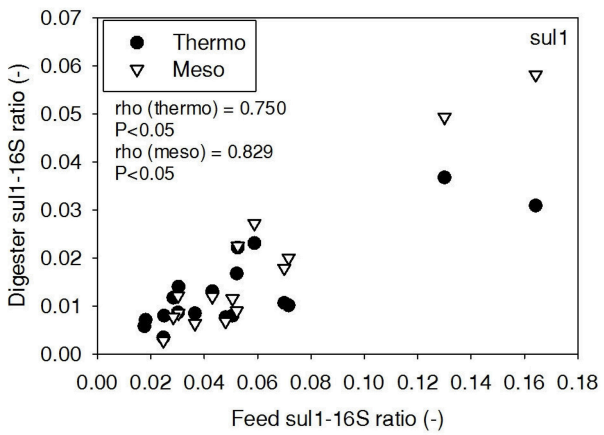


FIGURE 4 | Spearman rho correlation coefficients and *P*-values for *sul1* ARG ratios (normalized to 16S rRNA) in raw sludge feed compared to *sul1* ARG ratios in thermophilic and mesophilic digester effluent. A subset of this data (79% of data points) were previously published in Miller et al. (2013) as bar chart ARG concentration averages in a study of the effects of antibiotic concentration on ARGs in digestion or as ARG trends with time as a function of raw sludge storage temperature.

Interestingly, *intI1* and *sul1* ratios were positively correlated in thermophilic digesters as well as mesophilic digesters (**Figure 5**). This is consistent with current understanding that *sul1* is typically associated with Class 1 integrons (Mazel, 2006). In contrast, *tet*(O) and *tet*(W) ratios in the feed sludge were not correlated to *tet*(O) or *tet*(W) ratios in thermophilic or mesophilic digesters ($P > 0.05$, **Figures 6**, **7**, respectively).

DISCUSSION

Fate of Tetracycline Resistant Isolate (Iso M1-1) during Mesophilic Digestion

The central question to this study is to what extent raw sludge influences the antibiotic resistance level of an anaerobic digester and its effluent? It was observed that both ARBs and ARGs survived in batch mesophilic reactors. The Iso M1-1 ARB and *tet*(G) ARG associated with this host remained 1-log and 1.5-log above background, respectively, after 40 days of digestion. This finding has important implications for the management of upstream levels of ARBs and ARGs in primary and secondary sludge, suggesting that sludge pre-treatments that aim to minimize ARB in the influent could help reduce the spread of antibiotic resistance from wastewater sources.

Tetracycline-resistant CFUs rapidly declined following spiking of Iso M1-1 in the influent, indicating that they were initially at a level greater than its carrying capacity for the digester community. This is not surprising given that digesters rely on a symbiotic balance among communities of hydrolytic, fermentatative, and methanogenic microorganisms and any one group cannot survive or thrive without the others. However, the 1-log long term increase in tetracycline-resistant CFUs also suggests that the niche filled by Iso M1-1 within the mesophilic digester community was sufficiently large to accommodate a spike in its population. However, the tetracycline-resistant plate count method could not distinguish Iso M1-1 specifically from the background resistant microorganisms. Bearing this in mind, horizontal transfer of *tet*(G) to other hosts in the digester microbial communities could have contributed to the increase in tetracycline-resistant CFU.

These results may be specific to Iso M1-1, which was native to a digested sludge environment, whereas, exogenous bacteria typically present in raw sludge may have a different fate. To our knowledge, the overlap of ARB survival or microbe composition in raw sludge and digesters has not previously been reported. However, studies examining ARG survival have reported 1- to 2-log reduction of several ARGs relative to raw sludge

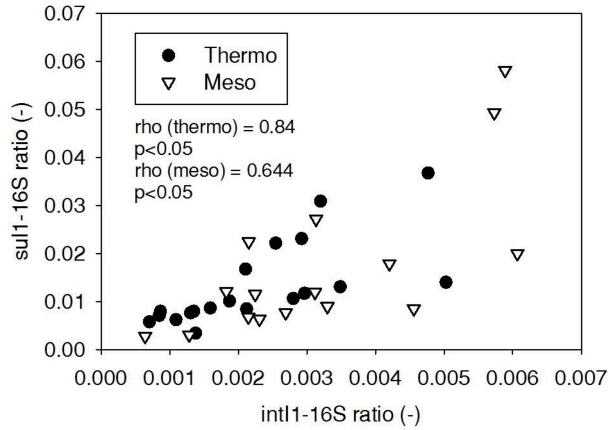


FIGURE 5 | Spearman rho correlation coefficients and *P*-values for *intI1* compared to *sul1* ARG ratios in thermophilic and mesophilic digester effluent. A subset of this data (79% of data points) were previously published in Miller et al. (2013) as bar chart ARG concentration averages in a study of the effects of antibiotic concentration on ARGs in digestion or as ARG trends with time as a function of raw sludge storage temperature.

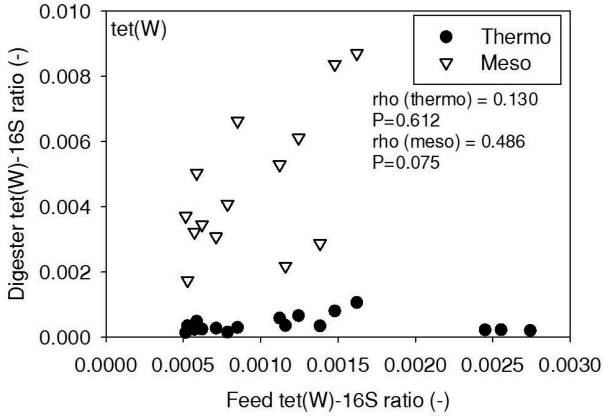


FIGURE 7 | Spearman rho correlation coefficients and *P*-values for *tet*(W) ARG ratios (normalized to 16S rRNA) in raw sludge feed compared to *tet*(W) ARG ratios in thermophilic and mesophilic digester effluent. A subset of this data (75% of data points) were previously published in Miller et al. (2013) as bar chart ARG concentration averages in a study of the effects of antibiotic concentration on ARGs in digestion.

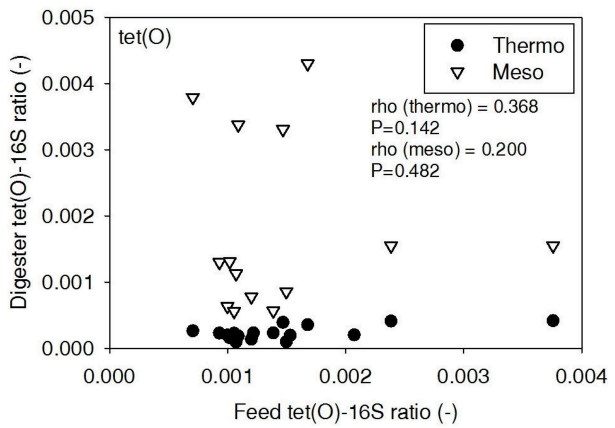


FIGURE 6 | Spearman rho correlation coefficients and *P*-values for *tet*(O) ARG ratios (normalized to 16S rRNA) in raw sludge feed compared to *tet*(O) ARG ratios in thermophilic and mesophilic digester effluent. A subset of this data (75% of data points) were previously published in Miller et al. (2013) as bar chart ARG concentration averages in a study of the effects of antibiotic concentration on ARGs in digestion.

during thermophilic digestion, while ARG fate reported under mesophilic digestion conditions has been more variable (Ghosh et al., 2009; Diehl and LaPara, 2010; Ma et al., 2011; Miller et al., 2013). These prior studies did not track or quantify specific ARBs or evidence of horizontal gene transfer.

Sequencing of the Iso M1-1 16S rRNA gene indicated that it was a *Pseudomonas* sp. *Pseudomonas* are generally characterized as being extremely metabolically diverse with respect to capability of utilizing a wide range of organic compounds as electron donors and carbon sources and as such have been identified as key players in anaerobic digestion (Kummerer, 2009; Zheng et al., 2013). *Pseudomonas* also tend to be adept at acquiring and disseminating ARGs, with certain multi-antibiotic resistant strains particularly problematic in hospitals (Obritsch et al., 2004). They are also thought to widely carry tetracycline resistance and have been found to house two or more tetracycline ARGs at a time, including *tet*(G) (Roberts, 2015). The fact that *Pseudomonas* are typically aerobic organisms, with some using nitrate as an electron acceptor (Madigan et al., 2010), supports the conclusion that they had a relatively narrow niche in the digester community, but were able to horizontally transfer ARGs as spiked levels attenuated. Interestingly, 16S rRNA gene levels lagged in their decline following CFU decline, suggesting that some time is required for the digester community to re-appropriate cell components (DNA) from dead Iso M1-1 cells into new biomass through growth.

Fate of *tet* ARG during Mesophilic Digestion

As discussed above, *tet*(G) ARGs were elevated by ~1.5 log in the effluents of the batch mesophilic digesters, presumably originating from the spiked Iso M1-1 known to be carrying it. In addition to survival of a significant fraction of IsoM1-1 cells and horizontal transfer of *tet*(G) to new hosts, persistence of extracellular *tet*(G) cannot be completely ruled out (Nielsen et al., 1996; Pietramellara et al., 2009; Dominiak et al., 2011). However, Zhang et al. (2013) quantified extracellular and intracellular DNA composition in livestock waste lagoon sludge and found that extracellular DNA accounted for less than 0.5% of the total DNA in DNA extracts from sludge, indicating that it degrades readily in sludge environments. Assuming similar distribution of DNA in digesters, *tet*(G) likely existed primarily in the intracellular form.

The maintenance of *tet*(G) at elevated levels above background combined with a substantial decrease from spiked levels in the CFU plate counts of Iso M1-1 suggest that

horizontal gene transfer was likely the primary mechanism of *tet*(G) persistence. Rizzo et al. (2013) employed a similar culture-based method to assess horizontal transfer of resistance plasmids where growth of donor bacteria and transconjugates on tetracycline-amended agar plates resulted in elevated tetracycline-resistant CFUs when compared with the plates where the donors and recipients were not inoculated together. Merlin et al. (2011) observed similar loss of donor bacteria with a concurrent relative stability of plasmid pB10 DNA in batch mesophilic (35°C) anaerobic digestion experiments and concluded that the ARG plasmid had entered the indigenous background population via conjugative transfer. Interestingly, the initial 5-log increase in tetracycline-resistant CFUs (an increase of $\sim 10^8$ CFU/ml) translated into only 2-log increase in *tet*(G) (an increase of $\sim 10^7$ gcn/ml) relative to the background. This suggests that the shock of the initial experimental set-up may stimulate Iso M1-1 to expel a portion of the *tet*(G) that it carried.

tet(G) has a broad host range and is commonly associated with mobile genetic elements, which supports the conclusion that significant horizontal gene transfer contributed to its persistence in the mesophilic digesters. *tet*(G) is found in Gram negative bacteria and encodes an energy-dependent, membrane-associated efflux protein. Currently, the following genera are known to be capable of carrying *tet*(G), including *Mannheimia*, *Ochrobactrum*, *Roseobacter*, *Salmonella*, *Acinetobacter*, *Brevundimonas*, *Enterobacter*, *Escherichia*, *Fusobacterium*, *Pasteurella*, *Proteus*, *Providencia*, *Pseudomonas*, *Shewanella*, and *Vibrio* (Roberts, 2015). It is likely that this list is not exhaustive, but limited by the studies investigating the distribution of tetracycline resistance (Roberts, 2005). Gram negative efflux ARGs [including *tet*(G)] are commonly associated with large, typically conjugative, plasmids that often carry other ARGs (Roberts, 1996). Conjugative plasmids can be highly promiscuous and can transfer between different genera or even domains (Ochman et al., 2000). *tet* genes have not yet been found on integrons (Roberts, 2005), although *tet* genes could be located on the same transposon or plasmid as integrons (Stalder et al., 2012).

Fate of Iso T10 and *tet* ARG during Thermophilic Digestion

In contrast to the mesophilic digestion condition, tetracycline-resistant CFUs returned almost immediately to background following spiking of the corresponding tetracycline-resistant ARB isolated from thermophilic sludge, Iso T10. This suggests that, in contrast to the mesophilic digestion condition, the niche for Iso T10 was not sufficient to accommodate an expanded population. This could be attributed to the fact that mesophilic digestion is characterized by a wider diversity of microorganisms that are able to assimilate, survive, and thrive in the digester environment. Likewise, it has been hypothesized that the extreme thermophilic environment limits available hosts for ARGs and may at least partially explain why thermophilic digesters often are more efficient for *tet* ARG removal (Diehl and LaPara, 2010; Ma et al., 2011; Miller et al., 2013).

Alternately, the decline in CFUs following the initial spike could be at least partially the result of bacteria entering the viable but non-culturable (VBNC) state. While it was the intent of this study to obtain isolates native to the mesophilic and thermophilic digester environments, spiking of the cultures back into the digestion tubes following several isolation steps could have affected their acclimation state to the digester environment. In particular, the thermophilic environment may have posed a shock to Iso T10, which was isolated and maintained at 37°C prior to spiking. It is also possible that Iso T10, while isolated from thermophilic digested sludge, was not a viable, active, or continuous member of the thermophilic community, but rather a transient microbe that was present during the initial plating of isolates.

Lab-Scale Semi-Continuous Feed Digester Study

The lab-scale digesters afforded the opportunity to observe the influence of raw sludge ARG levels on the ARG content of mesophilic and thermophilic digesters. Over the 9-month period, feed sludge ARG levels of *sul1*, *intI1*, *tet*(O), and *tet*(W) were monitored and compared with digester effluent concentrations. Consistent with the batch-scale studies, raw sludge ARG ratios of *intI1* and *sul*1 were positively correlated with *intI1* and *sul*1 ARG ratios in both the mesophilic and thermophilic digester effluents (**Figures 3**, **4**, respectively). This phenomenon could be explained by the survival or selective enrichment of influent raw sludge ARBs containing *intI1* or *sul*1 or by horizontal gene transfer between incoming raw sludge bacteria and the digester community. Thus, the semi-continuous feed digester study confirmed that the ARG content of raw sludge influences the ARG content of the digester effluent. Interestingly, the effect was observed for both mesophilic and thermophilic digesters.

There was no correlation of *tet*(O) and *tet*(W) ratios in raw sludge and ratios in thermophilic or mesophilic digester effluent (**Figures 6**, **7**). Also, the *tet*(O) and *tet*(W) ratios in the thermophilic digester remained consistently low over the entire monitoring period (**Figures 6**, **7**). These results suggest that the thermophilic digester community was relatively immune to transfer or intrusion of these *tet* ARGs or tetracycline-resistant ARBs.

In this study, raw sludge was stored in a refrigerator (4°C) to minimize biological activity (degradation of organics) prior to feeding to the digesters. Although cold storage has been shown to increase ARG concentrations in biosolids (Miller et al., 2014), the impact of cold storage on the ARB composition of the raw sludge and survivability in digestion has not been investigated. That being said, ARG concentrations reported herein were measured in raw sludge at the time of feeding (i.e., after refrigeration). Ghosh et al. (2009) monitored *tet* ARG and *intI1* in full-scale WWTPs with no cold storage of sludge prior to digestion and the *tet* ARG and *intI1* trends in thermophilic and mesophilic digestion relative to raw sludge are similar to those reported in the present study. That is, *tet* ARGs had a variable response in mesophilic digestion, whereas levels in thermophilic digestion were reduced by 0.5- to 2-log (Ghosh et al., 2009) and 1- to

2-log (Miller et al., 2013). Ghosh et al. (2009) reported that *intI1* levels were lower in mesophilic digested sludge and thermophilic digested sludge compared to raw sludge in two of three samples and three of three samples, respectively. Our work (Miller et al., 2013) has shown a consistent 1- to 1.5-log reduction of *intI1* by both mesophilic digestion and thermophilic digestion.

The variable response of different ARGs within the same digester may not be a surprise, considering *intI1* is the integrase gene associated with the highly mobile Class 1 integrons and *sul*1 is often co-located on Class 1 integrons (Paulsen et al., 1993; Zhang et al., 2009). *tet*(W) and *tet*(O) are found on plasmid and chromosomes, but have not been found within integron cassettes (Roberts, 2005). Thermophilic digestion may be able to achieve higher ARG content removal with respect to tetracycline genes because of the limited occurrence of *tet* genes within this particular thermophilic digester community, the incompatibility of the incoming raw sludge bacteria with the thermophilic digestion community for horizontal gene transfer, and/or the harsh environment restricting entrance into the digester community of new ARBs containing *tet* genes.

These results also suggest that limiting the ARG content of the initial seed community of a thermophilic digester may help limit the ARG content of digester effluent (i.e., biosolids intended for land application) because the microbial community is less prone to intrusion from raw sludge bacteria and horizontal gene transfer, thus retaining its initial ARG signature (or lack thereof). Again, these results highlight the importance of not only horizontal gene transfer, but also host survival. The limited diversity of a thermophilic digester (Wilson et al., 2008) and limiting (high temperature, high ammonia) conditions of a thermophilic digester likely limit opportunities for horizontal gene transfer and limit survival of incoming ARBs.

Implications for ARG Management via Sludge Digestion

The presence of a diverse bacterial, mobile genetic element, and ARG population in activated sludge systems has recently been highlighted by metagenomic studies (Zhang et al., 2011, 2015). The activated sludge environment is characterized by ambient temperatures (10−25°C), rich nutrient and organic loading, dense microbial populations. Similar conditions also exist in mesophilic digestion, albeit with a higher and more closely controlled temperature regime (∼37°C). In contrast, thermophilic regimes operate at high temperatures, endogenous conditions, with very high ammonia concentrations. Thermophilic digestion environments have been shown to support a less diverse microbial population than mesophilic digestion (Wilson et al., 2008). Moreover, to gain critical time-temperature requirements for production of Class A biosolids classification, thermophilic digesters are operated with minimum retention times prior to effluent withdrawal and shorter SRTs. This may allow longer times of uninterrupted degradation between raw sludge additions and shorter solids retention times for HGT to occur. These environmental and operating conditions may give thermophilic digestion a competitive advantage over activated sludge or mesophilic digestion in the reduction of ARG content of biosolids. Although, Zhang et al. (2015) reported similar total tetracycline ARG abundance in thermophilic and mesophilic digested sludge in a metagenomic analysis that gave a broad picture of ARG behavior, this statement is supported by our work, along with other QPCR-based studies reporting enhanced reductions of ARGs by thermophilic digestion relative to mesophilic digestion (Ghosh et al., 2009; Diehl and LaPara, 2010; Ma et al., 2011; Miller et al., 2013). Moreover, although horizontal gene transfer continues to be an important mechanism of ARG persistence for genes associated with Class 1 integrons (*sul*1, *intI1*), we also report that thermophilic digestion may allow for the reduction of other ARGs [(*tet*(O), *tet*(W)] through incoming ARB death and narrowed host range for horizontal gene transfer.

One possibility would be to intentionally seed a digester with ARG-free microorganisms to support an ARG-free effluent (biosolid). However, it is evident that horizontal gene transfer still does occur, particularly with integrons that are typically associated with mobile genetic elements, so that complete reduction of all ARGs may not be possible with ARG-free digester seed alone. Further investigation into pre-treatments designed for complete cell lysis and DNA degradation in raw sludge are warranted for both digestion methods to reduce intrusive ARBs and transformable DNA. It seems plausible that due to the apparent limitations on microbial diversity and the resultant limitations on horizontal gene transfer, that thermophilic digestion could achieve a higher rate of ARG reductions than mesophilic digestion particularly in the face of inefficient pre-treatments. In conclusion, influent ARB and ARG composition and sludge digestion conditions are important for determining the fate of influent ARBs and ARGs and ultimately determining digester effluent ARG content.

AUTHOR CONTRIBUTIONS

JM conducted the experiments, wrote the original manuscript, and edited the manuscript. AP provided technical oversight and critical manuscript review and editing. All authors discussed the results and implications and commented on the manuscript at all stages.

FUNDING

This work was supported by National Science Foundation Chemical, Bioengineering, and Transport Systems CAREER award #0852942 and Virginia Tech Institute for Critical Technology and Applied Science seed funding and award TSTS 11–26. JM was supported by the Charles E. Via, Jr. Department of Civil and Environmental Engineering Via Scholarship, Virginia Tech Graduate School Cunningham Fellowship, and Water Environment Research Foundation (WERF) Project U1R12. The findings of this study do not necessarily reflect the views of the supporting entities.

REFERENCES

Aminov, R. I., Garrigues-Jeanjean, N., and Mackie, R. I. (2001). Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *Appl. Environ. Microbiol.* 1, 22–32. doi: 10.1128/A. E. M.67.1.22-32.2001

APHA/AWWA/WEF (1995). *Standard Methods for the Examination of Water and Wastewater, 19th Edn.* Washington, DC: American Public Health Association/American Water Works Association/Water Environmental Federation.

Ashbolt, N. J., Amézquita, A., Backhaus, T., Borriello, P., Brandt, K. K., Collignon, P., et al. (2013). Human Health Risk Assessment (HHRA) for environmental development and transfer of antibiotic resistance. *Environ. Health Perspect.* 121, 993–1001. doi: 10.1289/ehp.1206316

Bonot, S., and Merlin, C. (2010). Monitoring the dissemination of the broad-host-range plasmid pB10 in sediment microcosms by quantitative PCApplied R and Environmental *Microbiology* 76, 378–382. doi: 10.1128/AEM.01125-09

da Silva, M. F., Vaz-Moreira, I., Gonzalez-Pajuelo, M., Nunes, O. C., and Manaia, C. M. (2007). Antimicrobial resistance patterns in Enterobacteriaceae isolated from an urban wastewater treatment plant. *FEMS Microbiol. Ecol.* 60, 166–176. doi: 10.1111/j.1574-6941.2006.00268.x

Davison, J. (1999). Genetic exchange between bacteria in the environment. *Plasmid* 42, 73–91. doi: 10.1006/plas.1999.1421

Diehl, D. L., and LaPara, T. M. (2010). Effect of temperature on the fate of genes encoding tetracycline resistance and the integrase of class 1 integrons within anaerobic and aerobic digesters treating municipal wastewater solids. *Environ. Sci. Technol.* 44, 9128–9133. doi: 10.1021/es102765a

Dominiak, D. M., Nielsen, J. L., and Nielsen, P. H. (2011). Extracellular DNA is abundant and important for microcolony strength in mixed microbial biofilms. *Environ. Microbiol.* 13, 710–721. doi: 10.1111/j.1462-2920.2010.02375.x

Dröge, M., Pühler, A., and Selbitschka, W. (2000). Phenotypic and molecular characterization of conjugative antibiotic resistance plasmids isolated from bacterial communities of activated sludge. *Mol.Gen. Genet.* 263, 471–482. doi: 10.1007/s004380051191

Ghosh, S., Ramsden, S. J., and LaPara, T. M. (2009). The role of anaerobic digestion in controlling the release of tetracycline resistance genes and class 1 integrons from municipal wastewater treatment plants. *Appl. Microbiol. Biotechnol.* 84, 791–796. doi: 10.1007/s00253-009-2125-2

Gillings, M. R., Gaze, W. H., Pruden, A., Smalla, K., Tiedje, J. M., and Zhu, Y. (2014). Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. *ISME J.* 9, 1269. doi: 10.1038/ismej.2014.226

Hardwick, S. A., Stokes, H. W., Findlay, S., Taylor, M., and Gillings, M. R. (2008). Quantification of class 1 integron abundance in natural environments using real-time quantitative PCR. *FEMS Microbiol. Lett.* 278, 207–212. doi: 10.1111/j.1574-6968.2007.00992.x

Helsel, D. R., and Hirsch, R. M. (2002). *Statistical Methods in Water Resources. Techniques of Water-Resources Investigations of the United States Geological Survey. Book 4, Hydrologic Analysis and Interpretation*. Washington, DC: United States Printing Office.

Kummerer, K. (2009). Antibiotics in the aquatic environment – A review – Part II. *Chemosphere* 75, 435–441. doi: 10.1016/j.chemosphere.2008.12.006

LaPara, T. M., Burch, T. R., McNamara, P. J., Tan, D. T., Yan, M., and Eichmiller, J. J. (2011). Tertiary-treated municipal wastewater is a significant point source of antibiotic resistance genes into Duluth-Superior harbor. *Environ. Sci. Technol.* 45, 9543–9549. doi: 10.1021/es202775r

Luby, E., Ibekwe, A. M., Zilles, J., and Pruden, A. (2016). Molecular methods for assessment of antibiotic resistance in agricultural ecosystems: prospects and challenges. *J. Environ. Qual.* 45, 1. doi: 10.2134/jeq2015.07.0367

Luczkiewicz, A., Jankowska, K., Fudala-Ksiazek, S., and Olanczuk-Neyman, K. (2010). Antimicrobial resistance of fecal indicators in municipal wastewater treatment plant. *Water Res.* 44, 5089–5097. doi: 10.1016/j.watres.2010.08.007

Lupo, A., Coyne, S., and Berendonk, T. U. (2012). Origin and evolution of antibiotic resistance: the common mechanisms of emergence and spread in water bodies. *Front. Microbiol.* 3:18. doi: 10.3389/fmicb.2012.00018

Ma, Y. J., Wilson, C. A., Novak, J. T., Riffat, R., Aynur, S., Murthy, S., et al. (2011). Effect of various sludge digestion conditions on sulfonamide, macrolide, and tetracycline resistance genes and Class I integrons. *Environ. Sci. Technol.* 45, 7855–7861. doi: 10.1021/es200827t

Madigan, M. T., Martinko, J. M., Stahl, D., and Clark, D. P. (2010). *Biology of Microorganisms. 13th Edn*. San Francisco, CA: Benjamin Cummings.

Mazel, D. (2006). Integrons: agents of bacterial evolution. *Nat. Rev. Microbiol.* 4, 608–620. doi: 10.1038/nrmicro1462

Merlin, C., Bonot, S., Courtois, S., and Block, J. C. (2011). Persistence and dissemination of the multiple-antibiotic-resistance plasmid pB10 in the microbial communities of wastewater sludge microcosms. *Water Res.* 45, 2897–2905. doi: 10.1016/j.watres.2011.03.002

Miller, J. H., Novak, J. T., Knocke, W. R., and Pruden, A. (2014). Elevation of antibiotic resistance genes at cold temperatures: implications for winter storage of sludge and biosolids. *Lett. Appl. Microbiol.* 59, 587–593. doi: 10.1111/lam.12325

Miller, J. H., Novak, J. T., Knocke, W. R., Young, K., Hong, Y. J., Vikesland, P. J., et al. (2013). Effect of silver nanoparticles and antibiotics on antibiotic resistance genes in anaerobic digestion. *Water Environ. Res.* 85, 411–421. doi: 10.2175/106143012X13373575831394

Moura, A., Oliveira, C., Henriques, I., Smalla, K., and Correia, A. (2012). Broad diversity of conjugative plasmids in integron-carrying bacteria from wastewater environments. *FEMS Microbiol. Lett.* 330, 157–164. doi: 10.1111/j.1574-6968.2012.02544.x

Muniesa, M., Imamovic, L., and Jofre, J. (2011). Bacteriophages and genetic mobilization in sewage and faecally polluted environments. *Microb. Biotechnol.* 4, 725–734. doi: 10.1111/j.1751-7915.2011.00264.x

Munir, M., Wong, K., and Xagoraraki, I. (2011). Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water Res.* 45, 681–693. doi: 10.1016/j.watres.2010.08.033

Munir, M., and Xagoraraki, I. (2011). Levels of antibiotic resistance genes in manure, biosolids, and fertilized soil. *J. Environ. Qual.* 40, 248–255. doi: 10.2134/jeq2010.0209

Nielsen, P. H., Frolund, B., and Keiding, K. (1996). Changes in the composition of extracellular polymeric substances in activated sludge during anaerobic storage. *Appl. Microbiol. Biotechnol.* 44, 823–830. doi: 10.1007/BF00178625

Obritsch, M. D., Fish, D. N., MacLaren, R., and Jung, R. (2004). National surveillance of antimicrobial resistance in Pseudomonas aeruginosa isolates obtained from intensive care unit patients from 1993 to 2002. *Antimicrob. Agents Chemother.* 48, 4606–4610. doi: 10.1128/AAC.48.12.4606-4610.2004

Ochman, H., Lawrence, J. G., and Groisman, E. A. (2000). Lateral gene transfer and the nature of bacterial innovation. *Nature* 405, 299–304. doi: 10.1038/35012500

Paulsen, I. T., Littlejohn, T. G., Rådström, P., Sundstrom, L., Skold, O., Skurray, R. A., et al. (1993). The 3′ conserved segment of integrons contains a gene associated with multidrug resistance to antiseptics and disinfectants. *Antimicrob. Agents Chemother*. 37, 761–768. doi: 10.1128/AAC.37.4.761

Pei, R., Kim, S. C., Carlson, K. H., and Pruden, A. (2006). Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Res.* 40, 2427–2435. doi: 10.1016/j.watres.2006.04.017

Pietramellara, G., Ascher, J., Borgogni, F., Ceccherini, M. T., Guerri, G., and Nannipieri, P. (2009). Extracellular DNA in soil and sediment: fate and ecological relevance. *Biol. Fertil. Soils* 45, 219–235. doi: 10.1007/s00374-008-0345-8

Poirel, L., Kämpfer, P., and Nordmann, P. (2002). Chromosome-encoded ambler class A beta-lactamase of Kluyvera georgiana, a probable progenitor of a subgroup of CTX-M extended-spectrum beta-lactamases. *Antimicrob. Agents Chemother.* 46, 4038–4040. doi: 10.1128/AAC.46.12.4038-4040.2002

Resende, J. A., Diniz, C. G., Silva, V. L., Otenio, M. H., Bonnafous, A., Arcuri, P. B., et al. (2014). Dynamics of antibiotic resistance genes and presence of putative pathogens during ambient temperature anaerobic digestion. *J. Appl. Microbiol.* 117, 1689–1699. doi: 10.1111/jam.12653

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M. C., et al. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. *Sci. Total Environ.* 447, 345–360. doi: 10.1016/j.scitotenv.2013.01.032

Roberts, M. (2015). *Distribution of Tet Resistance Genes among Gram-Negative Bacteria*. Available online at: http://faculty.washington.edu/marilynr/ (Accessed June 13, 2015).

Roberts, M. C. (1996). Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. *FEMS Microbiol. Rev.* 19, 1–24. doi: 10.1111/j.1574-6976.1996.tb00251.x

Roberts, M. C. (2005). Update on acquired tetracycline resistance genes. *FEMS Microbiol. Lett.* 245, 195–203. doi: 10.1016/j.femsle.2005.02.034

Rossolini, G. M., D'Andrea, M. M., and Mugnaioli, C. (2008). The spread of CTX-M-type extended-spectrum beta-lactamases. *Clin. Microbiol. Infect.* 14, 33–41. doi: 10.1111/j.1469-0691.2007.01867.x

Schlüter, A., Szczepanowski, R., Puhler, A., and Top, E. M. (2007). Genomics of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence for a widely accessible drug resistance gene pool. *FEMS Microbiol. Rev.* 31, 449–477. doi: 10.1111/j.1574-6976.2007.00074.x

Stalder, T., Alrhmoun, M., Louvet, J. N., Casellas, M., Maftah, C., Carrion, C., et al. (2013). Dynamic assessment of the floc morphology, bacterial diversity, and integron content of an activated sludge reactor processing hospital effluent. *Environ. Sci. Technol.* 47, 7909–7917. doi: 10.1021/es4008646

Stalder, T., Barraud, O., Casellas, M., Dagot, C., and Ploy, M. C. (2012). Integron involvement in environmental spread of antibiotic resistance. *Front. Microbiol.* 3:119. doi: 10.3389/fmicb.2012.00119

Stoddard, S. F., Smith, B. J., Hein, R., Roller, B. R. K., and Schmidt, T. M. (2015). *rrn*DB: improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development. *Nucleic Acids Res.* 43, D593–D598. doi: 10.1093/nar/gku1201

Storteboom, H., Arabi, M., Davis, J. G., Crimi, B., and Pruden, A. (2010). Tracking antibiotic resistance genes in the South Platte River Basin using molecular signatures of urban, agricultural, and pristine sources. *Environ. Sci. Technol.* 44, 7397–7404. doi: 10.1021/es101657s

Suzuki, M. T., Taylor, L. T., and DeLong, E. F. (2000). Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5′-nuclease assays. *Appl. Environ. Microbiol.* 66, 4605–4614. doi: 10.1128/AEM.66.11.4605-4614.2000

Szczepanowski, R., Linke, B., Krahn, I., Gartemann, K. H., Gutzkow, T., Eichler, W., et al. (2009). Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiology* 155, 2306–2319. doi: 10.1099/mic.0.028233-0

Wellington, E. M. H., Boxall, A. B. A., Cross, P., Feil, E. J., Gaze, W. H., Hawkey, P. M., et al. (2013). The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. *Lancet Infect. Dis.* 13, 155–165. doi: 10.1016/S1473-3099(12)70317-1

Wilson, C. A., Murthy, S. M., Fang, Y., and Novak, J. T. (2008). The effect of temperature on the performance and stability of thermophilic anaerobic digestion. *Water Sci. Technol.* 57, 297–304. doi: 10.2166/wst.2008.027

Yang, Y., Li, B., Zou, S. C., Fang, H. H. P., and Zhang, T. (2014). Fate of antibiotic resistance genes in sewage treatment plant revealed by metagenomic approach. *Water Res.* 62, 97–106. doi: 10.1016/j.watres.2014.05.019

Zhang, T., Yang, Y., and Pruden, A. (2015). Effect of temperature on removal of antibiotic resistance genes by anaerobic digestion of activated sludge revealed by metagenomic approach. *Appl. Microbiol. Biotechnol.* 99, 7771–7779. doi: 10.1007/s00253-015-6688-9

Zhang, T., Zhang, X. X., and Ye, L. (2011). Plasmid metagenome reveals high levels of antibiotic resistance genes and mobile genetic elements in activated sludge. *PLoS ONE* 6:e26041. doi: 10.1371/journal.pone.0026041

Zhang, X. X., Zhang, T., Zhang, M., Fang, H. H. P., and Cheng, S. P. (2009). Characterization and quantification of class 1 integrons and associated gene cassettes in sewage treatment plants. *Appl. Microbiol. Biotechnol.* 82, 1169–1177. doi: 10.1007/s00253-009-1886-y

Zhang, Y. P., Snow, D. D., Parker, D., Zhou, Z., and Li, X. (2013). Intracellular and extracellular antimicrobial resistance genes in the sludge of livestock waste management structures. *Environ. Sci. Technol.* 47, 10206–10213. doi: 10.1021/es401964s

Zheng, X., Su, Y., Li, X., Xiao, N., Wang, D., and Cheng, Y. (2013). Pyrosequencing reveals the key microorganisms involved in Sludge. Alkaline fermentation for efficient short-chain fatty acids production. *Environ. Sci. Technol.* 47, 4262–4268. doi: 10.1021/es400210v

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 12 May 2014
doi: 10.3389/fmicb.2014.00152

Various pAQU plasmids possibly contribute to disseminate tetracycline resistance gene *tet*(M) among marine bacterial community

Lisa Nonaka[1]*, Fumito Maruyama[2], Yuki Onishi[3], Takeshi Kobayashi[4], Yoshitoshi Ogura[5], Tetsuya Hayashi[5], Satoru Suzuki[3] and Michiaki Masuda[1]

[1] *Department of Microbiology, Dokkyo Medical University School of Medicine, Mibu, Tochigi, Japan*
[2] *Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan*
[3] *Division of Aquatic Biosciences, Center for Marine Environmental Studies, Ehime University, Matsuyama, Ehime, Japan*
[4] *Department of Ophthalmology and Regenerative Medicine, Ehime University Graduate School of Medicine, Shitsukawa, Toon, Ehime, Japan*
[5] *Division of Microbial Genomics, Department of Genomics and Bioenvironmental Science, Frontier Science Research Centre, University of Miyazaki, Kiyotake, Miyazaki, Japan*

Edited by:
Marko Virta, University of Helsinki, Finland

Reviewed by:
Atte Von Wright, University of Eastern Finland, Finland
Veljo Kisand, University of Tartu, Estonia

****Correspondence:***
Lisa Nonaka, Department of Microbiology, Dokkyo Medical University School of Medicine, Kitakobayashi 880, Mibu, Tochigi 321-0293, Japan
e-mail: nonaka@dokkyomed.ac.jp

Emergence of antibiotic-resistant bacteria in the aquaculture environment is a significant problem for disease control of cultured fish as well as in human public health. Conjugative mobile genetic elements (MGEs) are involved in dissemination of antibiotic resistance genes (ARGs) among marine bacteria. In the present study, we first designed a PCR targeting *tral* gene encoding essential relaxase for conjugation. By this new PCR, we demonstrated that five of 83 strains isolated from a coastal aquaculture site had *tral*-positive MGEs. While one of the five strains that belonged to *Shewanella* sp. was shown to have an integrative conjugative element of the SXT/R391 family (ICE*Vch*Mex-like), the MGEs of the other four strains of *Vibrio* spp. were shown to have the backbone structure similar to that of previously described in pAQU1. The backbone structure shared by the pAQU1-like plasmids in the four strains corresponded to a ∼100-kbp highly conserved region required for replication, partition and conjugative transfer, suggesting that these plasmids constituted "pAQU group." The pAQU group plasmids were shown to be capable of conjugative transfer of *tet*(M) and other ARGs from the *Vibrio* strains to *E. coli*. The pAQU group plasmid in one of the examined strains was designated as pAQU2, and its complete nucleotide sequence was determined and compared with that of pAQU1. The results revealed that pAQU2 contained fewer ARGs than pAQU1 did, and most of the ARGs in both of these plasmids were located in the similar region where multiple transposases were found, suggesting that the ARGs were introduced by several events of DNA transposition into an ancestral plasmid followed by drug selection in the aquaculture site. The results of the present study indicate that the "pAQU group" plasmids may play an important role in dissemination of ARGs in the marine environment.

Keywords: pAQU group, pAQU2, transferable plasmid, *tet*(M), antimicrobial resistance genes, SXT/R391 ICEs, aquaculture, *tral*

INTRODUCTION

Wide ranges of antimicrobial agents have been used not only for treatment of human infectious diseases but also in the fish farming industry for therapeutic and prophylactic purposes (Cabello et al., 2013). Among these, tetracyclines (TCs) are broad-spectrum agents, exhibiting activity against various microorganisms, such as Gram-positive and -negative bacteria, chlamydiae, mycoplasmas, and rickettsiae. In some countries, TCs are added to animal feeds at subtherapeutic levels so that they can act as growth promoters (Chopra and Roberts, 2001). In Japan, oxytetracyline and doxycyline have been included in the drugs permitted to use in the process of aquaculture, and the former is one of the drugs available to use for the largest variety of fish species (Ministry of Agriculture, Forestry and Fisheries, Japan, 2011). When the antibiotics are used in the aquaculture, they are usually mixed with feed and administered to fish, and the extensive use of TCs has led emergence of resistant bacteria (Nonaka et al., 2007). To date, 46 different TC resistance genes have been submitted to the nomenclature center (http://faculty.washington.edu/marilynr/), of which 30 genes code for efflux proteins, 12 genes for ribosomal protection proteins and four genes for other categories. Emergence and dissemination of drug resistance in the marine environment have a large impact on the fish farming industry. It is also an important issue for clinical medicine because a number of antibiotics resistance genes (ARGs) are shared between marine bacteria and human pathogens (Cabello et al., 2013) and the gene flow between these environments has been suggested. Further, transfer of opportunistic human pathogen from aquatic environments possible to generate antimicrobial resistance infections (Cabello et al., 2013).

A TC resistance gene, *tet*(M), is among the ARGs shared between marine bacteria and human pathogens and has extensively been studied in terms of the molecular ecology in the environment. The gene is known to be distributed among numerous bacterial species (Chopra and Roberts, 2001; Roberts, 2005) derived from diverse sources, such as human pathogens (Jones et al., 2006), domestic animals (Aminov et al., 2001; Chee-Sanford et al., 2001), food (Gevers et al., 2003; Bertrand et al., 2005), and farmed fish (Kim et al., 2007). In our previous study (Kim et al., 2004; Nonaka et al., 2007), we also found that *tet*(M) was distributed among several bacterial species isolated from fishes or the marine aquaculture environment. Other recent studies have also reported identification of *tet*(M) in the bacteria from the aquaculture environments (Nikolakopoulou et al., 2008; Seyfried et al., 2010; Di Cesare et al., 2012, 2013). Thus, the marine bacteria have been suggested to be a reservoir of *tet*(M).

While detailed mechanisms by which *tet*(M) has propagated in the marine environment are unknown, it has previously been demonstrated that *tet*(M) is frequently carried by mobile genetic elements (MGEs), such as plasmid and transposon (Chopra and Roberts, 2001). Previously, 73 strains of *tet*(M)-positive marine bacteria from an aquaculture site were examined, and it was shown that five of them, strains 04Ya001, 04Ya016, 04Ya090, 04Ya108, and 04Ya311, were able to transfer TC resistance to *E. coli* (Neela et al., 2009). Among these strains, 04Ya311 was characterized in detail, and it was demonstrated that this strain had a novel self-transferable plasmid, which we designated as pAQU1, and that conjugative transfer of pAQU1 conferred TC resistance on *E. coli* (Nonaka et al., 2012). Determination of its complete nucleotide sequence revealed that pAQU1 encoded *tet*(M) and other ARGs, such as *tet*(B), *sul*2, *flo*R and genes similar to bla_{CARB-9}, *mph*(A) and *mef*(A). Phylogenetic analysis of pAQU1's *traI* gene encoding relaxase (Nonaka et al., 2012), an only protein component of the conjugative machinery common to all MGEs using a conjugative system (Garcillan-Barcia et al., 2009; Smillie et al., 2010), indicated that the plasmid had a close relationship with IncA/C plasmid and SXT/R391 family of integrative conjugative elements (SXT/R391 ICEs) which belong to the MOB_{H12} clade in the MOB_H family (Nonaka et al., 2012). Since IncA/C plasmid and SXT/R391 ICEs are distributed among many aquatic isolates of genera *Enterobacteriaceae* and *Vibrionaceae* (Welch et al., 2007; McIntosh et al., 2008; Fricke et al., 2009), we hypothesized that pAQU1 found in 04Ya311 was a novel plasmid that might play a role in dissemination of *tet*(M) in the marine environment. In present study, since several pAQU1-related plasmids were found, we aimed to reveal that these pAQU1- related plasmids constituted a novel "pAQU group" characterized by a common backbone structure and conferred to transfer *tet*(M) and other ARGs to *E. coli* by conjugation.

MATERIALS AND METHODS

BACTERIAL STRAINS

For screening of *traI*, 83 tetracycline-resistant strains of bacteria isolated from the sediment or seawater of a coastal aquaculture site in Japan were used (Supplementary Table1). At the site, oxytetracycline and flumequine had been used (Nonaka et al., 2007). Theses environmental strains were cultured at 25°C in the brain heart infusion (BHI) medium (Becton, Dickinson and Company, Sparks, MD, USA) with 0.2% NaCl and 10 μg/ml of TC (Nakalai tesque, Kyoto, Japan). All of the strains were confirmed by PCR to have *tet*(M) (Nonaka et al., 2007), and strains 04Ya001, 04Ya016, 04Ya090, and 04Ya108 have previously been shown to transfer *tet*(M) to *E. coli* (Neela et al., 2009). *E. coli* W3110 obtained from the National BioResource Project (National Institute of Genetics, Japan) and its rifampicin-resistant derivative, W3110Rif^r (Nonaka et al., 2012), were cultured at 37°C in the Luria-Bertani medium (BD) with or without 10 μg/ml (for liquid media) or 20 μg/ml (solid media) of TC and 100 μg/ml of rifampicin (Sigma-Aldrich, Saint-Louis, MO).

DNA EXTRACTION AND PCR

Total bacterial DNA was extracted by using a QuickGene DNA tissue kit (Fujifilm Corporation, Tokyo, Japan) according to the manufacturer's protocol.

A set of degenerate primers (MOBH1_traI062-1F, MOBH1_traI062-2R) listed in **Table 1** were designed and used for detecting *traI* gene of MGEs of MOB_{H12} clade of MOB_H family. Namely, the *traI* nucleotide sequences of pAQU1 (GenBank accession number AB571865), IncA/C plasmids including pYR (CP000602), pIP1202 (CP000603), pP99-018 (AB277723), pP91278 (AB277724), pSN254 (CP000604) (Welch et al., 2007; Fricke et al., 2009), SXT/R391 ICEs including ICE*Vch*Mex (DQ180350, GQ463143), ICE*Vch*Ind4 (GQ463141), ICE*Vch*Ind5 (GQ463142), ICE*Vch*Ban5 (GQ463140), ICE*Pal*Ban1 (GQ463139), SXT^{MO10} (AY090559), ICE*Pda*Spa1 (AJ870986), and R391(AY090559) (Wozniak et al., 2009) were aligned using the multiple-sequence alignment program Clustal W (Thompson et al., 1994; Larkin et al., 2007) and conserved regions were selected for the designing of the primers. The PCR mixture (total volume, 10 μl) contained 5 μM each of the primers, 1 × PCR buffer (Life Technologies Corporation, Carlsbad, CA, USA), 2.5 mM each of dNTPs, 0.5 U of AmpliTaq DNA polymerase (Life Technologies Corporation) and the template DNA (20–50 ng). PCR amplification was performed with initial denaturation at 94°C for 1 min followed by 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s and a final extension step at 72°C for 3 min.

For PCR detection of ARGs [*tet*(B), *tet*(M), *sul*2, *flo*R, bla_{CARB-9}-like, *mph*(A)-like and *mef*(A)-like], genes related to the type IV secretion system (*repA*, *traA2*, *traB*, *traC*, *traF*, *traN*, *traI*, and *traL)* and ICE*Vch*Mex-related genes (*int*, *traF*, and *traN*), respective primers shown in **Table 1** were used. PCR amplification was performed with initial denaturation at 94°C for 1 min followed by 30 cycles of denaturation at 94°C for 30 s, 1 min of annealing at the temperatures shown in **Table 1**, and extension at 72°C for 1 min and a final extension step at 72°C for 3 min.

Additional 12 sets of primers (**Table 2**) were designed for PCR to identify and characterize the backbone structure of the "pAQU group" plasmids. The PCR mixture (total volume, 10 μl) contained 0.4 μM each of the primers, 1 × LA PCR buffer II (TAKARA BIO INC, Ohtsu, Japan), 0.4 mM each of dNTPs, 2.5 mM of $MgCl_2$, 0.5 U of LA Taq DNA polymerase (TAKARA

Table 1 | PCR primers used in this study.

Gene (s)	MGE (s)	Primer name	Primer sequence (5′ -> 3′)	Annealing temperature (°C)	Size (bp)	References
traI	pAQU1 (GenBank accession number AB571865) IncA/C plasmids* SXT/R391 family of ICEs**	MOBH1_traI062-1F MOBH1_traI062-2R	CAYYTRYTRCCAGCWTCYGA SRCGYCWSGCTSATTGAC	57	645	This study
tet(B)	pAQU1 (AB571865)	TetB-FW TetB-RV	TACGTGAATTTATTGCTTCGG ATACAGCATCCAAAGCGCAC	65	226	This study
tet(M)		tet(M)-1 tet(M)-2	GTTAAATAGTGTTCTTGGAG CTAAGATATGGCTCTAACAA	55	676	Nonaka et al., 2007
bla_{CARB-9}-like		cds084-1f cds084-672r	ATGAGTACATTCAAGACATT TTATTTGACAGCATACTCTT	55	672	This study
mph(A)-like		cds165-1f cds165-885r	ATGAAAAATAGAGATATTCA CTACTCAACACCTAACTGTA	55	885	This study
mef(A)-like		cds166-1f cds166-1124r	ATGGAAAACCGTAAATGGTT TTAAATATTTTTGATTTTAC	55	1,224	This study
*sul*2		sul2-F sul2-R	CTCAATGATATTCGCGGTTTYCC AAAAACCCCATGCCGGGRTC	58	245	Heuer and Smalla, 2007
*flo*R		FloR283f FloR519r	GGAGCAGCTTGGTCTTCAAC AATGAATATCGCCTGCCATC	55	226	This study
repA		CDS001(AB571865)-446f CDS001(AB571865)-646r	GGAAGAAGCATCCAGAGCAC AGCCAGAGACGCTGTTGATT	55	236	This study
traA2		traA89f traA246r	AAGCTCACGCTGGTACTGGT CATTGCGAATGCCATGATAC	67	177	This study
traB		traB343f traB591r	CGCGATGTTAAGGAGCTTTC CTTACCCGTTGGTTTGCTGT	55	268	This study
traC		traC1742f traC2110r	CCGCGTATACCGCTTATGTT CAGCAGGATCGTCAAAGTGA	55	388	This study
traF		traF1f traF1r	GTGGGATAACCCAACCATTG GGAGCCTGTATTTCGCACAT	55	298	This study
traN		traN535f traN1600r	CCAGGCGATGTGGTTAGTTT CTTTGAGAGCCTGAATTGCC	67	1,085	This study
traI		traI1976f traI2225r	ATGTTGATGTGCGTGGTCAT CTTCCGGTTTGGATGAACTG	55	270	This study
traL		TraL70f TraL270r	CTGGCTCCTATGCTTCTTGG CCTCCGGATAAATGGGTTTT	55	220	This study
Int	ICE*Vch*Mex (DQ180350)	int575f int803r	TCACAGCAAAGAAAGTCGGC CGGATCTCTATGGGCACTGT	57	229	This study
traF		traF517f traF708r	CGTTCGGATTGCCCTTACTG AACTAAGAACACGGCAGGGA	57	192	This study
traN		traN1087f traN1288r	ATTCAATGCACGGATCAGCC CACAGGAAGGATTCGCTTCG	57	202	This study

**IncA/C plasmid including pYR (CP000602), pIP1202 (CP000603), pP99-018 (AB277723), pP91278 (AB277724), pSN254 (CP000604) (Welch et al., 2007; Fricke et al., 2009).*

***SXT/R391 ICEs including ICEVchMex (DQ180350, GQ463143), ICEVchInd4 (GQ463141), ICEVchInd5 (GQ463142), ICEVchBan5 (GQ463140), ICEPalBan1 (GQ463139), SXT^{MO10} (AY090559), ICEPdaSpa1 (AJ870986) and R391(AY090559) (Wozniak et al., 2009).*

BIO INC) and the template DNA (20–50 ng). For amplicons of 2–10 kb, PCR amplification was performed with initial denaturation at 94°C for 1 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 5 min and a final extension step at 72°C for 3 min. For amplicons longer than 10 kb, two-step PCR was performed with 30 cycles of denaturation at 96°C for 20 s and extension at 69°C for 16 min.

Table 2 | PCR primers for detection of the conserved region between pAQU1 and pAQU2 (pAQU backbone).

Region number	Primer name	Primer sequence (5′ -> 3′)	Product size (bp)	Relevant genes contained in the amplicon
1	AB571865-1	ATGACAACAACACTCCACAATGAGG	12,606	*rep*A
	170-16f	GCCGCAACGATGACAAACTTAACGA		
2	AB571865-30966	ACAGTTCACCTAAACATTGATG	3,785	*parA*, *parB*
	AB571865-34750	ACATACAACCAGAAACAGAACC		
3	170-11r	GCTTTGGCGTGTTCTCGGTTATGAT	9,017	*traI*, *traD*
	170-11f	CGCATGGAACACTACACCGCTATAT		
4	170-10r	GGTTGCTTCACTGCTTCCTATCATG	9,128	*S043*, (*traJ*), *traL*, *traE*, *traK*, *traB*, *traV*, *traA1*, *traA2*
	170-10f	CAGCTCGATCCGTTCTTCACCATTA		
5	170-9r	CGCACATTATCTCCAAGCACCGTTT	9,003	*DsbC*, *traC*
	170-9f	CAGCAACCCAAAGAAGAGCAAGTAC		
6	AB571865-68599	AAGGAAGTACGGTGGAAG	6,975	*trhF*, *traW*, *traU*, *traN*
	AB571865-75573	CAGGATCGTCAAAGTGAC		
7	170-5r	GCCGCAATGGATCGTTATCGTTTCT	9,143	
	170-5f	TGCTCTGCCGACAACATTGGTTTAC		
8	170-4r	ATTGGGTTGAAGGTTGGAAGGAGTG	9,141	
	170-4f	CGGCATACTCAGGTTCTCCTTCTTT		
9	170-3r	GGCTGGTTGTGGCGATATTGATGAT	9,242	
	170-3f	GCCTTCTGCCAAACCTAATACTCCT		
10	170-21r	GCCAAGTTCGGTAAGAGTGAGAGTT	9,003	*ter*
	170-21f	AGCGTTCTCAATGGTTGGGTTATCC		
11	170-20r	CCTCAAGATCCTTTCCCTATGGTTC	9,142	*traF*, *traH*, *traG*
	170-20f	CGACTGCCGTAATTTCTTGCTCTAG		
12	170-19r	CGGAAAGATACGAAGACGGATTACC	9,390	
	170-19f	CTCCAACTCCAATGACCTTCAAGAC		

For determination of the phylogenetic position of the strains were identified by the nucleotide sequences of the bacterial 16S rRNA gene which were amplified using primers 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1510R (5′-GGCTACCTTGTTACGA-3′). PCR reaction was performed as previously described (Nonaka et al., 2007).

SEQUENCING OF THE PCR PRODUCTS

When required, the nucleotide sequences of the PCR products were determined using the dideoxy chain termination method with a Big Dye terminator V 3.1 cycle sequencing kit (Life Technologies Corporation) and an appropriate primer according to the manufacture's protocol following to the purification of the PCR products using the QIAquick PCR purification kit (QIAGEN GmbH, Hilden Germany). For identification of the sequences of 16S rRNA genes, primers 9F, 339F (5′ CTCCTACGGGAGGCAG CAG 3′), 785F, (5′-GGATTAGATACCCTGGTAGTC-3′), 1099F (5′-GCAACGAGCGCAACCC-3′), 536R (5′-GTATTACCGCGGC TGCTG-3′), 802R (5′-TACCAGGGTATCTAATCC-3′), 1242R (5′-CCATTGTAGCACGTGT-3′) and 1510R were used.

FILTER MATING

The donor marine bacteria and recipient (*E. coli* W3110) were cultured to the mid-log phase (OD600 = 0.5–0.8) and mixed with each other (500 μl each) in triplicates. The mixture was then collected onto a sterile 25 mm in a diameter, 0.2 μm-pore size nitrocellulose membrane filter (Merck millipore, Billerica, MA) by using a KG-25 filter holder (Advantec, Tokyo, Japan) and a vacuum pump at 20 kPa. The filter with the collected cells were rinsed by 10 ml of BHI medium with 2% NaCl or LB and placed on the Marine Broth 2216 plate (Becton, Dickinson and Company) for incubation at 25°C for 20 h. Each filter was then transferred into 500 μl of phosphate-buffered saline (PBS) and agitated vigorously with a Vortex mixer to detach the bacteria. The suspension was serially diluted by 10-fold in PBS and plated onto the media. To select for the transconjugants, LB agar plates with 20 μg/ml of TC were used and incubated for 20 h at the temperature (42°C) which inhibits growth of the donors. For counting the total number of donor cells and the transconjugants, the cell suspension was spread on a BHI agar plate with 0.2% NaCl and 10 μg/ml of TC and incubated at 25°C. When the transconjugants were used as donors, *E. coli* W3110Rif^r was used as a recipient and 2nd transconjugants were counted on LB agar plates with 20 μg/ml of tetracycline and 100 μg/ml of rifampicin after incubation at 37°C for 20 h. For counting the total number of the donor cells and the transconjugants, LB plates with 20 μg/ml tetracycline were used and incubated at 37°C for 20 h. The number of the donor cells was obtained by subtracting the number of the transconjugants from the total number of the donor cells and the transconjugants. Transfer rate was calculated as a ratio of the number of transconjugants per donor cell.

PULSED-FIELD GEL ELECTROPHORESIS (PFGE) AND SOUTHERN HYBRIDIZATION

To determine whether *tet*(M) is carried by the plasmid or integrated in the chromosome, PFGE and Southern hybridization

were performed. First, a DNA-containing agar plug of each sample was prepared by the previously reported method (Kijima-Tanaka et al., 2007) with the following modifications. Culture fluid containing about 10^8 cells was put into a 15-ml centrifuge tube and centrifuged at 9,000 rpm for 10 min. The cell pellet was re-suspended in 120 μl of cell suspension buffer [100 mM Tris-HCl, 100 mM EDTA (pH 8.0)] and mixed with 120 μl of melted 2% InCert® Agarose (Lonza, Basel, Switzerland) and 12 μl of lysozyme (20 mg/ml; Sigma-Aldrich) dissolved in TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)]. The mixture was quickly dispensed into the plug mold (Bio-Rad, Hercules, CA). After solidification, the plug was transferred into 500 μl of TE buffer containing 1% SDS and 12 μl of lysozyme (20 mg/ml), and incubated at 37°C for 1 h. Then, the liquid portion in each tube was replaced with 500 μl of lysis buffer [100 mM Tris-HCl, 100 mM EDTA (pH 8.0), 1% N-lauroylsarcosine] and 50 μl of proteinase K (20 mg/ml; TAKARA BIO INC.), and the plug was incubated at 50°C for overnight. On the next day, the plug was washed three times with 500 μl of TE buffer at room temperature for 15 min, pretreated with 1 ml of S1 nuclease buffer [30 mM sodium acetate (pH 4.6), 1 mM zinc acetate, 5%(v/v) glycerol] at room temperature for 30 min, and treated with 10 U of S1 nuclease (Life Technologies Corporation, Carlsbad, CA) in 200 μl of the S1 nuclease buffer at 37°C for 15 min. The plugs was then washed twice with 1 ml of ice-cold high concentration EDTA-Tris [10 mM Tris-HCl, 100 mM EDTA (pH 8.0)]. The plug was cut into half and one of the pieces was embedded in a 1% Seakem® Gold Agarose (Lonza) gel and PFGE was performed on a CHFE MAPPER™ (Bio-Rad) under the conditions described previously (Nonaka et al., 2012). The DNA size standard lambda ladder (Bio-Rad) was used as a size marker. The DNA in the gel was stained with SYBR Gold (Life Technologies Corporation) at the concentration recommended by the manufacturer and visualized under UV light. Subsequent hybridization was performed according to the standard method (Sambrook and Russell, 2001). PCR-amplified DNA fragments of *tet*(M), *traI* in pAQU1 (*traI*-pAQU1), *traI* in ICE*Vch*Mex (*traI*-ICE*Vch*Mex) and *rep* in pAQU1 (*rep*-pAQU1) were labeled with digoxigenin by using the primers shown in **Table 1** and a PCR DIG probe synthesis kit (Roche Diagnostics, Mannheim, Germany). Hybridizations were performed at 41°C for overnight.

SEQUENCING AND ANNOTATION

The entire sequence of the total DNA of TJ090W1 was determined by the pyrosequencing method with the Titanium chemistry and the Genome Sequence FLX system (Roche Diagnostics), and a set of data consisting of 225,251 shotgun and 271,612 paired-end (average insert size: 7 kb) sequences with an average length of 684 and 398 nucleotides were generated, respectively. The volume of the data was estimated to cover 60 times of the entire DNA sequence. The plasmid-derived sequences were obtained by subtracting the genomic sequence of *E. coli* W3110 from the entire set of data and then assembled *de novo* by using the GS assembler (Newbler 2.8) (Roche Diagnostics), generating 1 scaffold and 19 sets of assembled contigs (over 500 bp), of which five selected contigs were used for assembling the whole structure of the plasmid molecule according to the previously described method (Nonaka et al., 2012). Preliminary identification of protein coding regions in the plasmid was carried out by using MetaGeneAnnotator (Noguchi et al., 2008). Then, the results were subjected to BLAST searches and compared with the sequences listed in the NCBI non-redundant protein database for their length, identity and coverage, and final annotation was manually performed by using In Silico Molecular Cloning Genomics Edition® (In Silico Biology, Inc., Yokohama, Japan) with the threshold *E*-value of 10^{-5}.

NUCLEOTIDE SEQUENCE ACCESSION NUMBER

The entire sequence of pAQU2 was deposited in GeneBank/ DDBJ/ EMBL databases under the accession no. AB856327.

RESULTS

SCREENING BY PCR DETECTION AND SEQUENCING OF *traI* GENE FOR THE MARINE BACTERIAL STRAINS WHICH HARBOR *tet*(M)-BEARING pAQU1-LIKE PLASMID OR ICE*Vch*MEX-LIKE MGE

For screening for the bacterial strains carrying the pAQU1-like plasmids, we designed degenerate primers, MOBH1_traI062-1F and MOBH1_traI062-2R, based on the *traI* nucleotide sequences of pAQU1 (GenBank accession number AB571865), five of IncA/C plasmids (Welch et al., 2007; Fricke et al., 2009) and seven of SXT/R391 ICEs (Wozniak et al., 2009) which belong to the MOB_{H12} clade of the MOB_H family (**Table 1**). When 83 *tet*(M)-positive strains of marine bacteria from the aquaculture site were examined, amplified *traI* DNA of 645 base pairs (bps) was detected in strains 04Ya001, 04Ya016, and 04Ya090 (**Table 3**) that had been known to carry transferable *tet*(M) gene (Neela et al., 2009). The 645-bp *traI* PCR product was also detected in strains 04Ya007 and 04Ya265 whose ability to transfer *tet*(M) had been unclear (**Table 3**). Although strain 04Ya108 had been shown to conjugatively transfer *tet*(M) to *E. coli* (Neela et al., 2009), *traI* was not detected in this strain (**Table 3**). *traI* was not detected in 77 other strains, either; 66 strains whose *tet*(M) was shown not to be transferable (Neela et al., 2009) and 11 strains that had not been examined before. Therefore, five of 83 strains examined appeared to carry MGE related to pAQU1 or IncA/C plasmid or SXT/R391 ICE. Then, nucleotide sequences of the PCR-amplified 645-bp *traI* DNA of the plasmids in these five strains were determined. The results indicated that the *traI* sequence of four strains (04Ya001, 04Ya016, 04Ya090, and 04Ya265) were identical to that of pAQU1, whereas the sequence of one strain (04Ya007) was distinct from that of pAQU1, but similar to that of ICE*Vch*Mex (97% identity), which belongs to SXT/R391 ICEs found in *V. cholerae* isolated from sewage in Mexico (Burrus et al., 2006). The ICE*Vch*Mex-specific *int*, *traF* and *traN* genes were also detected by PCR in 04Ya007. Therefore, strains 04Ya001, 04Ya016, 04Ya090, and 04Ya265 were shown to have pAQU1 or closely related pAQU1-like plasmids, whereas 04Ya007 likely had another member of the MOB_{H12} clade, ICE*Vch*Mex-like MGE.

To examine whether *tet*(M) is also plasmid-borne in strains 04Ya001, 04Ya016, 04Ya090, and 04Ya265 carrying the pAQU1-like plasmids, 04Ya007 with ICE*Vch*Mex-like MGE and 04Ya108 without *traI*, PFGE followed by Southern hybridization was performed. Hybridization with the *tet*(M) probe generated extrachromosomal DNA bands ranging from 150 to 350-kbp in all

Table 3 | Strains harboring *traI* gene belonging to the $MOBH_{12}$ clade in the MOB_H family on the basis of a PCR screening.

Strain ID	Species	Origin	Isolation date	TC resistance	*tet*(M)	*traI**	References
04Ya001	*Vibrio splendidus*	Sediment	Apr 26/2004	Yes	+	+	Nonaka et al., 2007; Neela et al., 2009
04Ya007	*Shewanella fidelis*	Sediment	Apr 26/2004	Yes	+	+	This study
04Ya016	*Vibrio splendidus*	Sediment	Apr 26/2004	Yes	+	+	Nonaka et al., 2007; Neela et al., 2009
04Ya090	*Vibrio splendidus*	Sediment	May 21/2004	Yes	+	+	Nonaka et al., 2007; Neela et al., 2009
04Ya108	*Vibrio ponticus*	Sediment	May 21/2004	Yes	+	−	Nonaka et al., 2007; Neela et al., 2009
04Ya265	*Photobacterium aphoticum*	Sediment	Sep 24/2004	Yes	+	+	This study
04Ya311	*Photobacterium damselae* subsp. *damselae*	Seawater	Sep 24/2004	Yes	+	+	Nonaka et al., 2007, 2012; Neela et al., 2009
W3110	*E. coli*	Laboratory strain	–	No	−	−	Paigen, 1966
W3110Rif^r	*E. coli*	Laboratory strain	–	No	−	−	Nonaka et al., 2012

Using degenerate primers MOBH1_traI062-1F and MOBH1_traI062-2R in* ***Table 1**.*

of the strains examined (**Figure 1A**, lanes 1–5 and **Figure 2A**, lane 1). When the *traI*-pAQU1 or *rep*-pAQU1 probe was used, bands of the same sizes were detected for 04Ya001, 04Ya016, 04Ya090, and 04Ya265 (**Figures 1B,C**, lanes 1–3 and 5), but not 04Ya108 (**Figures 1B,C**, lane 4) compatible with the PCR results. While when a strain 04Ya007 was used, the *traI*-ICE*Vch*Mex probe detected a signal at the position corresponding to the chromosomal DNA (**Figure 2B**, lane 1). The results indicated that in four strains 04Ya001, 04Ya016, 04Ya090, and 04Ya265, *tet*(M) was most likely carried on the pAQU1-like plasmids, whereas in strain 04Ya108, *tet*(M) appeared to be in the plasmid of non-MOB_{H12} clade. In strain 04Ya007, *tet*(M) appeared to be plasmid-borne while ICE*Vch*Mex-like MGE was integrated in the chromosome.

CONJUGATIVE TRANSFER OF *tet*(M) GENE MEDIATED BY THE pAQU1-LIKE PLASMIDS

To confirm and examine the ability of the six strains to transfer TC resistance, filter mating experiments were performed by using *E. coli* as a recipient. As reported previously (Neela et al., 2009), strains 04Ya001, 04Ya016, and 04Ya090 were able to transfer TC resistance to *E. coli* (**Table 4**). Strains 04Ya007 and 04Ya265 were also shown to transfer TC resistance to *E. coli* with the frequencies of 1.37×10^{-4} and 1.08×10^{-4}, respectively, at the levels comparable to the positive control strain 04Ya311 (**Table 4**). Strain 04Ya108, which did not appear to carry MOB_{H12} MGE, was also able to transfer TC resistance, but with a much lower frequency (**Table 4**).

Then, a single clone of TC-resistant transconjugant was selected for each donor strain and analyzed by PCR with MOBH1_traI062-1F and MOBH1_traI062-2R primers. The *traI* gene was detected in five transconjugants whose donors were 04Ya001, 04Ya007, 04Ya016, 04Ya090, and 04Ya265, but not in the transconjugant whose donor was 04Ya108 (**Table 5**). Nucleotide sequence analysis of the PCR products revealed that the *traI* sequences derived from the transconjugants whose donors were 04Ya001, 04Ya016, 04Ya090, and 04Ya265 were identical to that of pAQU1. On the other hand, the *traI* sequences of transconjugant TJ007W1 was identical to that of its donor 04Ya007, with 97% identity to ICE*Vch*Mex.

To examine whether *tet*(M) genes in the transconjugants were also plasmid-borne, PFGE followed by Southern hybridization by using the *tet*(M) probe was performed. For transconjugants TJ001W1, TJ016W1, TJ090W1, and TJ265W1, 150 to 350-kbp long extrachromosomal DNA reactive with the *tet*(M) probe was detected similar to their corresponding donors (**Figure 1A**, lanes 7–9 and 11). The *traI*-pAQU probe or rep-pAQU1 probe generated signals at the same positions as *tet*(M) (**Figures 1B,C**, lanes 7–9 and 11). For transconjugant TJ108W1, *tet*(M) hybridization signal was detected at the position of chromosomal DNA (**Figure 1A**, lane 10) although its donor had plasmid-borne *tet*(M) (**Figure 1A**, lane 4). Although transconjugant TJ007W1 was TC-resistant, no *tet*(M) signal was detected (**Figure 2A**, lane 2), while the *traI*-ICE*Vch*Mex probe hybridized with the chromosomal DNA (**Figure 2B**, lane 2).

These results strongly suggested that the *tet*(M)-bearing pAQU1-like plasmids could be conjugatively transferred from marine bacteria to *E. coli*, conferring TC resistance. The mechanism by which *tet*(M) gene was transferred from 04Ya108 to TJ108W1 is unclear.

TRANSFER OF OTHER ARGs ASSOCIATED WITH CONJUGATIVE TRANSFER OF THE pAQU1-LIKE PLASMIDS

PCR analysis revealed that 04Ya001, 04Ya016, 04Ya090, and 04Ya265 had *tet*(B) in addition to *tet*(M) (**Table 5**). Strain 04Ya265 was shown by PCR to have other ARGs, such as *sul*2, bla_{CARB-9}-like, *mph*(A)-like and *mef*(A)-like (**Table 5**). Generally, all of the ARGs were transferred to the transconjugants when these strains, capable of conjugative transfer of the pAQU1-like plasmids, were used as donors (**Table 5**).

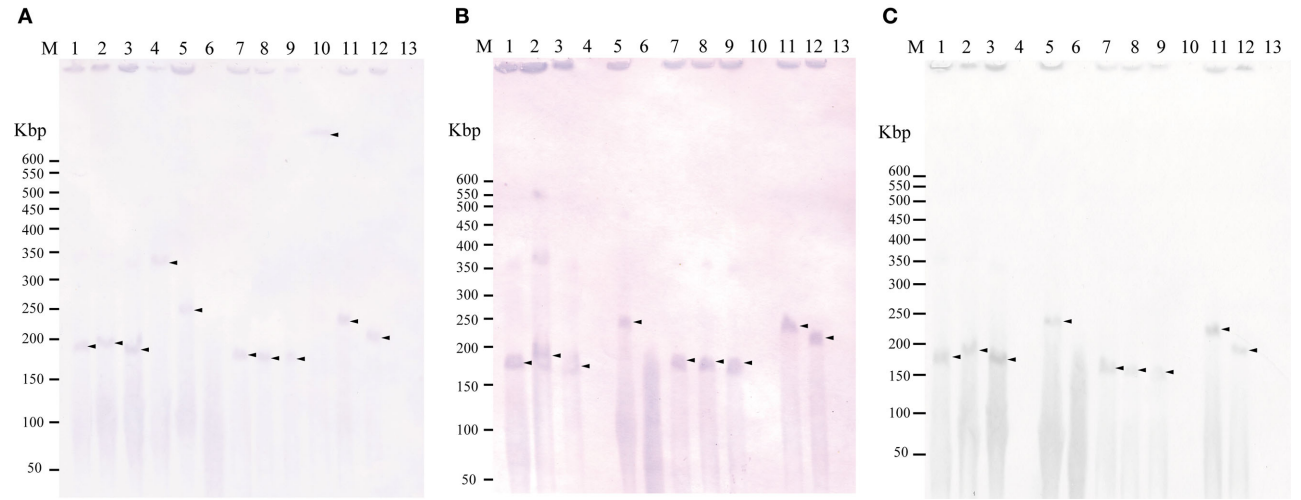


FIGURE 1 | Detection of plasmid(s) in the donors and transconjugants using Southern hybridization following to pulsed field gel electrophoresis with the *tet*(M) probe (A), *tral*-pAQU1 probe (B), and *rep*-pAQU1 probe (C). Lane: M, DNA size standard (lambda ladder); 1, 04Ya001; 2, 04Ya016; 3, 04Ya090; 4, 04Ya108; 5, 04Ya265; 6, 04Ya311; 7, TJ001W1; 8, TJ016W1; 9, TJ090W1; 10, TJ108W1; 11, TJ265W1; 12, TJ311W2 and 13, W3110. Approximately, 20 ng of DNA was loaded in each lane. Arrows indicate the positions of the bands.

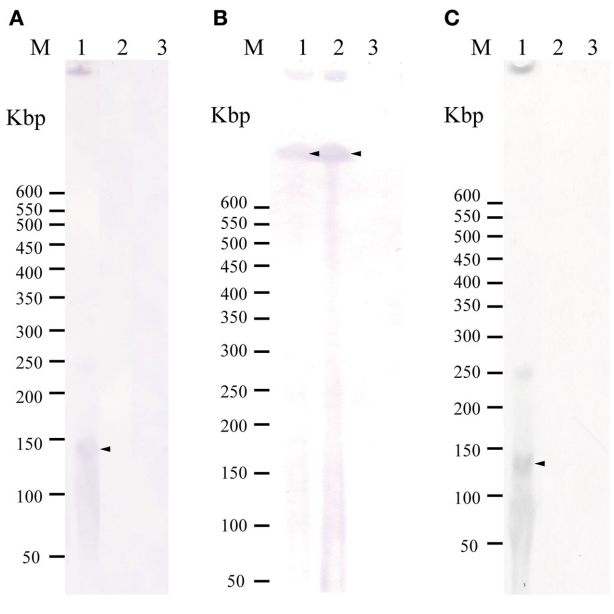


FIGURE 2 | Detection of a plasmid or ICE(s) in the donors and transconjugants using Southern hybridization following to pulsed field gel electrophoresis with the *tet*(M) probe (A), *tral*-ICE*Vch*Mex probe (B), and *rep*-pAQU1 probe. (C) Lane: M, DNA size standard (lambda ladder); 1, 04Ya007; 2, TJ007W1; 3, W3110. Approximately, 20 ng of DNA was loaded in each lane. Arrows indicate the positions of the bands.

Incidentally, both of 04Ya007 and 04Ya108 without the pAQU1-like plasmid were shown by PCR to have all of the ARGs mentioned above (**Table 5**). While 04Ya007, which harbored ICE*Vch*Mex-like MGE, transferred none of these ARGs, 04Ya108 with unclassified plasmid transferred all of them (**Table 5**).

Table 4 | Transfer frequencies of tetracycline resistance by filter mating methods.

	Strain	Transfer frequencies
Donors	04Ya001	$(1.64 \pm 0.56) \times 10^{-5}$
	04Ya007	$(1.37 \pm 0.18) \times 10^{-4}$
	04Ya016	$(1.90 \pm 0.73) \times 10^{-5}$
	04Ya090	$(7.71 \pm 3.84) \times 10^{-5}$
	04Ya108	$(1.78 \pm 0.16) \times 10^{-7}$
	04Ya265	$(1.08 \pm 0.18) \times 10^{-4}$
	04Ya311	$(4.11 \pm 0.84) \times 10^{-4}$
Recipient	*E. coli* W3110	
Donors	TJ001W1	$(3.90 \pm 1.21) \times 10^{-5}$
	TJ007W1	$(1.01 \pm 0.08) \times 10^{-3}$
	TJ016W1	$<(2.12 \pm 0.48) \times 10^{-10}$
	TJ090W1	$(1.00 \pm 0.32) \times 10^{-4}$
	TJ108W1	$<(4.88 \pm 0.28) \times 10^{-10}$
	TJ265W1	$(1.08 \pm 1.03) \times 10^{-4}$
	TJ311W2	$(6.60 \pm 3.01) \times 10^{-5}$
Recipient	*E. coli* W3110Rif^r	

SELF-TRANSFERABILITY OF THE pAQU1-LIKE PLASMIDS

To investigate whether the pAQU1-like plasmids are self-transferable, additional filter mating experiments were performed by using the transconjugants as donors and *E. coli* W3110Rif^r as a donor. TJ001W1, TJ090W1 and TJ265W1, which had the *tet*(M)-bearing pAQU1-like plasmid, transferred TC resistance at the frequencies (3.90×10^{-5} to 1.08×10^{-4}) comparable to that of the positive control TJ311W2 (**Table 4**). Although TJ016W1 also had a *tet*(M)-bearing pAQU1-like plasmid, it was unable to transfer TC resistance at a detectable level.

Table 5 | Detection of *tral* and other antimicrobial resistance genes in donors and transconjugants by PCR.

	Relaxase*		Antimicrobial resistance genes						
Strain	***tral* (pAQU1)**	***tral* (ICE*Vch*Mex)**	***tet*(B)**	***tet*(M)**	***bla*$_{CARB-9}$-like**	***mph*(A)-like**	***mef*(A)-like**	***sul*2**	***flo*R**
04Ya001	+	−	+	+	−	−	−	−	−
TJ001	+	−	+	+	−	−	−	−	ND
04Ya007	−	+	+	+	+	+	+	+	−
TJ007	−	+	−	−	−	−	−	−	ND
04Ya016	+	−	+	+	−	−	−	−	−
TJ016	+	−	+	+	−	−	−	−	ND
04Ya090	+	−	+	+	−	−	−	−	−
TJ090	+	−	+	+	−	−	−	−	ND
04Ya108	−	−	+	+	+	+	+	+	−
TJ108	−	−	+	+	+	+	+	+	ND
04Ya265	+	−	+	+	+	+	+	+	−
TJ265	+	−	+	+	+	+	+	+	ND
04Ya311	+	−	+	+	+	+	+	+	+
TJW2	+	−	+	+	+	+	+	+	+
W3110	−	−	−		−	−	−	−	−

*By the identification of the nucleotide sequence of amplicons obtained by PCR using MOBH1_tral062-1F and MOBH1_tral062-2R.

We also tested transconjugants TJ007W1 and TJ108W1 which did not harbor the pAQU1-like plasmid. Ten times higher frequency (1.01×10^{-3}) of TC resistance transfer was observed for TJ007W1 which had no detectable *tet*(M), whereas the transfer frequency of TJ108W1 was below the detection limit (**Table 4**).

CHARACTERIZATION OF A NOVEL pAQU1-LIKE PLASMID, pAQU2

As shown above, strains 04Ya001, 04Ya016, 04Ya090, and 04Ya265 and their transconjugants, TJ001W1, TJ016W1, TJ090W1, and TJ265W1, had a pAQU1-like plasmid. Among these, we chose the plasmid in TJ090W1, which was shown to be self-transferable, and determined its entire nucleotide sequence. The novel plasmid, which we designated as pAQU2, was 160,406-bp long and contained 195 predicted coding sequences (CDSs). The CDSs were asymmetrically distributed; i.e., 163 CDSs were oriented clockwise, whereas only 32 CDSs were oriented counterclockwise (**Figure 3**).

Comparison of the pAQU1 and pAQU2 sequences indicated that the regions containing the genes required for basic plasmid functions, such as replication, partition and conjugative transfer, were highly conserved between the two plasmids. Thus, it was suggested that these conserved regions, which totally sum up to ∼100 kbps, might constitute the backbone structure common to the pAQU1-like plasmids. In our previous study (Nonaka et al., 2012), pAQU1 was shown to have a high G+C content region with a cluster of ARGs and transposases. In pAQU2, a region with a cluster of ARGs and transposases were also found at the corresponding position, but with a low G+C content (**Figure 3**).

pAQU GROUP PLASMIDS CHARACTERIZED BY A COMMON BACKBONE STRUCTURE

To test whether the backbone structure shared by pAQU1 and pAQU2 also exists in the other pAQU1-like plasmids, we characterized the donor strains 04Ya001, 04Ya016, 04Ya090, and 04Ya265 and their transconjugants by PCR with 12 sets of primers covering the entire backbone structure. The results showed that the backbone structure was maintained with no apparent deletion in the pAQU1-like plasmids harbored in all of the donors and the transconjugants examined (**Table 6**). Additional PCR analysis with the primers specific to some of the genes (*rep*, *traA2*, *traB*, *traC*, *traF*, *traN* and *traL*) in the backbone structure revealed that these genes were also retained in all of the donors and the transconjugants examined (**Table 6**). These results indicated that the backbone structure common to pAQU1 and pAQU2 existed in all of the other pAQU1-like plasmids examined. Therefore, there may be a novel group (pAQU group) of plasmids characterized by this backbone structure.

Interestingly, strain 04Ya007 in which ICE*Vch*Mex-like *traI*, but not pAQU1-like *traI*, was shown by PCR to harbor part of the pAQU backbone structure and some backbone-specific genes (**Table 6**). In addition, Southern hybridization with the *rep*-pAQU1 probe generated a band corresponding to the *tet*(M)-bearing plasmid in this strain (**Figure 2A**, lane 1, **Figure 2C**, lane 1). Therefore, it is possible that 04Ya007 carry plasmid similar to that of the pAQU group although the plasmid did not appear to be transferred to TJ007W1 (**Figures 2A,C** lane 2 and **Table 6**). Neither the pAQU backbone structure nor the backbone-specific genes were detected in 04Ya108 or its transconjugant (**Table 6**).

DISCUSSION

Recently, we discovered a novel *tet*(M)-bearing plasmid pAQU1 (204,052 bp) whose TraI primary structure was similar to that of the MOB$_{H12}$ MGE of the MOB$_H$ family and proposed that pAQU1 might play a role in dissemination of *tet*(M) and other ARGs in the marine environment (Nonaka et al., 2012). In this study, in order to investigate this possibility, we first designed degenerate PCR primers for detecting *traI* gene of the MOB$_{H12}$ MGEs including pAQU1. With these *traI* primers, we could

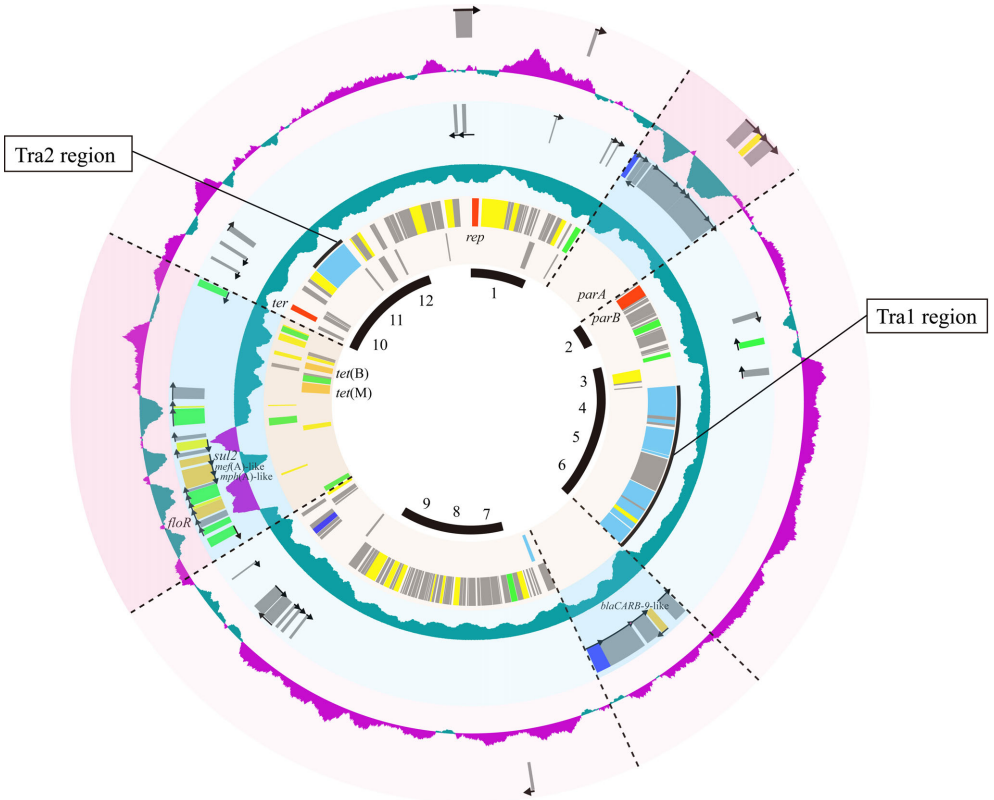


FIGURE 3 | Circular representation of pAQU1 backbone and specific genes for pAQU2 and pAQU1, respectively. Circles from inside to outside: 1, Putative pAQU back bone regions, 2 and 3, shared CDSs between pAQU1 and pAQU2; 5 and 6, CDSs specific for pAQU1; and 8 and 9, CDSs specific for pAQU2. Each of CDSs inside and outside of the circle coded counter-clockwise and clockwise, respectively. The 4th and 7th circle indicates GC content of pAQU1 and pAQU2 where purple shows upper GC value above the center line and green shows lower GC value below the center line. Putative functions of the products of the CDSs are indicated in color: red, replication, partition and termination; light blue, conjugative transfer; blue, integration; green, transposition; orange, antibiotic resistance; yellow, other functions and gray, unknown function.

rapidly identify the bacterial strains which have pAQU1-like plasmids or IncA/C plasmids or SXT/R391 ICEs. Compared with the filter mating usually used for identifying the strains with transferable MGEs, the *traI*-PCR developed in this study may serve as a specific screening for the MOB_{H12} MGE-harboring bacterial strains that are frequently found in the marine environment (Welch et al., 2007; McIntosh et al., 2008; Fricke et al., 2009; Nonaka et al., 2012). The filter mating requires an appropriate recipient strain with known markers to distinguish from donor and the experiment is time consuming. On the other hand, the primers for *traI*- PCR could rapidly identify the sequence of pAQU1-like plasmids or Inc A/C plasmids or SXT/R391 ICEs.

With the *traI*-PCR method, we examined 83 strains of *tet*(M)-positive marine bacteria and found 4 *Vibrio* spp. strains (04Ya001, 04Ya016, 04Ya090, and 04Ya265) that harbored the pAQU1-like plasmids. PFGE and following Southern hybridization showed that the plasmids were 150–350 kb long and carried *tet*(M). It was also shown that the plasmids could conjugatively be transferred to *E. coli* at the frequencies of 10^{-5} to 10^{-4}, generating TC-resistant transconjugants. From three (TJ001W1, TJ090W1, and TJ265W1) of the four transconjugants, the pAQU1-like plasmids were further transferred to another strain of *E. coli* at comparable frequencies, suggesting that the plasmids were self-transferable.

The entire nucleotide sequence of a *tet*(M)-bearing pAQU1-like plasmid, pAQU2, originally possessed by 04Ya090 isolated from marine sediment was determined and compared with that of pAQU1. It was shown that pAQU2 shared conserved regions, which contained the genes required for plasmid replication, partition and transfer, with pAQU1. These regions, which totally sum up to ∼100 kbp, were defined as the pAQU backbone structure. The other pAQU1-like plasmids harbored in 04Ya001, 04Ya016, and 04Ya265 also shared the pAQU backbone, suggesting that these plasmids together with pAQU1 and pAQU2 could be categorized as the "pAQU group."

In addition to *tet*(M), multiple ARGs [*tet*(B), *sul2*, *flo*R and genes similar to bla_{CARB-9}, *mph*(A) and *mef*(A)] were accumulated upstream of the Tra2 region in pAQU1 where multiple transposase genes were localized, as previously described (Nonaka et al., 2012). Multiple, but fewer, ARGs were also found in pAQU2 in the corresponding region. Therefore, it was suggested that the ARGs may have been introduced through transposon-mediated DNA insertion events into the backbone structure of a prototypic plasmid. Insertion of ARGs upstream of the Tra2 region

Table 6 | Detection of the conserved region (pAQU backbone) and *tra* genes specific for pAQU1 and ICE*Vch*Mex in donors and transconjugants by PCR.

	Region number												Representative genes required for conjugative transfer									
													pAQU1							ICE*Vch*Mex		
Strain	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	***rep***	***traA2***	***traB***	***traC***	***traF***	***traN***	***traL***	***int***	***traN***	***traF***
04Ya001	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	−	−	ND
TJ001	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	−	−	ND
04Ya007	+	+	*	−	+	−	−	−	+	+	+	+	+	−	−	−	+	−	−	+	+	+
TJ007	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+
04Ya016	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	ND
TJ016	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	ND
04Ya090	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	ND
TJ090	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	ND
04Ya108	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	ND
TJ108	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
04Ya265	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	ND
TJ265	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	ND
04Ya311	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
TJ311	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
W3110	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

**Amplicon sizes were smaller than expected sizes.*

has also been reported for IncA/C plasmids (Welch et al., 2007; Fricke et al., 2009; Fernandez-Alarcon et al., 2011). Also, arrangement of *tra* genes in the pAQU backbone was quite similar to that in IncA/C plasmids and SXT/R391 ICEs, suggesting that these MGEs shared synteny. These observations are compatible with the previous study showing a close evolutionary relationship among pAQU1, IncA/C and SXT/R391 ICEs (Nonaka et al., 2012). It is possible that these MGEs had a common ancestor and evolved through acquisition of different replication systems.

Among 83 bacterial strains examined in this study, six strains were able to conjugatively confer TC resistance upon *E. coli*, of which four strains were shown to harbor the pAQU group plasmid. Therefore, it is possible that the pAQU group plasmid played a significant role in dissemination of *tet*(M) in the aquaculture site. In addition to *tet*(M), most of the ARGs carried on pAQU1 were also found in the four strains, and all of them were transmitted to *E. coli* in association with transfer of the plasmid. Therefore, it is suggested that all of the pAQU group plasmids found in this study carried not only *tet*(M) but also other ARGs. The bacterial strains found to harbor the pAQU group plasmids were isolated from the same aquaculture site, but in different times, suggesting that the pAQU group plasmids were distributed and maintained in the aquaculture environment contributing to dissemination of *tet*(M) and other ARGs among marine bacteria.

Of six bacterial strains that were able to confer TC resistance on *E. coli*, two strains did not harbor pAQU group plasmid. One of them, marine sediment strain 04Ya007 which belonged to *Shewanella* sp. and was able to transfer TC resistance at a high frequency, was shown by nucleotide sequencing of the PCR-amplified *traI* gene and Southern hybridization to have chromosomal ICE*Vch*Mex-related MGE instead. In the transconjugant obtained by filter mating by using 04Ya007 as a donor, chromosomal *traI*, as well as three other essential genes of ICE*Vch*Mex, was detected, indicating the transfer of chromosomal ICE*Vch*Mex-related MGE of 04Ya007 to the chromosome of the recipient *E. coli*. Strain 04Ya007 also had partial sequence characteristic to the pAQU backbone and plasmid-borne ARGs including *tet*(M). However, none of these pAQU group-related sequences were detected in the transconjugant. While the original ICE*Vch*Mex was reported to have no ARGs (Burrus et al., 2006), other SXT/R391 ICEs in the same group were shown to have *tet*A and *tet*R (Wozniak et al., 2009). Therefore, it is possible that the TC resistance of the transconjugant was due to an ARG(s) other than *tet*(M) and *tet*(B) that was transferred in an ICE*Vch*Mex-related MGE-dependent manner, while the *tet*(M)-bearing plasmid with the partial pAQU backbone structure had suffered from the mutation which caused loss of the transferability. Further characterization of ICE*Vch*Mex-like MGE and the plasmid in this strain is necessary to test this possibility.

The remaining strain, 04Ya108 isolated from marine sediment, which conferred TC resistance on *E. coli* at a very low frequency, appeared to have *tet*(M)-bearing plasmid of non-pAQU group, and the transconjugant was shown to have chromosome-encoded *tet*(M). Although chromosomal integration of conjugatively transferred plasmid has been reported (Jaoua et al., 1990; Ka and Tiedje, 1994; Ghosh et al., 2005), additional studies are required to elucidate the mechanism by which plasmid-born *tet*(M) of 04Ya108 integrated in the chromosome of its transconjugant.

In conclusion, PCR targeting *traI* identified four marine bacterial strains that harbor the pAQU group plasmids closely related to previously described pAQU1 (Nonaka et al., 2012). The four

strains were shown by filter mating to be able to serve as donors for TC resistance. The results raise a possibility that the pAQU group plasmids might mediate conjugative transfer of *tet*(M) and other ARGs in the aquaculture site and play a major role in dissemination of drug resistance among marine bacteria.

ACKNOWLEDGMENTS

This work was supported by JSPS KAKENHI Grant Numbers 23780202, Fundamental Research Developing Association for Shipbuilding and Offshore (REDAS), The Japan Prize Foundation and The Foundation for Earth Environment. We thank Takashi Namatame, Misako Ishiguchi, Yuki Shinozaki, Satomi Kato, and Kayoko Kobayashi (Dokkyo Medical University, School of Medicine) for technical and secretarial assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00152/abstract

REFERENCES

Aminov, R. I., Garrigues-Jeanjean, N., and Mackie, R. I. (2001). Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *Appl. Environ. Microbiol.* 67, 22–32. doi: 10.1128/aem.67.1.22-32.2001

Bertrand, S., Huys, G., Yde, M., D'haene, K., Tardy, F., Vrints, M., et al. (2005). Detection and characterization of *tet*(M) in tetracycline-resistant *Listeria* strains from human and food-processing origins in Belgium and France. *J. Med. Microbiol.* 54, 1151–1156. doi: 10.1099/jmm.0.46142-0

Burrus, V., Quezada-Calvillo, R., Marrero, J., and Waldor, M. K. (2006). SXT-related integrating conjugative element in New World *Vibrio cholerae*. *Appl. Environ. Microbiol.* 72, 3054–3057. doi: 10.1128/aem.72.4.3054-3057.2006

Cabello, F. C., Godfrey, H. P., Tomova, A., Ivanova, L., Dolz, H., Millanao, A., et al. (2013). Antimicrobial use in aquaculture re-examined: its relevance to antimicrobial resistance and to animal and human health. *Environ. Microbiol.* 15, 1917–1942. doi: 10.1111/1462-2920.12134

Chee-Sanford, J. C., Aminov, R. I., Krapac, I. J., Garrigues-Jeanjean, N., and Mackie, R. I. (2001). Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Appl. Environ. Microbiol.* 67, 1494–1502. doi: 10.1128/aem.67.4.1494-1502.2001

Chopra, I., and Roberts, M. (2001). Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.* 65, 232–260. doi: 10.1128/mmbr.65.2.232-260.2001

Di Cesare, A., Luna, G. M., Vignaroli, C., Pasquaroli, S., Tota, S., Paroncini, P., et al. (2013). Aquaculture can promote the presence and spread of antibiotic-resistant *Enterococci* in marine sediments. *PLoS ONE* 8:e62838. doi: 10.1371/journal.pone.0062838

Di Cesare, A., Vignaroli, C., Luna, G. M., Pasquaroli, S., and Biavasco, F. (2012). Antibiotic-resistant enterococci in seawater and sediments from a coastal fish farm. *Microb. Drug Resist.* 18, 502–509. doi: 10.1089/mdr.2011.0204

Fernandez-Alarcon, C., Singer, R. S., and Johnson, T. J. (2011). Comparative genomics of multidrug resistance-encoding IncA/C plasmids from commensal and pathogenic *Escherichia coli* from multiple animal sources. *PLoS ONE* 6:e23415. doi: 10.1371/journal.pone.0023415

Fricke, W. F., Welch, T. J., Mcdermott, P. F., Mammel, M. K., Leclerc, J. E., White, D. G., et al. (2009). Comparative genomics of the IncA/C multidrug resistance plasmid family. *J. Bacteriol.* 191, 4750–4757. doi: 10.1128/jb.00189-09

Garcillan-Barcia, M. P., Francia, M. V., and De La Cruz, F. (2009). The diversity of conjugative relaxases and its application in plasmid classification. *FEMS Microbiol. Rev.* 33, 657–687. doi: 10.1111/j.1574-6976.2009.00168.x

Gevers, D., Danielsen, M., Huys, G., and Swings, J. (2003). Molecular characterization of *tet*(M) genes in *Lactobacillus* isolates from different types of fermented dry sausage. *Appl. Environ. Microbiol.* 69, 1270–1275. doi: 10.1128/AEM.69.2.1270-1275.2003

Ghosh, S., Mahapatra, N. R., Nandi, S., and Banerjee, P. C. (2005). Integration of metal-resistant determinants from the plasmid of an *Acidocella* strain into the chromosome of *Escherichia coli* DH5α. *Curr. Microbiol.* 50, 28–32. doi: 10.1007/s00284-004-4370-z

Heuer, H., and Smalla, K. (2007). Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. *Environ. Microbiol.* 9, 657–666. doi: 10.1111/j.1462-2920.2006.01185.x

Jaoua, S., Letouvet-Pawlak, B., Monnier, C., and Guespin-Michel, J. F. (1990). Mechanism of integration of the broad-host-range plasmid RP4 into the chromosome of *Myxococcus xanthus*. *Plasmid* 23, 183–193. doi: 10.1016/0147-619X(90)90050-M

Jones, C. H., Tuckman, M., Murphy, E., and Bradford, P. A. (2006). Identification and sequence of a *tet*(M) tetracycline resistance determinant homologue in clinical isolates of *Escherichia coli*. *J. Bacteriol.* 188, 7151–7164. doi: 10.1128/jb.00705-06

Ka, J. O., and Tiedje, J. M. (1994). Integration and excision of a 2,4-dichlorophenoxyacetic acid-degradative plasmid in *Alcaligenes paradoxus* and evidence of its natural intergeneric transfer. *J. Bacteriol.* 176, 5284–5289.

Kijima-Tanaka, M., Kawanishi, M., Fukuda, Y., Suzuki, S., and Yagyu, K. (2007). Molecular diversity of *Photobacterium damselae* ssp. *piscicida* from cultured amberjacks (Seriola spp.) in Japan by pulsed-field gel electrophoresis and plasmid profiles. *J. Appl. Microbiol.* 103, 381–389. doi: 10.1111/j.1365-2672.2006.03257.x

Kim, S. R., Nonaka, L., and Suzuki, S. (2004). Occurrence of tetracycline resistance genes *tet*(M) and *tet*(S) in bacteria from marine aquaculture sites. *FEMS Microbiol. Lett.* 237, 147–156. doi: 10.1016/j.femsle.2004.06.026

Kim, Y. H., Jun, L. J., Park, S. H., Yoon, S. H., Chung, J. K., Kim, J. C., et al. (2007). Prevalence of *tet*(B) and *tet*(M) genes among tetracycline-resistant *Vibrio* spp. in the aquatic environments of Korea. *Dis. Aquat. Organ.* 75, 209–216. doi: 10.3354/dao075209

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., et al. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* 23, 2947–2948. doi: 10.1093/bioinformatics/btm404

McIntosh, D., Cunningham, M., Ji, B., Fekete, F. A., Parry, E. M., Clark, S. E., et al. (2008). Transferable, multiple antibiotic and mercury resistance in Atlantic Canadian isolates of *Aeromonas salmonicida* subsp. *salmonicida* is associated with carriage of an IncA/C plasmid similar to the *Salmonella enterica* plasmid pSN254. *J. Antimicrob. Chemother.* 61, 1221–1228. doi: 10.1093/jac/dkn123

Ministry of Agriculture, Forestry and Fisheries, Japan. (2011). *Protocols for the Use of Antimicrobial Agents in Aquaculture (version 24)* (in Japanese).

Neela, A. F., Nonaka, L., Rahman, M. H., and Suzuki, S. (2009). Transfer of the chromosomally encoded tetracycline resistance gene *tet*(M) from marine bacteria to *Escherichia coli* and *Enterococcus faecalis*. *World J. Microbiol. Biotechnol.* 25, 1095–1101. doi: 10.1007/s11274-009-0004-8

Nikolakopoulou, T. L., Giannoutsou, E. P., Karabatsou, A. A., and Karagouni, A. D. (2008). Prevalence of tetracycline resistance genes in Greek seawater habitats. *J. Microbiol.* 46, 633–640. doi: 10.1007/s12275-008-0080-8

Noguchi, H., Taniguchi, T., and Itoh, T. (2008). MetaGeneAnnotator: detecting species-specific patterns of ribosomal binding site for precise gene prediction in anonymous prokaryotic and phage genomes. *DNA Res.* 15, 387–396. doi: 10.1093/dnares/dsn027

Nonaka, L., Ikeno, K., and Suzuki, S. (2007). Distribution of tetracycline resistance gene, *tet*(M), in Gram-positive and Gram-negative bacteria isolated from sediment and seawater at a coastal aquaculture site in Japan. *Microbes Environ.* 22, 355–364. doi: 10.1264/jsme2.22.355

Nonaka, L., Maruyama, F., Miyamoto, M., Miyakoshi, M., Kurokawa, K., and Masuda, M. (2012). Novel conjugative transferable multiple drug resistance plasmid pAQU1 from *Photobacterium damselae* subsp. *damselae* isolated from marine aquaculture environment. *Microbes Environ.* 27, 263–272. doi: 10.1264/jsme2.ME11338

Paigen, K. (1966) Phenomenon of transient repression in *Escherichia coli*. *J. Bacteriol.* 91, 1201–1209.

Roberts, M. C. (2005). Update on acquired tetracycline resistance genes. *FEMS Microbiol. Lett.* 245, 195–203. doi: 10.1016/j.femsle.2005.02.034

Sambrook, J., and Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual, 3rd Edn*. New York, NY: Cold Spring Harbor Laboratory Press.

Seyfried, E. E., Newton, R. J., Rubert, K. F. T., Pedersen, J. A., and Mcmahon, K. D. (2010). Occurrence of tetracycline resistance genes in aquaculture facilities with varying use of oxytetracycline. *Microb. Ecol.* 59, 799–807. doi: 10.1007/s00248-009-9624-7

Smillie, C., Garcillan-Barcia, M. P., Francia, M. V., Rocha, E. P., and De La Cruz, F. (2010). Mobility of plasmids. *Microbiol. Mol. Biol. Rev.* 74, 434–452. doi: 10.1128/mmbr.00020-10

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). "CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice." *Nucleic Acids Res.* 22, 4673–4680. doi: 10.1093/nar/22.22.4673

Welch, T. J., Fricke, W. F., Mcdermott, P. F., White, D. G., Rosso, M. L., Rasko, D. A., et al. (2007). Multiple antimicrobial resistance in plague: an emerging public health risk. *PLoS ONE* 2:e309. doi: 10.1371/journal.pone.0000309

Wozniak, R. A., Fouts, D. E., Spagnoletti, M., Colombo, M. M., Ceccarelli, D., Garriss, G., et al. (2009). Comparative ICE genomics: insights into the evolution of the SXT/R391 family of ICEs. *PLoS Genet* 5:e1000786. doi: 10.1371/journal.pgen.1000786

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 27 December 2013; accepted: 21 March 2014; published online: 12 May 2014.
*Citation: Nonaka L, Maruyama F, Onishi Y, Kobayashi T, Ogura Y, Hayashi T, Suzuki S and Masuda M (2014) Various pAQU plasmids possibly contribute to disseminate tetracycline resistance gene tet(M) among marine bacterial community. Front. Microbiol. **5**:152. doi: 10.3389/fmicb.2014.00152*
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology.

frontiers in Microbiology

REVIEW
published: 27 May 2015
doi: 10.3389/fmicb.2015.00494

Insight into the mobilome of *Aeromonas* strains

*Marta Piotrowska and Magdalena Popowska**

Department of Applied Microbiology, Institute of Microbiology, Faculty of Biology, University of Warsaw, Warsaw, Poland

The mobilome is a pool of genes located within mobile genetic elements (MGE), such as plasmids, IS elements, transposons, genomic/pathogenicity islands, and integron-associated gene cassettes. These genes are often referred to as "flexible" and may encode virulence factors, toxic compounds as well as resistance to antibiotics. The phenomenon of MGE transfer between bacteria, known as horizontal gene transfer (HGT), is well documented. The genes present on MGE are subject to continuous processes of evolution and environmental changes, largely induced or significantly accelerated by man. For bacteria, the only chance of survival in an environment contaminated with toxic chemicals, heavy metals and antibiotics is the acquisition of genes providing the ability to survive in such conditions. The process of acquiring and spreading antibiotic resistance genes (ARG) is of particular significance, as it is important for the health of humans and animals. Therefore, it is important to thoroughly study the mobilome of *Aeromonas* spp. that is widely distributed in various environments, causing many diseases in fishes and humans. This review discusses the recently published information on MGE prevalent in *Aeromonas* spp. with special emphasis on plasmids belonging to different incompatibility groups, i.e., IncA/C, IncU, IncQ, IncF, IncI, and ColE-type. The vast majority of plasmids carry a number of different transposons (Tn*3*, Tn*21*, Tn*1213*, Tn*1721*, Tn*4401*), the 1st, 2nd, or 3rd class of integrons, IS elements (e.g., IS*26*, IS*Pa12*, IS*Pa13*, IS*Kpn8*, IS*Kpn6*) and encode determinants such as antibiotic and mercury resistance genes, as well as virulence factors. Although the actual role of *Aeromonas* spp. as a human pathogen remains controversial, species of this genus may pose a serious risk to human health. This is due to the considerable potential of their mobilome, particularly in terms of antibiotic resistance and the possibility of the horizontal transfer of resistance genes.

Keywords: *Aeromonas*, mobilome, plasmid, transposon, integron, virulence factor, antibiotic resistance gene, horizontal gene transfer

OPEN ACCESS

Edited by:
Amy Pruden, Virginia Tech, USA

Reviewed by:
Peiying Hong, King Abdullah University of Science and Technology, Saudi Arabia
Michael J. Rothrock, United States Department of Agriculture – Agricultural Research Service, USA

****Correspondence:***
Magdalena Popowska, Department of Applied Microbiology, Institute of Microbiology, Faculty of Biology, University of Warsaw, Ilji Miecznikowa 1, 02-096 Warsaw, Poland
magdapop@biol.uw.edu.pl

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *17 February 2015*
Accepted: *05 May 2015*
Published: *27 May 2015*

Citation:
Piotrowska M and Popowska M (2015) Insight into the mobilome of Aeromonas strains. Front. Microbiol. 6:494. doi: 10.3389/fmicb.2015.00494

Introduction

Bacteria of the genus *Aeromonas* are common in a variety of environments. They have been isolated from water, mammals, fish, invertebrates, birds, insects, soil (Palumbo et al., 1985; Ceylan et al., 2009) as well as from food (Neyts et al., 2000; Kingombe et al., 2004). However, they mainly inhabit all kinds of aquatic environments, such as rivers, lakes, ponds, estuaries of marine waters, drinking water and groundwater as well as wastewater at various stages of purification (Gordon et al., 2008; Reith et al., 2008; Moura et al., 2012). The genus *Aeromonas* is composed of a large

number of species (31 species and 12 subspecies Martin-Carnahan and Joseph, 2005; http://www.bacterio.net/aeromonas.html) but only a few of them have been found to be primarily pathogens of fish and warm-blooded animals, including humans. In fish, mainly mesophilic *A. hydrophila*, *A. veronii* bv. *sobria* and psychrophilic strains of *A. salmonicida* are predominantly responsible for fish infections, e.g., furunculosis (Burr et al., 2005; Dallaire-Dufresne et al., 2014), but *A. caviae*, *A. jandaei*, *A. sobria*, *A. bestiarum* have also been reported to cause several known types of diseases as well as unusual infections, e.g., epizootic ulcerative syndrome (Rahman et al., 2002). Mesophilic *A. hydrophila*, *A. caviae,* and *A. veronii* bv. *sobria* strains are important human pathogens, responsible for a variety of infectious complications in both immunocompetent and immunocompromised individuals. They cause various types of infections of the digestive system, such as gastroenteritis (Holmberg and Farmer, 1984; Figueras, 2005; Edberg et al., 2007), respiratory (Bossi-Küpfer et al., 2007) and genitourinary infections (Al-Benwan et al., 2007; Huang et al., 2007), wound infections, infections of skin and soft tissue (Jorge et al., 1998; Vally et al., 2004; Chim and Song, 2007), sepsis (Ko et al., 2000; Lau et al., 2000; Tsai et al., 2006), eye infections (Khan et al., 2007), and meningitis (Seetha et al., 2004).

The mechanisms of the pathogenicity of *Aeromonas* spp. are not yet well understood, this being a concern because of recent reports of antibiotic resistant clinical strains (Ghenghesh et al., 2008; Wu et al., 2015). Moreover, cases of isolation of pathogenic strains from the environment are increasingly frequent, which can pose a serious threat to public health during natural disasters (Lin et al., 2013). Taking into account the pathogenicity potential of *Aeromonas* spp. and differences in antibiotic resistance profiles it seems reasonable to study the genetic background of these phenomena. In our study we have decided to focus on the mobile part of the *Aeromonas* spp. genome, that is the mobilome, since this topic has not yet been comprehensively reviewed. Based on the current knowledge we have focused in this review on plasmids, which carry a number of transposons, integron-associated gene cassettes and IS elements, and encode such determinants as antibiotic and heavy metal resistance, as well as virulence factors.

Mobile Genetic Elements and Mechanisms of Horizontal Gene Transfer

The mobilome is the total pool of mobile genes in the genome and consists of mobile genetic elements (MGE), such as plasmids, insertion sequences (IS), transposons, integron-associated gene cassettes and bacteriophages. Plasmids are mostly double-stranded and circular independent replicons of extrachromosomal DNA. They cover a variety of sizes from small, often cryptic plasmids to large megaplasmids with many features allowing them to adapt to different environmental conditions (**Table 1**). Other MGE are transposable elements (TE) such as the insertion sequences (IS) and transposons (Tn) (**Table 2**). IS are the most simple TE that reach about 0.5–3 kb and very often are flanked by short sequences of inverted repeats (IR). A transposase gene, encoding the transposition of an IS, is usually located between IRs. Transposons have a more complex structure, because in addition to the transposase, they also harbor various genes responsible for specific phenotypes (Oliver et al., 2013). Integrons are non-replicative genetic elements, which are able to capture and incorporate gene cassettes by site-specific recombination. They are composed of three main elements: the *intI* gene, coding for a site-specific recombinase of the integrase family, specific recombination site *attI*, where a gene cassette may be inserted, and the Pc promoter, managing the transcription of the captured gene (Stokes and Hall, 1989).

MGE are ubiquitous among all prokaryotes and play a significant role in horizontal gene transfer (HGT) and interspecies dissemination of resistance and virulence determinants (Brouwer et al., 2011; Oliveira et al., 2014). HGT occurs mainly by three mechanisms: DNA transformation, conjugative transfer involving plasmids, and other conjugative elements (conjugative transposons) and transduction by phages (Thomas and Nielsen, 2005). In the case of *Aeromonas* spp. until now gene transfer by transduction has never been observed. However, *Aeromonas* spp. phages have been identified in various environments, such as *A. hydrophila* phages Aeh1 and Aeh2 from sewage (Chow and Rouf, 1983), *A. hydrophila* phage CC2 from sewage in China (Shen et al., 2012), *A. salmonicida* phage phiAS4 from river in Korea (Kim et al., 2012a), and *A. salmonicida* phage PAS-1 from aquaculture in Korea (Kim et al., 2012b). Additionally, prophages have been also detected in *Aeromonas* spp.: ΦO18P (*Myoviridae*) in *A. media* isolated from a pond in Germany (Beilstein and Dreiseikelmann, 2008), AH1, AH2, AH3, AH4, and AH5 in *A. hydrophila* isolated from epidemic outbreak of catfish in the USA (Hossain et al., 2013). Hossain et al. (2013) detected five putative prophages (AH1-5) located in epidemic-associated regions in the genome. Unfortunately, their studies have not shown the ability of the prophages to transduce any bacterial genes. The other two mechanisms of HGT (DNA transformation and conjugative transfer by plasmids) are ubiquitous among *Aeromonas* spp. and will be the subject of this review. Various MGE, such as plasmids, transposons, or insertion sequences have been isolated from aeromonads, and many of them, regardless of the strain origin, have been found to carry resistance or virulence determinants (Sørum et al., 2003; Dallaire-Dufresne et al., 2014).

Antibiotic Resistance Genes on MGE

Over the years, there has been very little research on the *in vitro* susceptibility of *Aeromonas* bacteria isolated from clinical material to various chemotherapeutic agents. Most of the available information is focused on antibiotics used to treat infections caused by *A. hydrophila*, *A. caviae,* and *A. veronii* bv. *sobria*, and it is not yet certain whether these results can be extrapolated to other species of this genus (Fricke et al., 2009; Girlich et al., 2011; Maravić et al., 2013). The Clinical and Laboratory Standards Institute (CLSI) has recently published guidelines for the assessment of the sensitivity of clinical isolates of *Aeromonas* spp. using disk diffusion and MIC tests, but these data are based on testing of the three above- mentioned-, most clinically relevant species of *Aeromonas* (Jorgensen and Hindler,

TABLE 1 | Plasmids of *Aeromonas* spp., their origin and virulence features, and the presence of resistance determinants and mobile elements.

Plasmid	Size (kb)	Inc[a] group	Antibiotic resistance genes	Other MGE located within plasmids	Conjugative transfer	Special features	Source of isolation	References
pRA1	144 kb	IncA/C	*sul2*, *tetA*, *tetR*	- Two IS*26* elements - A truncated IS*Vsa3* element	+ (BHR[b])	- T4SS - *hip*AB toxin-antitoxin gene cluster	Sample type ND (*A. hydrophila*, Japan)	Hedges et al., 1985; Fricke et al., 2009
pR148	165.9 kb	IncA/C	bla_{OXA-10}, *aadA*1, *sul1*, *tet*A, *tetR*, *catA*2 (MDR[c])	- Tn*21*: *qacH*, bla_{OXA-10}, *aadA*1, *sul1*, *intI* (class 1 integron) - Tn*1721*: *tetA, tetR*	–	- T4SS - Mercuric resistance operon (*mer*)	Sample type ND (*A. hydrophila*, tilapia farm, Thailand)	del Castillo et al., 2013
ND[d]	ND	IncA/C	bla_{CMY-2}, *floR*, *tetA*, *strA-B, aadA7*, *sul2*	- Class 1 integron (*intI*, *aadA7*) - Transposon-like element	+ (BHR)	- Mercuric resistance operon (*mer*)	Fish [*A. salmonicida* subsp. *salmonicida*, atlantic salmon (*Salmo salar*), aquaculture, New Brunswick and Nova Scotia, Canada]	McIntosh et al., 2008
pSN254b	152.2 kb	IncA/C	*floR, sul1,* bla_{CMY-2}, *aadA*, *tetA, tetR, sul2*	- Tn*21*: IS*4321*, IS*26*, *intI1, aadA, sul1, mer*		-T4SS -Mercuric resistance genes (*merA, merB, merD, merE, merP, merR, merT*)	Fish (*A. salmonicida* subsp. *salmonicida*, brook trout, New Brunswick, Canada)	Vincent et al., 2014
pAsa4	166.7 kb	IncA/C - related	*tetE-tetR*, *sul2*	- Tn*21*: mercury resistance, In2 integron encoding resistance to streptomycin/spectinomycin, quaternary ammonia compounds, sulfonamides (*sul2*) and chloramphenicol	± (genes of conjugative transfer)	- T6SS – three genes (*vgrG*, *icmF*, *hcp*)	Fish (*A. salmonicida* subsp. *salmonicida*, brown trout, Eure river, France)	Reith et al., 2008
RA3	45.9 kb	IncU	*sul1*, *catA*2, *aadA*2 (MDR)	- 10.5 kb class I complex integron (*intI*, *sul1*, *catA2*, *aadA2*, *qacEΔ1*, IS*6100)*	+ (BHR)	–	Fish (*A. hydrophila*, diseased fish Japan)	Sørum et al., 2003; Kulinska et al., 2008
pAR-32	ND	IncU	*sul1*, *catA*2, *aadA*2 (MDR)	- In6-like integron (*intI*, *sul1*, *catA*2, *aadA*2, *qacEΔ1*)	+ (BHR)	- pAr-32 is identical to plasmid RA3	Fish (*A. salmonicida,* diseased biwamasu, trout hatchery, Shiga, Japan)	Sørum et al., 2003; Kulinska et al., 2008
pRAS1	45 kb	IncU	*tetA*, *dfrA16*, *sul1* (MDR)	- Truncated Tn*1721*—5.4 kb *Eco*RI fragment (*tetA*) - In4-like integron (*intI*, *dfrA16, qacEΔ1, sul1,* IS*6100*)	+ (BHR)	–	Fish [*A. salmonicida*, atlantic salmon (*Salmo salar*) with furunculosis, fish farm, Bergen, Norway]	Sandaa and Enger, 1994; Sørum et al., 2003

(Continued)

TABLE 1 | Continued

Plasmid	Size (kb)	Inc[a] group	Antibiotic resistance genes	Other MGE located within plasmids	Conjugative transfer	Special features	Source of isolation	References
pASOT, pASOT2, pASOT3	47 kb, 39 kb	IncU	*tetA*	- Tn*1721* – 5.4 kb *Eco*RI fragment (*tetA*)	+ (BHR)	–	Fish (*A. salmonicida*, furunculosis outbreaks, Scotland)	Adams et al., 1998; Rhodes et al., 2000
pFBAOT1-17	large plasmid	IncU – 3,4,5,7,9, 11	*tetA*	- Tn*1721* (*tetA*) in all plasmids	+ (pFBAOT3 to -7, -9, and -11 BHR)	- pFBAOT7 is identical to plasmid pFBAOT11	Environment - untreated hospital effluent, fish farm (*A. hydrophila* and *A. veronii*, United Kingdom)	Rhodes et al., 2000
pFBAOT6	84.7 kb	IncU	*tetA*, *sul1*, *aadA*2 (MDR)	- IS*630* family - In4-like integron (*intI*, *aadA*2, *qacEΔ1*, *sul1*, IS*6100*) - 43-kb Tn*1721*-based transposone - 6.5-kb Tn*3*	+	- First 31 kb (core genes) – Putative replication, maintence and transfer functions - 54 kb – genetic load	Environment - untreated hospital effluent (*A. punctata*, United Kingdom)	Rhodes et al., 2004
pAS37, p42	55 kb, 20 kb	IncU	*qnrS*2	- Mobile insertion cassette (mic) - transposone-like structure - (*qnrS2)*	ND	–	Environment – river (*A. punctata* and *A. media*, Seine River, Paris, France)	Cattoir et al., 2008
p34	80 kb	IncU	*qnrS*2, bla_{OXA-1}, *aac(6')-Ib-cr, catB3* (MDR)	- Mobile insertion cassette (mic) - transposone-like structure - (*qnrS2)* - IN37-like integron *(intI*, *aac(6')-Ib-cr*, bla_{OXA-1}, *catB3*)	ND	–	Environment - lake (*A. allosaccharophila*, Lugano lake, Lugano, Switzerland)	Picão et al., 2008
pP2G1	26.6 kb	IncU	*aac(6˜)-Ib-cr,* bla_{OXA-1}*, catB3, mphA-mrx-mphR, qnrS2, arr-3, sul1* (MDR)	- IS*Kpn9* present upstream of *qnrS2* - In37-like integron (*intI, aac(6¢)-Ib-cr,* bla_{OXA-1}*, catB3* and *arr-3*, *qacEΔ1*, *sul1*) - IS*6100* present upstream of *mphA-mrx-mphR*	–	-Resistance to quaternary ammonium compounds (*qacEΔ1*)	Environment – river (*Aeromonas* sp., Ter River, Ripoll, Spain)	Marti and Balcázar, 2012
ND	20 kb, 35 kb, 40 kb	IncU	*qnrS2*, *aac(6˜)-Ib-cr, sul1*, *dfrA22* (MDR)	- Class 1 integrons (*intI1*): 0.75 kb (*sul1*)	ND	- *qnrS2*, *aac(60)-Ib-cr* (20 kb plasmid) - MDR; - *qnrS2*, *sul1*, *dfrA22* (35 kb plasmid) - *qnrS2* (40 kb plasmid)	Fish (*A. sorbia* and *A. hydrophila*, koi carps - Czech Republic, ornamental fish -Vietnam and Thailand)	Dobiasova et al., 2014
pJA8102-1	11.8 kb	IncQ-2	*tetC*	–	± (Genes of conjugative transfer)	–	Sample type ND (*A. salmonicida*, Japan)	Aoki et al., 1986

(Continued)

TABLE 1 | Continued

Plasmid	Size (kb)	Inc[a] group	Antibiotic resistance genes	Other MGE located within plasmids	Conjugative transfer	Special features	Source of isolation	References
pRAS3.1 pRAS3.2	11.8 kb, 12 kb	IncQ-2	*tetC*	–	± (Mobilizable - *mobA*, *mobC*, *mobD*, *mobE*)	- pRAS3.1 and pRAS3.2. putatively variants of the same plasmid pRAS3	Fish [*A. salmonicida* subs. *salmonicida*, atlantic salmon (*Salmo salar*) with furunculosis, fish farm, Norway]	L'Abée-Lund and Sørum, 2002; Loftie-Eaton and Rawlings, 2009
pBRST7.6	7.6 kb	IncQ-3	*qnrS2*	–	± (Mobilizable - *mobB*, *mobC*)	–	Fish (*A. hydrophila*, UDS-affected *Channa punctatus*)	Majumdar et al., 2011
pAHH04	7.2 kb	IncQ	*qnrS2*	–	± (Mobilizable - *mobA*, *mobC*)	–	Fish, environment [*A. hydrophila*, water and diseased Glowlight tetra (*Hemigrammus erythrozonus*),South Korea]	Han et al., 2012c
pAQ2-1, pAQ2-2	6.9 kb	ColE – type	*qnrS2*	- Mobile insertion cassette (mic) element (*qnrS2*)	–	–	Fish [*A. sobria* and *A. hydrophila*, diseased fish Medaka (*Oryzias latipes*) and Congo tetra (*Phenacogrammus interruptus*), South Korea]	Han et al., 2012a
pAHH01	8.9 kb	ColE2 – type	*tetE, tetR*	–	± (Mobilizable - *mob*)	– *relE/B* (toxin-antitoxin)	Fish [*A. hydrophila*, diseased fish cherry salmon (*Oncorhynchus masou masou*), South Korea]	Han et al., 2012b
ND	40 kb	IncFIB	$bla_{CTX-M-15}$	- Class 1 (*intI1*) and class 2 (*intI2*) integrons	+	–	Mussel [*A. caviae*, Mediterranean mussel (*Mytilus galloprovincialis*), Adriatic Sea, Kaštela Bay, Croatia]	Maravić et al., 2013
ND	Large plasmids (>30 kb)	ND	*tetA, tetD, tetE, dhfr, catB, aadA1a*	- Class 1 integrons (*intI1*) (in 27 plasmids) - 10 plasmids with *tetA* and *intI* integrons with two or more inserted ARG	+ (- 17 plasmids 110–160 kb)	Plasmid profiles: - A - at least one plasmid >30 kb; - B - one plasmid 2.3–20 kb - C - two plasmids 6.5–15 kb - D - three to nine plasmids 3–25 kb	Fish, environment – water, sediment (*Aeromonas* spp., Denmark)	Schmidt et al., 2001

(Continued)

TABLE 1 | Continued

Plasmid	Size (kb)	Inc[a] group	Antibiotic resistance genes	Other MGE located within plasmids	Conjugative transfer	Special features	Source of isolation	References
pTf28	60 kb	ND	bla_{GES-7}, *aacA4*	- Class 1 integrons (*intI1*, bla_{GES-7}, *aacA4*)	± (Mobilizable – *mobA*)	–	Environment – water (*A. veronii*, Seine River, Paris, France)	Girlich et al., 2011
pAB5S9	24.7 kb	ND	*floR, sul2, strA-strB, tetR-tetY* (MDR)	- 7.5 kb sequence showed 100% identity to three non-contiguous segments of the SXT element of *Vibrio cholera* (comprised *floR* gene flanked upstream by a complete and downstream by a truncated IS*CR2* element)	+	–	Environment – river sediment (*A. bestiarum*, freshwater stream, North Brittany, France; New Brunswick and Quebec, Canada)	Gordon et al., 2008; Vincent et al., 2014
pAsa5	155 kb	ND	not detected	–	± (Genes of conjugative transfer)	- T3SS - effector proteins (AopH, AopO, Ati2), chaperones (SycH, SycO, Ati1)	Fish (*A. salmonicida* subsp. *salmonicida*, brown trout, Eure river, France)	Reith et al., 2008; Daher et al., 2011; Dallaire-Dufresne et al., 2013; Vanden Bergh et al., 2013
pAsa6	18.5 kb	ND	not detected	- Eight transposases: six complete and two partial IS sequences	– (Also not mobilizable)	- Two putative truncated genes of sulfatases - *aopH* and *sycH* (T3SS) – virulence factors	Fish [*A. salmonicida* subsp. *salmonicida*, diseased turbot (*Scophthalmus maximus*), Portugal]	Najimi et al., 2009
pAsal1	6.4 kb	ND	–	- IS*AS11*	± (Mobilizable – *mobA*)	- T3SS effector protein (AopP)	Fish (*A. salmonicida* subsp. *salmonicida*, arctic char, Switzerland)	Fehr et al., 2006; Jones et al., 2012; Trudel et al., 2013
pAsal1B	9.0 kb	ND	–	- 2614 bp IS belongs to the IS*21* family inserted in the *mobA* gene	–	- Combination of pAsal1 and IS*AS5*	Sample type ND (*A. salmonicida* subsp. *salmonicida*, France)	Trudel et al., 2013
pASvirA	140 kb	ND	ND	–	ND	- T3SS genes - AexT toxin	Fish [*A. salmonicida*, arctic char (*Savelinus alpines*) with furunculosis]	Stuber et al., 2003
ND	>55 kb	ND	ND	- Class 1 integrons (*intI1*)	+	–	Fish, environment – water, sediment (*Aeromonas* spp., catfish, fish farm, Vietnam)	Nguyen et al., 2014

[a]*Inc group, Incompatibility group;* [b]*BHR, broad host range plasmid;* [c]*MDR, multidrug resistance;* [d]*ND, not determined; "±"- conjugative transfer not determined or mobilizable.*

TABLE 2 | Other than plasmid borne mobile genetic elements of *Aeromonas* spp.

Mobile genetic element	Characteristics	References
Tn*3*	- Tn*3*-*tnpR*/IS*Kpn8*/bla_{KPC-2}/IS*Kpn6*	Picão et al., 2013
Tn*21*	- *qacH*, bla_{OXA-10}, *aadA1, sul1, intI* - IS*4321*, IS*26*, *intI, aadA, sul1, mer* - *mer*	Reith et al., 2008; del Castillo et al., 2013; Vincent et al., 2014
Tn*1721*	- *tetA, tetR*	Rhodes et al., 2000; Sørum et al., 2003; del Castillo et al., 2013
Tn*4401*	- bla_{KPC-2}	Picão et al., 2013
IS*26*	- Sulfonamide resistance gene - bla_{PER-1} (between IS*Pa12* and IS*Pa13* which composed part of Tn*1213*)	Fricke et al., 2009; Girlich et al., 2011
IS*6100*	*mphA-mrx-mphR*	Marti and Balcázar, 2012
ISV*sa3*	- *sul2*	Fricke et al., 2009
IS*Kpn9*	- *qnrS2*	Marti and Balcázar, 2012
Class I integrons	- 10.5 kb class I complex integron (*intI, sul1, catA2, aadA2, qacEΔ1,* IS*6100*) - 0.75 kb integron (*sul1*) - bla_{GES-7}, *aacA4* - *aadA7* - bla_{VEB-1} - bla_{SHV-12}	Kulinska et al., 2008; McIntosh et al., 2008; Girlich et al., 2011; Dobiasova et al., 2014
IN2-like integron (class I)	- Resistance to streptomycin/spectinomycin, quaternary ammonia compounds, sulfonamides (*sul2*) and chloramphenicol	Reith et al., 2008
IN4-like (class I)	- *intI, dfrA16, qacEΔ1, sul1*, IS*6100* - *intI, aadA2, qacEΔ1, sul1*, IS*6100*	Sørum et al., 2003; Rhodes et al., 2004
IN6-like (class I)	*intI, sul1, catA2, aadA2, qacEΔ1*	Kulinska et al., 2008
IN37-like (class I)	- *intI*, *aac(6')-Ib-cr*, bla_{OXA-1}, *catB3* - *intI, aac(6¢)-Ib-cr,* bla_{OXA-1}*, catB3 and arr-3*, *qacEΔ1*, *sul1*	Picão et al., 2008; Marti and Balcázar, 2012
SXT	- *floR* gene flanked upstream by a complete and downstream by a truncated IS*CR2* element	Gordon et al., 2008
Mobile insertion cassette (mic)	- *qnrS2*	Cattoir et al., 2008; Picão et al., 2008; Han et al., 2012a

2007) plus *A. jandaei* and *A. schubertii* (Clinical and Laboratory Standards Institute, 2006). The most commonly administered antibiotics In the treatment of *Aeromonas* infections are ciprofloxacin, levofloxacin, sulfamethoxazole/trimethoprim, amikacin, gentamicin, ciprofloxacin, and trimethoprim (Jones and Wilcox, 1995). Sensitivity analysis of clinical strains demonstrated that more than half of the strains tested were resistant to antibiotics of the following groups: antifolates (sulfamethoxazole), cephalosporins, penicillins (amoxicillin, ampicillin, ampicillin-sulbactam, oxacillin, penicillin, ticarcillin. The susceptibilities were determined by agar dilution or disc diffusion method, respectively, according to CLSI guidelines, but the ARG were not pin-pointed (Lamy et al., 2009; Aravena-Román et al., 2012). However, the susceptibility profile of individual strains can also vary depending on the particular species, different geographical localization and environment in which they occur (Ghenghesh et al., 2008). Such differences could be related to the recommended approach for the treatment of *Aeromonas* infections in different countries.

On the basis of fairly abundant literature data concerning the antibiotic resistance of environmental *Aeromonas* strains, it can be concluded that this phenomenon mostly concerns strains isolated from various water environments, including wastewater (Figueira et al., 2011), natural waters such as rivers (Girlich et al.,

2011), lakes (Picão et al., 2008), and estuaries (Fiorentini et al., 1998; Henriques et al., 2006), aquacultures (Schmidt et al., 2001; Jacobs and Chenia, 2007; Yi et al., 2014a,b), and urban drinking water (Carvalho et al., 2012). ARG recently found in water strains encoded resistance to four major groups of antibiotics: β-lactams, quinolones, aminoglycosides, tetracyclines and less frequently to sulfonamides and trimethoprim, chloramphenicol, florfenicol, macrolides, streptogramins, streptothricin, and ansamycins. In general, the resistance profile and the presence of specific resistance genes depends on the particular aquatic environment (Piotrowska and Popowska, 2014). Given the risk to human health, the incidence of ARG is alarming, particularly among *A. hydrophila*, *A. caviae,* and *A. sorbia*, which are considered opportunistic pathogens responsible for infections in both fish and humans (Alcaide et al., 2010; Ottaviani et al., 2011; Shak et al., 2011; Dias et al., 2012; Yi et al., 2014b). The localization of ARG and virulence determinants of *Aeromona*s spp. on MGE such as plasmids, insertion sequences, transposons and mobile integron gene cassettes have been determined by many environmental studies. There is some literature data on the localization of ARG among clinical *Aeromonas* strains. However, in the recent publications, the presence of ARG on plasmids (e.g., MOX, TEM, PSE, and CTX-M β-lactamase genes, *sul1* and *sul2*) has been confirmed, but no characteristics have been provided (Ye et al., 2010; Puah et al., 2013). Moreover, among clinical strains three cases of β-lactamases genes located within integrons: bla_{VIM} from *A. caviae* (Adler et al., 2014), $bla_{\mathrm{VIM-4}}$ from *A. hydrophila* (Libisch et al., 2008) bla_{IMP} which also was located on 35-kb plasmid from *A. caviae* have been identified (Neuwirth et al., 2007). Also (Wu et al., 2011) identified the ESBL gene $bla_{\mathrm{PER-3}}$ in two *A. caviae* isolates. The gene was located in both chromosomes and plasmids. Additionally, there is only one clinical report of gene *qnrS2* that has been found on a plasmid isolated from *A. veronii* (Sánchez-Céspedes et al., 2008). However, all these reports are still sufficient enough to look for any gene-MGE correlation and consequently research on a larger scale should be conducted.

Plasmids

Analysis of a number of studies on *Aeromonas* spp. showed the prevalence of different incompatibility groups of plasmids, i.e., IncA/C, IncU, IncQ, IncF, IncI, and ColE-type, with the greatest frequency of the first two groups (**Table 1**). The vast majority of plasmids carry a number of different determinants such as antibiotic and metal resistance genes or virulence factors. *Aeromonas* spp. very often carry resistance plasmids (R-plasmids) of various length belonging to different incompatibility groups and of worldwide spread. Furthermore, numerous R-plasmids contain multidrug-resistance (MDR) to three or more antimicrobial classes (according to the European Centre of Disease Prevention and Control, Magiorakos et al., 2012). Of particular concern is the fact that most of the isolated plasmids are broad-host-range (BHR), capable of conjugative transfer (*tra* genes) or capable of mobilization (*mob* genes).

Plasmids of the IncA/C incompatibility group have been described as conjugative and BHR, and capable of spreading multidrug-resistance. A variety of isolates among different bacterial genera, such as *Escherichia*, *Salmonella*, *Vibrio,* or *Yersinia* (Winokur et al., 2001; Pan et al., 2008; Guo et al., 2014) of environmental, animal and human origin have been reported to carry these plasmids, which increases public health concerns worldwide (Mataseje et al., 2010). The first member of the IncA/C family was the pRA1 plasmid isolated in 1971 from *A. hydrophila* derived from Japan (Hedges et al., 1985). The complete DNA sequence of this large plasmid (144 kb) revealed that pRA1 and other members of the IncA/C family shared 100 kb of a highly conserved plasmidic backbone with more than 80% nucleotide sequence identity. Among the most important core genes are those that encode type IV secretion-like conjugative transfer operons. A complete set of the type IV secretion system operons was also found on pR148 (MDR plasmid) of IncA/C group isolated from diseased fish from Thailand (del Castillo et al., 2013). Another interesting feature was the *hipAB* toxin-antitoxin gene cluster, which was also partially (*hipAB*-related gene cluster) described in the IncA/C-related plasmid pAsa4, isolated from the fish pathogen *A. salmonicida* subsp. *salmonicida* (Fricke et al., 2009). According to the BLAST database, integrating conjugative elements (ICE) were identified as the closest relatives of IncA/C plasmids (Fricke et al., 2009). Antimicrobial resistance profile of pRA1 is reduced compared with all other IncA/C plasmids sequenced of *Aeromonas* spp., as it is limited to tetracyclines (*tetRA* cluster) and sulfonamides (*sul2*) (McIntosh et al., 2008; del Castillo et al., 2013). The *sul2* gene was located next to the truncated IS*Vsa3* element, which has been observed previously in an IncA/C plasmid isolated from a Spanish *S. enterica* strain (García et al., 2011). Class D *tetRA* gene cluster was found within two IS*26* elements that played a key role in the distribution of ARG on different plasmids (Cullik et al., 2010). In addition to antibiotics, heavy metals are also implicated as potential substances that can co-select antibiotic resistance in the environment, resulting in a frequent presence of heavy metal resistance genes on the same MGE as ARG (Lazar et al., 2002). Mercury resistance operons (*mer*) have been found on IncA/C plasmids such as pR148 or pAsa4 in *Aeromonas* spp. The plasmid isolated from *A. salmonicida* subsp. *salmonicida* showed 100% nucleotide sequence homology to the *mer* operon carried by *S. enterica* IncA/C plasmid pSN254 (McIntosh et al., 2008; del Castillo et al., 2013). The pR148 plasmid also carries genes encoding resistance to quaternary ammonium compounds. Moreover, phylogenetic comparative studies revealed that pR148 is the most closely related to human pathogenic *E. coli* and *Acinetobacter baumanii*. This similarity indicates that the IncA/C group of plasmids was transferred between different genera (McIntosh et al., 2008; Moura et al., 2012). Furthermore, the IncA/C MDR plasmid isolated by McIntosh et al., 2008 carried *floR*, *tetA*, *sul2,* and *strA*/*strB* sequences on a cassette that had 99.9% nucleotide sequence homology to that of the pSN254 plasmid isolated from *S. enterica*. Recently Vincent et al. (2014) identified a 152-kb pSN254b plasmid which is a different variant of pSN254. This MDR plasmid provides resistance to chloramphenicol (*floR*), florfenicol (*floR*), streptomycin (*aadA*), spectinomycin (*aadA*), tetracycline (*tet*), sulfonamide (*sul1*), beta-lactam antibiotics ($bla_{\mathrm{CMY-2}}$), quaternary ammonium compounds (*sugE2*), and mercury (*merA*, *merB*, *merD*, *merE*, *merP*, *merR*, *merT*). There

is no strong correlation between IncA/C plasmids and other MGE, but suggestive associations have been observed and will be described in the next chapter.

Plasmids of the IncU group have been isolated from many clinical and environmental strains of *Esherichia coli* and *Aeromonas* spp. Conjugative and BHR plasmids are members of this group and are also involved in the dissemination of antibiotic resistance among *Aeromona*s spp. This group of plasmids is widely distributed around the world (**Table 1**) and it has been postulated that they share a conserved backbone structure with a variable region limited to resistance-determining genes (Rhodes et al., 2000; Sørum et al., 2003). The RA3 plasmid (45.9 kb) was isolated from *A. hydrophila* in Japan and serves as the reference plasmid of the IncU group (Kulinska et al., 2008). Functional analysis demonstrated that RA3, as a BHR plasmid, could self-transfer, replicate and be stably maintained in Alpha-, Beta-, and Gammaproteobacteria. RA3, similarly to other members of the IncU group, is a MDR plasmid and contains a 10.5-kb antibiotic resistance region that comprises class I integron with *sul1*, *catA2*, *aadA2*, *qacE* resistance genes. The pAr-32 plasmid, isolated from *A. salmonicida* in Japan in 1970 is very similar to the RA3 plasmid and carries the same integron cassette, which is highly similar to the In6 integron of the pSa plasmid (Sørum et al., 2003). Another IncU plasmid (pRAS1) was isolated from *A. salmonicida* from Norway in 1989 and had the same backbone structure as pAR-32. The region controlling drug resistance in pRAS1 contains two main elements: the complete class 1 In4-like integron with *dfrA16*, *qacE*, *sul1* gene cassette and a fragment of the Tn*1721* transposon carrying *tetA* resistance gene. The study of Schmidt et al. (2001) demonstrated a positive correlation between oxytetracycline resistant strains of *Aeromonas* spp. containing large plasmids, and the presence of *tetA* genes. Among IncU R-plasmids, such as pRAS1, pASOT, or pFBOAT, tetracycline resistance determinants were observed in the complete or truncated Tn*1721* (Adams et al., 1998; Rhodes et al., 2000). In all cases the TetA determinant was located within a 5.5-kb *Eco*RI restriction fragment. Based upon RFLP assessment, antibiotic resistance, and frequency of transfer all these tetracycline resistance encoding plasmids are considered to be closely related to plasmid pIE420 isolated from a German hospital strain of *E. coli* (Rhodes et al., 2000). The study of Rhodes et al. (2004) showed that plasmids pRAS1 and pIE420 are probably identical. These results support the hypothesis that IncU is an evolutionarily narrow group. However, Rhodes et al. (2004) also characterized plasmid pFBOAT6 (84.7 kb), which had a 31-kb region of core genes and a 54-kb region of genetic load, which made this plasmid almost twice as large as the other IncU plasmids. This was due to the presence of a 43-kb resistance region flanked by Tn*1721*. This region is highly similar to those of the pXF51, pIPO2, and pSB102 plasmids found in a plant-associated bacterial hosts. Nevertheless, only several nucleotide differences in the core genes were found between RA3 and pFBAOT6 plasmids.

Many IncU plasmids also harbor quinolone resistance determinants–*qnrS2* and *aac(6′)-Ib-cr* (**Table 1**). Plasmid-mediated *qnr* genes have been identified in many *Enterobacteriaceae* isolates (Nordmann and Poirel, 2005; Kehrenberg et al., 2006; Pasom et al., 2013) and recently also in *Pseudomonas* spp. (Cayci et al., 2014). In addition, the *qnrS2* gene has been recently detected in *A. caviae* clinical strain isolated from a stool sample collected from a patient with gastroenteritis (Arias et al., 2010). Among the environmental isolates of *Aeromonas* spp., the *qnrS2* gene was found in the following plasmids: pAS37 and p42 from French strains of *A. punctata* and *A. media* (Cattoir et al., 2008), p34 from a Swiss strain of *A. allasacharophila* (Picão et al., 2008), pP2G1 from a Spanish strain (Marti and Balcázar, 2012) and recently isolated unnamed plasmids from Thai strains of *A. sorbia* and *A. hydrophila* (Dobiasova et al., 2014). All of the described plasmids are medium to large in size (20–80 kb) and have an interesting genetic descent. The first two plasmids, pAS37 and p34, contain the *qnrS2* gene as a part of a novel transposon-like genetic structure called the mobile insertion cassette (mic) instead of the transposase gene. Moreover, this specific mobile element has been previously found in a *Bacillus cereus* strain, which makes mic a possible vector of ARG between environmental and clinical pathogens (de Palmenaer et al., 2004). Furthermore, the study of Han et al. (2012a) revealed two small (6.9 kb) plasmids (pAQ2-1 and pAQ2-2) carrying the mic-*qnr2S* structure. These ColE-type plasmids were 99% identical and genes for plasmid replication were organized in a similar way to ColE2-type cryptic plasmids pAsa1, pAsa2, and pAsa3, isolated from *A. salmonicida* subsp. *salmonicida* (Boyd et al., 2003). This observation suggests that these mic-type structures are potential vehicles of plasmid-mediated quinolone resistance determinants among different groups of plasmids in various geographical locations, and more importantly in clinically relevant strains.

Plasmids of the IncQ group are also strongly associated with quinolone resistance that have been identified among *Aeromonas* spp. These small, mobilizable plasmids (5.1–14.2-kb) are BHR and are found in many bacterial species worldwide (Loftie-Eaton and Rawlings, 2009). Among *Aeromonas* spp., two plasmids (pBRST7.6 and pAHH04) isolated from *A. hydrophila* strains from diseased fish and water samples harbored *qnrS2* genes (Majumdar et al., 2011; Han et al., 2012c). In addition, the exogenous pGNB2 plasmid obtained from the wastewater treatment plant in Germany also harbored the *qnrS2* gene (Bönemann et al., 2006). In contrast to IncU plasmid-mediated *qnr* genes, quinolone determinants of the IncQ plasmids were not associated with any mic or integron, and did not harbor any additional resistance determinants. Moreover, the pJA8102-1 plasmid found in *A. salmonicida* from Japan, and pRAS3.1 and pRAS3.2 of Norwich *A. salmonicida* strains carried *tetAR*(C) genes (L'Abée-Lund and Sørum, 2002). However, it is worth to emphasize that pRAS3.1 and pRAS3.2 are considered variants of the same plasmid pRAS3, which was also identified in a Scottish *A. salmonicida* strain and appears to be identical to the R-plasmid pJA8102-2.

In addition to the frequently occurring BHR plasmids that were discussed earlier (IncA/C, IncU, IncQ), plasmids belonging to IncFrepB, IncFIB, IncFIC and IncI groups were also observed in the genus *Aeromona*s (Han et al., 2012b; Moura et al., 2012; Maravić et al., 2013). The study of Maravić et al. (2013) found 40-kb conjugative plasmids described as narrow host range

IncFIB group in 11 *A. caviae* strains isolated from Croatian mussels. All the vectors carried the $bla_{CTX-M-15}$ gene, encoding ESBL β-lactamase. The same β-lactamase was also identified in IncFIB plasmids isolated from *E. coli*, and a large (210 kb), non-conjugative IncFIA plasmid identified in an *A. hydrophila* strain (Dolejska et al., 2011; Gómez-Garcés et al., 2011).

Insertion Sequences and Transposons

Aeromonas spp. often contain transposons located on plasmids and chromosomes (Tn*3*, Tn*21*, Tn*1213*, Tn*1721*, Tn*4401*) as well as insertion sequences (e.g., IS*26*, IS*Pa12*, IS*Pa13*, IS*Kpn8*, IS*Kpn6*) (**Tables 1, 2**). This makes the mobilome an even more complex structure that likely plays an important role in the dissemination of various resistance and virulence determinants.

McIntosh et al. (2008) found a transposon-like element that contained the bla_{CMY-2} β-lactamase gene. This element is known to be widely distributed among foodborne and clinical *Salmonella* strains as well as other *Enterobacteriaceae* in Asia and the United States. Other transposons (e.g., Tn*21*) are involved in the dissemination of ARG and mercury resistance genes (as in pAsa4) between gram-negative bacteria (Liebert et al., 1999). Transposon Tn*21* located on the pR148 plasmid carried *qacH*, bla_{OXA-10}, *aadA1,* and *sul1* cassette, which showed 100% similarity (when the last gene is excluded) to *Acinetobacter baumanii* AYE genome. A Tn*1721*-like transposon, conferring tetracycline resistance via *tetA/R* genes, was identified on the same plasmid. Tn*1721* belongs to the Tn*501* subfamily and the Tn*3* family of transposons. The involvement of Tn*1721* and Tn*1721*-like elements in the dissemination of the *tetA* gene has been observed in many other studies (Rhodes et al., 2000; Pasquali et al., 2005; Girlich et al., 2010). This transposon is also ubiquitous among IncU plasmids, which form another important group within *Aeromonas* spp.

Furthermore, many transposons and various insertion sequences were identified among *Aeromonas* spp. in association with β-lactamases. The study of Girlich et al. (2011) revealed many ESBL β-lactamases of different genetic backgrounds. The bla_{SHV-12} gene was preceded by IS*26* while the bla_{PER-1} gene was located between IS*Pa12* and IS*Pa13*, thus forming a part of a composite transposon Tn*1213*. It is also alarming that bla_{KPC-2} genes encoding carbapenemases have been isolated from *Aeromonas* spp. recovered from hospital sewage (Picão et al., 2013). The bla_{KPC-2} gene was found on the Tn*4401* transposon and Tn*3*-*tnpR*/IS*Kpn8*/bla_{KPC-2}/IS*Kpn6* array.

Integrons

Integrons are widely distributed bacterial genetic elements that are able to acquire gene cassettes frequently containing ARG. Most of them belong to the 1st, 2nd, or 3rd class of integrons and contain *intI1*, *intI2,* or *intI3* integrase genes, respectively (Hall et al., 1999). Integrons harbored by plasmids, transposons and other mobile structures are called "mobile integrons" (MI), because MGE promote their dissemination. For this reason, MI are also involved in spreading antibiotic resistance in the environment (Laroche et al., 2009). Integrons found in *Aeromonas* spp. mainly belong to the class 1 and carry a number of antibiotic resistance gene cassettes (**Tables 1, 2**). Schmidt et al. (2001) demonstrated that class 1 integrons frequently occurred on oxytetracycline resistance plasmids (most often *tetA*), but they did not observe any strong correlations between integrons and tet genes or any other group of plasmids. Similar results were reported by Jacobs and Chenia (2007), who observed class 1 integrons and tet genes in 68.4% isolates that also harbored different types of plasmid profiles. However, the study of Moura et al. (2012) demonstrated a positive correlation between integrons and FrepB and I1 plasmids isolated from *Aeromonas* spp. from wastewater. Furthermore, Schmidt et al. (2001) reported a close association of sulfadiazine/trimethoprim resistance and class 1 integrons, which manifested in the presence of *sul1* and *dfr* gene cassette inserts in class 1 integrons. Henriques et al. (2006), detected *intI* genes in 21% of *Aeromonas* and 29.6% of *Enterobacteriaceae* isolates. The most often found resistance gene cassettes contained various *aadA* genes, which were also observed in later studies (Moura et al., 2007; Koczura et al., 2014; Sarria-Guzmán et al., 2014). Integrons are also correlated with β-lactamase genes, e.g., genes bla_{VEB-1} and bla_{SHV-12}were located in class 1 integrons on plasmids of different sizes (30–170 kb) (Jacobs and Chenia, 2007; Carvalho et al., 2012). Integrons of class 2 were found in a couple of studies that indicated putative chromosomal location of these integrons (Carvalho et al., 2012). Other integrons belonging to class 1, such as IN2-like, IN4-like, IN6-like, IN37-like, or SXT, as well as many undescribed integrons, have been also reported on *Aeromonas* spp. plasmids (**Table 1**).

Virulence Factors on MGE

The role of potential virulence factors in the pathogenesis of *Aeromonas* spp. is not yet fully understood. However, several factors probably play important roles in the host infection process. In addition to adhesive factors, the capsular polysaccharide of *Aeromonas* (Khan et al., 2008), bacterial flagella and pili are needed for the first stage of infection. Lysis proteases, which are involved in the second step (metalloproteases, serine proteases, and aminopeptidases) are capable of degrading complex proteins present in the serum and connective tissue (Merino et al., 1996; Han et al., 2008; Imamura et al., 2008; Ottaviani et al., 2011; Puthucheary et al., 2012; John and Hatha, 2013). Many other factors are also likely to play important roles in infections, including specific proteins required for adaptive acid tolerance, biofilm formation and synthesis of autoinducers (e.g., acyl-homoserine lactone) in the quorum sensing process (Jangid et al., 2007) and type three secretion systems (Vanden Bergh et al., 2013). S-layers are also important, as they have the ability to bind different proteins, such as the extracellular matrix proteins fibronectin, laminin, and vitronectin, which provide a defense against the components of the serum and protease digestion (Noonan and Trust, 1997). The main pathogenic factors of *Aeromonas* spp. have also been observed on several plasmids (**Table 1**). Genes coding for the AexT toxin and three types of secretion systems—type III (T3SS), type IV (T4SS), and type VI (T6SS) have been identified on virulence plasmids (**Table 1**).

Six virulence plasmids were isolated from strains of the fish pathogen *A. salmonicida* i.e., pASvirA from diseased fish, pAsa4 and pAsa5 from the same French strain, pAsa6 from

diseased fish in Portugal (Stuber et al., 2003; Reith et al., 2008; Najimi et al., 2009) and pAsal1 and pAsal1B (Fehr et al., 2006; Jones et al., 2012; Trudel et al., 2013). Three plasmids were large (140–166.7 kb), i.e., pASvirA, pAsa4 and pAsa5, and in addition the latter two harbored conjugative transfer genes. Plasmid pAsa5 contained most of the T3SS genes that have been shown to be required for virulence in *A. salmonicida*. Three putative effector proteins (AopH, AopO, Ati2) and their associated chaperones (SycH, SycO, Ati1) were identified in it. The recommended temperature for growth of *A. salmonicida* by Bergey's Manual of Systematic Bacteriology is 22–28°C. However, several studies demonstrated that culturing at 25°C resulted in a lack of virulence due to the loss of virulence factors, such as the A-layer (Ishiguro et al., 1981), T3SS region (Stuber et al., 2003), or AexT toxine (Najimi et al., 2009). The mechanism of this phenomenon is not fully understood, but Stuber et al. (2003) explained it as a result of the loss of a virulence plasmid. As an example, the loss of plasmid pASvirA is accompanied by the inability of *A. salmonicida* to secrete AexT and loss of virulence toward RTG-2 cells. However, Tanaka et al. (2013) proposed a mechanism of the loss of virulence mediated by IS, wherein rearrangements are caused by recombination of three IS from thermolabile plasmids, e.g., pAsa5 or pASvirA. A consequence of the recombination of IS*AS1*, IS*AS2,* and IS*AS11* is the deletion of the T3SS region in *A. salmonicida*, resulting in the loss of virulence. This is consistent with a previous study that showed a large number of IS in *A. salmonicida* genome being involved in virulence gene disruption with the formation of pseudogenes (Reith et al., 2008). Sequencing and analysis studies of the total genome of *A. salmonicida* subsp. *salmonicida* A449 revealed the occurrence of 88 complete IS sequences of different types: IS*As1*, IS*As2*, IS*As3*, IS*As4*, IS*As5*, IS*As6*, IS*As7*, IS*As8*, IS*As9*, IS*As10*, ISAs11 and a significant number of pseudogenes (170). Many putative transposons and IS sequences, as well as AopH and SycH proteins are also present on pAsa6. This vector is a non-mobilizable 18-kb plasmid with characteristic strong homology to many pAsa5 genes (Najimi et al., 2009). Comparative analyses suggested that pAsa6 might be derived from pAsa5 through a deletion of numerous genes, or conversely, pAsa5 might have been formed as a fusion of a pAsa6-like plasmid with another megaplasmid. This is even more interesting when one considers the fact that both plasmids were isolated in different countries. Three genes of T6SS (*vgrG, icmF, hcp*) were found on pAsa4, but the majority of them were located on the chromosome of *A. salmonicida* subs. *salmonicida* or *A. hydrophila* (Reith et al., 2008). It has been demonstrated that T6SS plays an important role in the pathogenesis of *Aeromonas* strains, in the translocation of hemolysin protein (Hcp) into the host cells (Suarez et al., 2008). In addition, two small mobilizable plasmids, pAsal1 and pAsal1B (6.7 and 9.0 kb, respectively) encoding T3SS effector protein AopP, were recently discovered (Trudel et al., 2013). AopP has been reported to have a inhibitory activity against the NF-κB pathway in cultured cells (Fehr et al., 2006) and has potent pro-apoptotic activity when expressed in cultured mammalian macrophage or epithelial cells (Jones et al., 2012). Trudel et al. (2013) showed that pAsalB is a combination of pAsal1 and ISAS5, where this 2614 bp IS belongs to the IS*21* family inserted in the *mobA* gene sequence of the pAsal1 plasmid.

Conclusions

Bacteria from the genus *Aeromonas* have a complex mobilome consisting of many different MGE. This review presents the characteristics of more than 26 plasmids belonging to different incompatibility groups, all of which were isolated from environmental strains. Resistance genes have been detected in 21 of them, and 7 meet the MDR criteria for the isolated *Aeromonas* strains. Of particular note are the conjugative broad-host-range plasmids, belonging to the incompatibility group IncA/C, IncU, IncQ, IncF, IncI, and ColE-type. These plasmids are primarily responsible for multi-drug resistance among bacteria both in clinical and natural environments. They harbor resistance genes against antibiotics of key importance in clinical therapy, such as the quinolones, β-lactams, aminoglycosides, tetracyclines and sulfonamide. *Aeromonas* strains causing infections in humans may transfer MGEs carrying resistance genes to pathogenic or opportunistic bacteria in the human microbiome, and thus pose a threat to public health. This *in vivo* transfer has been reported from two clinical outbreaks in France where a 180-kb plasmid carrying the $bla_{\mathrm{TEM-24}}$ gene has been isolated from *Enterobacter aerogenes* and two *Aeromonas* species: *A. hydrophila* and *A. caviae* (Marchandin et al., 2003; Fosse et al., 2004). The same plasmid has been previously characterized in the case of *Klebsiella pneumonia* and *Pseudomonas aeruginosa* (Giraud-Morin and Fosse, 2003; Marchandin et al., 2000) indicative of their broad host range potential among pathogenic bacterial species. Studies of antibiotic resistance in clinical strains focus solely on the determination of susceptibility using disk diffusion tests and subsequent classification to the R (resistant) or S (susceptible) groups according to the guidelines of such organizations as CLSI (Clinical and Laboratory Standards Institute, 2006). Publications concerning such strains rarely explain the molecular mechanisms of resistance, i.e., the identification of specific genes by PCR amplification or hybridization. There are also no studies on the correlation between the presence of these genes and MGE, in contrast to studies on environmental *Aeromonas* strains. Hence, the comparison of the mobilome of environmental and clinical isolates of *Aeromonas* at this stage of research is virtually impossible. One can only compare the phenotypic profiles of resistance. The resistance profile of *Aeromonas* clinical and environmental strains is very similar, but additional resistance to chloramphenicol and florfenicol can be found in the latter (Michel et al., 2003). However, resistant profiles differ depending on species and geographical localization. The main reason for this is the serious lack of sufficient data about the contribution of antibiotic resistant clinical strains of *Aeromonas* that are not under epidemiological surveillance in most parts of the world. At this point it is worthwhile to refer to the term "clinical strain" itself, and answer the question what the difference between "clinical" and "environmental" strain is. While in the case of environmental strains an explanation arises spontaneously, it is no longer so obvious for clinical strains. Based on an analysis of the literature data, it can be said that the site of isolation is

the essence of the definition of a clinical strain and the ability to cause disease in humans. Thus, clinical strains may be the same as environmental ones, while the opposite is not always true. The aquatic environment seems to be a "hot spot" for the transmission of antibiotic resistance caused by the selective pressure associated with excessive use of antimicrobial compounds. A wide range of ARG have been found in *Aeromonas* spp., as described in the preceding chapters. Although numerous ARG have been found on plasmids and other MGE, sulfonamide (*sul*), tetracycline (*tet*), quinolone (*qnr*), and β-lactam (*bla*) resistance genes are most common. Nevertheless, there is no correlation between one definite group of plasmids and any particular ARG. In addition, among heavy metal resistance genes, only mercury resistance genes (*mer*) have been found on MGE. They were identified among several R-plasmids that belong to IncA/C. It is worth noticing that the co-localization of heavy metal resistance and ARG on the same MGE can promote a co-selection mechanism (Pérez-Valdespino et al., 2014; Yi et al., 2014b).

It should also be noted that other MGE, such as IS, transposons or mobile integrons form a complex mobilome and may play a significant role in the dissemination of ARG. This can be explained by natural transformation, which is a general property of *Aeromonas* spp. in the environment (Huddleston et al., 2013). Frequent transformation of exogenous DNA may indicate different genetic structures of *Aeromonas* populations, including the participation of various MGE. This is consistent with previous observations in that there is no clear, detectable association between *Aeromonas* species, virulence pattern, source or origin (Tanaka et al., 2013; Martino et al., 2014). However, a review of the literature data shows a clear association between mobilome and ARG. This makes the genus *Aeromonas* a complex one and highlights the fact that there are many mechanisms of antibiotic resistance dissemination among prokaryotes. The situation is different for virulence factors. As knowledge about virulence factors and infection is incomplete, there is no clear evidence of their association with MGE. However, it has been demonstrated that the genomic plasticity of *A. salmonicida* is dependent on various IS. The majority of clinical strains, especially of *A. caviae* are considered pathogenic to humans, but they did not present all of the main known virulence factors (Janda and Kokka, 1991; Khajanchi et al., 2010; Ottaviani et al., 2011). The low prevalence of these factors suggests that pathogenicity may not depend on these virulence markers, but primarily on adaptation toward specific habitats. It should be noted that these processes also play a significant role in the distribution of strains in the environment. Originally, *Aeromonas* spp. were described as fish pathogens. Currently, these bacteria are considered emerging human pathogens, but their effective role in virulence toward humans remains controversial. This does not change the fact that the mobilome of *Aeromonas* has a considerable potential, particularly in terms of antibiotic resistance, the possibility of horizontal transfer of resistance genes, and the threat it may pose to humans.

Acknowledgments

The authors' research was financed by a grant from the National Center of Science, Poland (741/N-COST/2010/0). The authors would like to thank EU for the support provided through COST Action ES1403 "New and emerging challenges and opportunities in wastewater reuse (NEREUS)."

References

Adams, C. A., Austin, B., Meaden, P. G., and McIntosh, D. (1998). Molecular characterization of plasmid-mediated oxytetracycline resistance in *Aeromonas salmonicida*. *Appl. Environ. Microbiol.* 64, 4194–4201.

Adler, A., Assous, M. V., Paikin, S., Shulman, A., Miller-Roll, T., Hillel, S., et al. (2014). Emergence of VIM-producing *Aeromonas caviae* in Israeli hospitals. *J. Antimicrob. Chemother.* 69, 1211–1214. doi: 10.1093/jac/dkt505

Al-Benwan, K., Abbott, S., Janda, J. M., Huys, G., and Albert, M. J. (2007). Cystitis caused by *Aeromonas caviae*. *J. Clin. Microbiol.* 45, 2348–2350. doi: 10.1128/JCM.00480-07

Alcaide, E., Blasco, M. D., and Esteve, C. (2010). Mechanisms of quinolone resistance in *Aeromonas* species isolated from humans, water and eels. *Res. Microbiol.* 161, 40–45. doi: 10.1016/j.resmic.2009.10.006

Aoki, T., Mitoma, Y., and Crosa, J. H. (1986). The characterization of a conjugative R-plasmid isolated from *Aeromonas salmonicida*. *Plasmid* 16, 213–218. doi: 10.1016/0147-619X(86)90059-4

Aravena-Román, M., Inglis, T. J. J., Henderson, B., Riley, T. V., and Chang, B. J. (2012). Antimicrobial susceptibilities of *Aeromonas* strains isolated from clinical and environmental sources to 26 antimicrobial agents. *Antimicrob. Agents Chemother.* 56, 1110–1112. doi: 10.1128/AAC.05387-11

Arias, A., Seral, C., Navarro, F., Miró, E., Coll, P., and Castillo, F. J. (2010). Plasmid-mediated QnrS2 determinant in an *Aeromonas caviae* isolate recovered from a patient with diarrhoea. *Clin. Microbiol. Infect.* 16, 1005–1007. doi: 10.1111/j.1469-0691.2009.02958.x

Beilstein, F., and Dreiseikelmann, B. (2008). Temperate bacteriophage PhiO18P 790 from an *Aeromonas media* isolate: characterization and complete genome sequence. *Virology* 30, 25–29. doi: 10.1016/j.virol.2007.11.016

Bönemann, G., Stiens, M., Pühler, A., and Schlüter, A. (2006). Mobilizable IncQ-related plasmid carrying a new quinolone resistance gene, *qnrS2*, isolated from the bacterial community of a wastewater treatment plant. *Antimicrob. Agents Chemother.* 50, 3075–3080. doi: 10.1128/AAC.00378-06

Bossi-Küpfer, M., Genini, A., Peduzzi, R., and Demarta, A. (2007). Tracheobronchitis caused by *Aeromonas veronii* biovar *sobria* after near-drowning. *J. Med. Microbiol.* 56, 1563–1564. doi: 10.1099/jmm.0.47202-0

Boyd, J., Williams, J., Curtis, B., Kozera, C., Singh, R., and Reith, M. (2003). Three small, cryptic plasmids from *Aeromonas salmonicida* subsp. *salmonicida* A449. *Plasmid* 50, 131–144. doi: 10.1016/S0147-619X(03)00058-1

Brouwer, M. S., Warburton, P. J., Roberts, A. P., Mullany, P., and Allan, E. (2011). Genetic organisation, mobility and predicted functions of genes on integrated, mobile genetic elements in sequenced strains of *Clostridium difficile*. *PLoS ONE* 6:e23014. doi: 10.1371/journal.pone.0023014

Burr, S. E., Pugovkin, D., Wahli, T., Segner, H., and Frey, J. (2005). Attenuated virulence of an *Aeromonas salmonicida* subsp. salmonicida type III secretion mutant in a rainbowtrout model. *Microbiology* 151, 2111–2118. doi: 10.1099/mic.0.27926-0

Carvalho, M. J., Martínez-Murcia, A., Esteves, A. C., Correia, A., and Saavedra, M. J. (2012). Phylogenetic diversity, antibiotic resistance and virulence traits of *Aeromonas* spp. from untreated waters for human consumption. *Int. J. Food Microbiol.* 159, 230–239. doi: 10.1016/j.ijfoodmicro.2012.09.008

Cattoir, V., Poirel, L., Aubert, C., Soussy, C. J., and Nordmann, P. (2008). Unexpected occurrence of plasmid-mediated quinolone resistance

determinants in environmental *Aeromonas* spp. *Emerg. Infect. Dis.* 14, 231–237. doi: 10.3201/eid1402.070677

Cayci, Y. T., Coban, A. Y., and Gunaydin, M. (2014). Investigation of plasmid-mediated quinolone resistance in *Pseudomonas aeruginosa* clinical isolates. *Indian J. Med. Microbiol.* 32, 285–289. doi: 10.4103/0255-0857.136567

Ceylan, E., Berktas, M., and Ağaoğlu, Z. (2009). The occurrence and antibiotic resistance of motile *Aeromonas* in livestock. *Trop. Anim. Health Prod.* 41, 199–204. doi: 10.1007/s11250-008-9175-9

Chim, H., and Song, C. (2007). *Aeromonas* infection in critically ill burn patients. *Burns* 33, 756–759. doi: 10.1016/j.burns.2006.10.389

Chow, M. S., and Rouf, M. A. (1983). Isolation and partial characterization of two *aeromonas hydrophila* bacteriophages. *Appl. Environ. Microbiol.* 45, 1670–1676.

Clinical and Laboratory Standards Institute. (2006). *Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; Approved Guideline. M45-A*. Wayne, PA: Clinical and Laboratory Standards Institute.

Cullik, A., Pfeifer, Y., Prager, R., von Baum, H., and Witte, W. (2010). A novel IS*26* structure surrounds bla_{CTX-M} genes in different plasmids from German clinical *Escherichia coli* isolates. *J. Med. Microbiol.* 59, 580–587. doi: 10.1099/jmm.0.016188-0

Daher, R. K., Filion, G., Tan, S. G., Dallaire-Dufresne, S., Paquet, V. E., and Charette, S. J. (2011). Alteration of virulence factors and rearrangement of pAsa5 plasmid caused by the growth of *Aeromonas salmonicida* in stressful conditions. *Vet. Microbiol.* 152, 353–360. doi: 10.1016/j.vetmic.2011.04.034

Dallaire-Dufresne, S., Barbeau, X., Sarty, D., Tanaka, K. H., Denoncourt, A. M., Lagüe, P., et al. (2013). *Aeromonas salmonicida* Ati2 is an effector protein of the type three secretion system. *Microbiology* 159, 1937–1945. doi: 10.1099/mic.0.067959-0

Dallaire-Dufresne, S., Tanaka, K. H., Trudel, M. V., Lafaille, A., and Charette, S. J. (2014). Virulence, genomic features, and plasticity of *Aeromonas salmonicida* subsp. salmonicida, the causative agent of fish furunculosis. *Vet. Microbiol.* 21, 1–7. doi: 10.1016/j.vetmic.2013.06.025

del Castillo, C. S., Hikima, J., Jang, H. B., Nho, S. W., Jung, T. S., Wongtavatchai, J., et al. (2013). Comparative sequence analysis of a multidrug-resistant plasmid from *Aeromonas hydrophila*. *Antimicrob. Agents Chemother.* 57, 120–129. doi: 10.1128/AAC.01239-12

de Palmenaer, D., Vermeiren, C., and Mahillon, J. (2004). IS*231*-MIC*231* elements from *Bacillus cereus* sensu lato are modular. *Mol. Microbiol.* 53, 457–467. doi: 10.1111/j.1365-2958.2004.04146.x

Dias, C., Mota, V., Martinez-Murcia, A., and José Saavedra, M. (2012). Antimicrobial resistance patterns of *Aeromonas* spp. Isolated from ornamental fish. *J. Aquacult. Res. Dev.* 3:3. doi: 10.4172/2155-9546.1000131

Dobiasova, H., Kutilova, I., Piackova, V., Vesely, T., Cizek, A., and Dolejska, M. (2014). Ornamental fish as a source of plasmid-mediated quinolone resistance genes and antibiotic resistance plasmids. *Vet. Microbiol.* 171, 413–421. doi: 10.1016/j.vetmic.2014.02.011

Dolejska, M., Duskova, E., Rybarikova, J., Janoszowska, D., Roubalova, E., Dibdakova, K., et al. (2011). Plasmids carrying $bla_{CTX-M-1}$ and *qnr* genes in *Escherichia coli* isolates from an equine clinic and a horseback riding centre. *J. Antimicrob. Chemother.* 66, 757–764. doi: 10.1093/jac/dkq500

Edberg, S. C., Browne, F. A., and Allen, M. J. (2007). Issues for microbial regulation: *Aeromonas* as a model. *Crit. Rev. Microbiol.* 33, 89–100. doi: 10.1080/10408410601172180

Fehr, D., Casanova, C., Liverman, A., Blazkova, H., Orth, K., Dobbelaere, D., et al. (2006). AopP, a type III effector protein of *Aeromonas salmonicida*, inhibits the NF-κB signalling pathway. *Microbiology* 152, 2809–2818. doi: 10.1099/mic.0.28889-0

Figueira, V., Vaz-Moreira, I., Silva, M., and Manaia, C. M. (2011). Diversity and antibiotic resistance of *Aeromonas* spp. in drinking and waste water treatment plants. *Water Res.* 45, 5599–5611. doi: 10.1016/j.watres.2011.08.021

Figueras, M. J. (2005). Clinical relevance of *Aeromonas* sM503. *Rev. Med. Microbiol.* 16, 145–153. doi: 10.1097/01.revmedmi.0000184410.98677.8a

Fiorentini, C., Barbieri, E., Falzano, L., Baffone, W., Pianetti, A., Katouli, M., et al. (1998). Occurrence, diversity and pathogenicity of mesophilic *Aeromonas* in estuarine waters of the Italian coast of the Adriatic *Sea. J. Appl. Microbiol.* 85, 501–511. doi: 10.1046/j.1365-2672.1998.853517.x

Fosse, T., Giraud-Morin, C., Madinier, I., Mantoux, F., Lacour, J. P., and Ortonne, J. P. (2004). *Aeromonas hydrophila* with plasmid-borne class A extended-spectrum β-lactamase TEM-24 and three chromosomal class B, C, and D β-lactamases, isolated from a patient with necrotizing fasciitis. *Antimicrob. Agents Chemother.* 48, 2342–2343. doi: 10.1128/AAC.48.6.2342-2343.2004

Fricke, W. F., Welch, T. J., McDermott, P. F., Mammel, M. K., LeClerc, J. E., White, D. G., et al. (2009). Comparative genomics of the IncA/C multidrug resistance plasmid family. *J. Bacteriol.* 191, 4750–4757. doi: 10.1128/JB.00189-09

García, P., Guerra, B., Bances, M., Mendoza, M. C., and Rodicio, M. R. (2011). IncA/C plasmids mediate antimicrobial resistance linked to virulence genes in the Spanish clone of the emerging *Salmonella enterica* serotype 4,[5],12:i:-. *J. Antimicrob. Chemother.* 66, 543–549. doi: 10.1093/jac/dkq481

Ghenghesh, K. S., Ahmed, S. F., El-Khalek, R. A., Al-Gendy, A., and Klena, J. (2008). *Aeromonas*-associated infections in developing countries. *J. Infect. Dev. Ctries.* 2, 81–98. doi: 10.3855/jidc.277

Giraud-Morin, C., and Fosse, T. (2003). A seven-year survey of *Klebsiella pneumoniae* producing TEM-24 extended-spectrum β-lactamase in Nice University Hospital (1994–2000). *J. Hosp. Infect.* 54, 25–31. doi: 10.1016/S0195-6701(03)00038-0

Girlich, D., Poirel, L., and Nordmann, P. (2010). PER-6, an extended-spectrum beta-lactamase from *Aeromonas allosaccharophila*. *Antimicrob. Agents Chemother.* 54, 1619–1622. doi: 10.1128/AAC.01585-09

Girlich, D., Poirel, L., and Nordmann, P. (2011). Diversity of clavulanic acid-inhibited extended-spectrum β-lactamases in *Aeromonas* spp. from the Seine River, Paris, France. *Antimicrob. Agents Chemother.* 55, 1256–1261. doi: 10.1128/AAC.00921-10

Gómez-Garcés, J. L., Saéz, D., Almagro, M., Fernández-Romero, S., Merino, F., Campos, J., et al. (2011). Osteomyelitis associated to CTX-M-15-producing *Aeromonas hydrophila*: first description in the literature. *Diagn. Microbiol. Infect. Dis.* 70, 420–422. doi: 10.1016/j.diagmicrobio.2011.03.004

Gordon, L., Cloeckaert, A., Doublet, B., Schwarz, S., Bouju-Albert, A., Ganière, J. P., et al. (2008). Complete sequence of the *floR*-carrying multiresistance plasmid pAB5S9 from freshwater *Aeromonas bestiarum*. *J. Antimicrob. Chemother.* 62, 65–71. doi: 10.1093/jac/dkn166

Guo, Y. F., Zhang, W. H., Ren, S. Q., Yang, L., Lü, D. H., Zeng, Z. L., et al. (2014). IncA/C plasmid-mediated spread of CMY-2 in multidrug-resistant *Escherichia coli* from food animals in China. *PLoS ONE* 9:e96738. doi: 10.1371/journal.pone.0096738

Hall, R. M., Collis, C. M., Kim, M. J., Partridge, S. R., Recchia, G. D., and Stokes, H. W. (1999). Mobile gene cassettes and integrons in evolution. *Ann. N. Y. Acad. Sci.* 870, 68–80. doi: 10.1111/j.1749-6632.1999.tb08866.x

Han, H. J., Taki, T., Kondo, H., Hirono, I., and Aoki, T. (2008). Pathogenic potential of a collagenase from *Aeromonas veronii*. *Can. J. Microbiol.* 54, 1–10. doi: 10.1139/W07-109

Han, J. E., Kim, J. H., Choresca, C. H. Jr., Shin, S. P., Jun, J. W., Chai, J. Y., et al. (2012a). First description of ColE-type plasmid in *Aeromonas* spp. carrying quinolone resistance (*qnrS2*) gene. *Lett. Appl. Microbiol.* 55, 290–294. doi: 10.1111/j.1472-765X.2012.03293.x

Han, J. E., Kim, J. H., Choresca, C. H. Jr., Shin, S. P., Jun, J. W., Chai, J. Y., et al. (2012b). Prevalence of *tet* gene and complete genome sequencing of *tet* gene-encoded plasmid (pAHH01) isolated from *Aeromonas* species in South Korea. *J. Appl. Microbiol.* 112, 631–638. doi: 10.1111/j.1365-2672.2012.05237.x

Han, J. E., Kim, J. H., Choresca, C. H. Jr., Shin, S. P., Jun, J. W., Chai, J. Y., et al. (2012c). A small IncQ-type plasmid carrying the quinolone resistance (*qnrS2*) gene from *Aeromonas hydrophila*. *Lett. Appl. Microbiol.* 54, 374–376. doi: 10.1111/j.1472-765X.2012.03208.x

Hedges, R. W., Smith, P., and Brazil, G. (1985). Resistance Plasmids of Aeromonads. *J. Gen. Microbiol.* 131, 2091–2095. doi: 10.1099/00221287-131-8-2091

Henriques, I. S., Fonseca, F., Alves, A., Saavedra, M. J., and Correia, A. (2006). Occurrence and diversity of integrons and β-lactamase genes among ampicillin-resistant isolates from estuarine waters. *Res. Microbiol.* 157, 938–947. doi: 10.1016/j.resmic.2006.09.003

Holmberg, S. D., and Farmer, III J. J. (1984). *Aeromonas hydrophila* and *Plesiomonas shigelloides* as causes of intestinal infection. *Rev. Infect. Dis.* 6, 633–639. doi: 10.1093/clinids/6.5.633

Hossain, M. J., Waldbieser, G. C., Sun, D., Capps, N. K., Hemstreet, W. B., Carlisle, K., et al. (2013). Implication of lateral genetic transfer in the emergence of

Aeromonas hydrophila isolates of epidemic outbreaks in channel catfish. *PLoS ONE* 20:e80943. doi: 10.1371/journal.pone.0080943

Huang, H. C., Yu, W. L., Huan, K. H., Cheng, K. C., and Chuang, Y. C. (2007). *Aeromonas sobria* prostatitis and septic shock in a healthy man with chronic alcoholic consumption. *Jpn. J. Infect. Dis.* 60, 400–401.

Huddleston, J. R., Brokaw, J. M., Zak, J. C., and Jeter, R. M. (2013). Natural transformation as a mechanism of horizontal gene transfer among environmental *Aeromonas* species. *Syst. Appl. Microbiol.* 36, 224–234. doi: 10.1016/j.syapm.2013.01.004

Imamura, T., Nitta, H., Wada, Y., Kobayashi, H., and Okamoto, K. (2008). Impaired plasma clottability induction through fibrinogen degradation by ASP, a serine protease released from *Aeromonas sobria*. *FEMS. Microbiol. Lett*. 284, 35–42. doi: 10.1111/j.1574-6968.2008.01184.x

Ishiguro, E. E., Kay, W. W., Ainsworth, T., Chamberlain, J. B., Austen, R. A., Buckley, J. T., et al. (1981). Loss of virulence during culture of *Aeromonas salmonicida* at high temperature. *J. Bacteriol.* 148, 333–340.

Jacobs, L., and Chenia, H. Y. (2007). Characterization of integrons and tetracycline resistance determinants in *Aeromonas* spp. isolated from South African aquaculture systems. *Int. J. Food Microbiol.* 114, 295–306. doi: 10.1016/j.ijfoodmicro.2006.09.030

Janda, J. M., and Kokka, R. P. (1991). The pathogenicity of *Aeromonas* strains relative to genospecies and phenospecies identification. *FEMS. Microbiol. Lett*. 90, 29–34. doi: 10.1111/j.1574-6968.1991.tb05120.x

Jangid, K., Kong, R., Patole, M. S., and Shouche, Y. S. (2007). *luxRI* homologs are universally present in the genus *Aeromonas*. *BMC. Microbiol.* 7:93. doi: 10.1186/1471-2180-7-93

John, N., and Hatha, A. A. M. (2013). Distribution, extracellular virulence factors and drug resistance of motile aeromonads in fresh water ornamental fishes and associated carriage water. *Int. J. Aquacult.* 3, 92–100. doi: 10.5376/ija.2013.03.0017

Jones, B. L., and Wilcox, M. H. (1995). *Aeromonas* infections and their treatment. *J. Antimicrob. Chemother*. 35, 453–461. doi: 10.1093/jac/35.4.453

Jones, R. M., Luo, L., and Moberg, K. H. (2012). *Aeromonas salmonicida*-secreted protein AopP is a potent inducer of apoptosis in a mammalian and a *Drosophila* model. *Cell Microbiol*. 14, 274–285. doi: 10.1111/j.1462-5822.2011.01717.x

Jorge, M. T., Nishioka Sde, A., de Oliveirá, R. B., Ribeiro, L. A., and Silveira, P. V. (1998). *Aeromonas hydrophila* soft-tissue infection as a complication of snake bite: report of three cases. *Ann. Trop. Med. Parasitol.* 92, 213–217. doi: 10.1080/00034989860067

Jorgensen, J. H., and Hindler, J. F. (2007). New consensus guidelines from the Clinical and Laboratory Standards Institute for antimicrobial susceptibility testing of infrequently isolated or fastidious bacteria. *Clin. Infect. Dis.* 44, 280–286. doi: 10.1086/510431

Kehrenberg, C., Friederichs, S., de Jong, A., Michael, G. B., and Schwarz, S. (2006). Identification of the plasmid-borne quinolone resistance gene *qnrS* in *Salmonella enterica* serovar *Infantis*. *J. Antimicrob. Chemother*. 58, 18–22. doi: 10.1093/jac/dkl213

Khajanchi, B. K., Fadl, A. A., Borchardt, M. A., Berg, R. L., Horneman, A. J., Stemper, M. E., et al. (2010). Distribution of virulence factors and molecular fingerprinting of Aeromonas species isolates from water and clinical samples: suggestive evidence of water-to-human transmission. *Appl. Environ. Microbiol*. 76, 2313–2325. doi: 10.1128/AEM.02535-09

Khan, M. I., Walters, G., and Metcalfe, T. (2007). Bilateral endogenous endophthalmitis caused by *Aeromonas hydrophila*. *Eye.* 21, 1244–1245. doi: 10.1038/sj.eye.6702926

Khan, R., Takahashi, E., Nakura, H., Ansaruzzaman, M., Banik, S., Ramamurthy, T., et al. (2008). Toxin production by *Aeromonas sobria* in natural environments: river water vs. seawater. *Acta. Med. Okayama* 62, 363–371.

Kim, J. H., Son, J. S., Choi, Y. J., Choresca, C. H., Shin, S. P., Han, J. E., et al. (2012a). Isolation and characterization of a lytic *Myoviridae* bacteriophage PAS-1 with broad infectivity in *Aeromonas salmonicida*. *Curr. Microbiol.* 64, 418–426. doi: 10.1007/s00284-012-0091-x

Kim, J. H., Son, J. S., Choi, Y. J., Choresca, C. H., Shin, S. P., Han, J. E., et al. (2012b). Complete genomic sequence of a T4-like bacteriophage, phiAS4, infecting *Aeromonas salmonicida* subsp. *salmonicida*. *Arch. Virol.* 157, 391–395. doi: 10.1007/s00705-011-1175-9

Kingombe, C. I. B., Huys, G., Howald, D., Luthi, E., Swing, J., and Jemmi, T. (2004). The usefulness of molecular techniques to assess the presence of *Aeromonas* spp. harbouring virulence markers in foods. *Int. J. Food Microbiol*. 94, 113–121. doi: 10.1016/S0168-1605(03)00105-3

Ko, W. C., Lee, H. C., Chuang, Y. C., Liu, C. C., and Wu, J. J. (2000). Clinical features and therapeutic implications of 104 episodes of monomicrobial *Aeromonas bacteraemia*. *J. Infect.* 40, 267–273. doi: 10.1053/jinf.2000.0654

Koczura, R., Semkowska, A., and Mokracka, J. (2014). Integron-bearing Gram-negative bacteria in lake waters. *Lett. Appl. Microbiol*. 59, 514–519. doi: 10.1111/lam.12307

Kulinska, A., Czeredys, M., Hayes, F., and Jagura-Burdzy, G. (2008). Genomic and functional characterization of the modular broad-host-range RA3 plasmid, the archetype of the IncU group. *Appl. Environ. Microbiol*. 74, 4119–4132. doi: 10.1128/AEM.00229-08

L'Abée-Lund, T. M., and Sørum, H. (2002). A global non-conjugative Tet C plasmid, pRAS3, from *Aeromonas salmonicida*. *Plasmid* 47, 172–181. doi: 10.1016/S0147-619X(02)00001-X

Lamy, B., Kodjo, A., colBVH Study Group, and Laurent, F. (2009). Prospective nationwide study of *Aeromonas* infections in France. *J. Clin. Microbiol*. 47, 1234–1237. doi: 10.1128/JCM.00155-09

Laroche, E., Pawlak, B., Berthe, T., Skurnik, D., and Petit, F. (2009). Occurrence of antibiotic resistance and class 1, 2 and 3 integrons in *Escherichia coli* isolated from a densely populated estuary (Seine, France). *FEMS Microbiol. Ecol*. 68, 118–130. doi: 10.1111/j.1574-6941.2009.00655.x

Lau, S. M., Peng, M. Y., and Chang, F. Y. (2000). Outcomes of *Aeromonas* bacteremia in patients with different types of underlying disease. *J. Microbiol. Immunol. Infect*. 33, 241–247.

Lazar, V., Cernat, R., Balotsen, C., Cotar, A., Coipan, E., and Cojocaru, C. (2002). Correlation between multiple antibiotic resistance and heavy metal tolerance among some *E. coli* strains isolated from polluted waters. *Bacteriol. Virusol. Parazitol. Epidemiol*. 47, 155–160.

Libisch, B., Gisk, C. G., Kovács, B., Tóth, T. G., and Füzi, M. (2008). Identification of the first VIM metallo-beta-lactamase-producing multiresistant *Aeromonas hydrophila* strain. *J. Clin. Microbiol*. 46, 1878–1880. doi: 10.1128/JCM.00047-08

Liebert, C. A., Hall, R. M., and Summers, A. O. (1999). Transposon Tn*21*, flagship of the floating genome. *Microbiol. Mol. Biol. Rev*. 63, 507–522.

Lin, W. T., Su, S. Y., Lai, C. C., Tsai, T. C., Gau, S. J., and Chao, C. M. (2013). Peritonitis caused by *Aeromonas* species at a hospital in southern Taiwan. *Intern. Med*. 55, 2517–2521. doi: 10.2169/internalmedicine.52.0180

Loftie-Eaton, W., and Rawlings, D. E. (2009). Comparative biology of two natural variants of the IncQ-2 family plasmids, pRAS3.1 and pRAS3.2. *J. Bacteriol*. 191, 6436–6446. doi: 10.1128/JB.00864-09

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., et al. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect*. 18, 268–281. doi: 10.1111/j.1469-0691.2011.03570.x

Majumdar, T., Das, B., Bhadra, R. K., Dam, B., and Mazumder, S. (2011). Complete nucleotide sequence of a quinolone resistance gene (*qnrS2*) carrying plasmid of *Aeromonas hydrophila* isolated from fish. *Plasmid* 66, 79–84. doi: 10.1016/j.plasmid.2011.05.001

Maravić, A., Skoèibušić, M., Samanić, I., Fredotović, Z., Cvjetan, S., Jutronić, M., et al. (2013). *Aeromonas* spp. simultaneously harbouring *bla*(*CTX-M-15*), *bla*(*SHV-12*), *bla*(*PER-1*) and *bla*(*FOX-2*), in wild-growing Mediterranean mussel (*Mytilus galloprovincialis*) from Adriatic Sea, Croatia. *Int. J. Food. Microbiol*. 166, 301–308. doi: 10.1016/j.ijfoodmicro.2013.07.010

Marchandin, H., Godreuil, S., Darbas, H., Jean-Pierre, H., Jumas-Bilak, E., Chanal, C., et al. (2003). Extended-spectrum beta-lactamase TEM-24 in an *Aeromonas* clinical strain: acquisition from the prevalent *Enterobacter aerogenes* clone in France. *Antimicrob. Agents Chemother*. 47, 3994–3995. doi: 10.1128/AAC.47.12.3994-3995.2003

Marchandin, H., Jean-Pierre, H., De Champs, C., Sirot, D., Darbas, H., Perigault, P. F., et al. (2000). Production of a TEM-24 plasmid-mediated extended-spectrum β-lactamase by a clinical isolate of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother*. 44, 213–216. doi: 10.1128/AAC.44.1.213-216.2000

Marti, E., and Balcázar, J. L. (2012). Multidrug resistance-encoding plasmid from *Aeromonas* sp. strain P2G1. *Clin. Microbiol. Infect*. 18, E366–E368. doi: 10.1111/j.1469-0691.2012.03935.x

Martin-Carnahan, A., and Joseph, S. W. (2005). *Genus I. Aeromonas Stanier 1943, 213AL, p. 557–578. Bergey's Manual of Systematic Bacteriology*. New York, NY: Springer.

Martino, M. E., Fasolato, L., Montemurro, F., Novelli, E., and Cardazzo, B. (2014). *Aeromonas* spp.: ubiquitous or specialized bugs? *Environ. Microbiol.* 16, 1005–1018. doi: 10.1111/1462-2920.12215

Mataseje, L. F., Baudry, P. J., Zhanel, G. G., Morck, D. W., Read, R. R., Louie, M., et al. (2010). Comparison of CMY-2 plasmids isolated from human, animal, and environmental *Escherichia coli* and *Salmonella* spp. from Canada. *Diagn. Microbiol. Infect. Dis*. 67, 387–391. doi: 10.1016/j.diagmicrobio.2010.02.027

McIntosh, D., Cunningham, M., Ji, B., Fekete, F. A., Parry, E. M., Clark, S. E., et al. (2008). Transferable, multiple antibiotic and mercury resistance in Atlantic Canadian isolates of *Aeromonas salmonicida* subsp. *salmonicida* is associated with carriage of an IncA/C plasmid similar to the *Salmonella enterica* plasmid pSN254. *J. Antimicrob. Chemother.* 61, 1221–1228. doi: 10.1093/jac/dkn123

Merino, S., Aguilar, A., Rubires, X., Simon-Pujol, D., Congregado, F., and Tomás, J. M. (1996). The role of the capsular polysaccharide of *Aeromonas salmonicida* in the adherence and invasion of fish cell lines. *FEMS Microbiol. Lett*. 142, 185–189. doi: 10.1111/j.1574-6968.1996.tb08428.x

Michel, C., Kerouault, B., and Martin, C. (2003). Chloramphenicol and florfenicol susceptibility of fish-pathogenic bacteria isolated in France: comparison of minimum inhibitory concentration, using recommended provisory standards for fish bacteria. *J. Appl. Microbiol.* 95, 1008–1015. doi: 10.1046/j.1365-2672.2003.02093.x

Moura, A., Henriques, I., Ribeiro, R., and Correia, A. (2007). Prevalence and characterization of integrons from bacteria isolated from a slaughterhouse wastewater treatment plant. *J. Antimicrob. Chemother*. 60, 1243–1250. doi: 10.1093/jac/dkm340

Moura, A., Oliveira, C., Henriques, I., Smalla, K., and Correia, A. (2012). Broad diversity of conjugative plasmids in integron-carrying bacteria from wastewater environments. *FEMS Microbiol. Lett*. 330, 157–164. doi: 10.1111/j.1574-6968.2012.02544.x

Najimi, M., Lemos, M. L., and Osorio, C. R. (2009). Identification of iron regulated genes in the fish pathogen *Aeromonas salmonicida* subsp. salmonicida: genetic diversity and evidence of conserved iron uptake systems. *Vet. Microbiol*. 133, 377–382. doi: 10.1016/j.vetmic.2008.07.008

Neuwirth, C., Siebor, E., Robin, F., and Bonnet, R. (2007). First occurrence of an IMP metallo-beta-lactamase in *Aeromonas caviae*: IMP-19 in an isolate from France. *Antimicrob. Agents Chemother*. 51, 4486–4488. doi: 10.1128/AAC.01462-06

Neyts, K., Huys, G., Uyttendaele, M., Swings, J., and Debevere, J. (2000). Incidence and identification of mesophilic *Aeromonas* spp. from retail foods. *Lett. Appl. Microbiol*. 31:359–363. doi: 10.1046/j.1472-765x.2000.00828.x

Nguyen, H. N., Van, T. T., Nguyen, H. T., Smooker, P. M., Shimeta, J., and Coloe, P. J. (2014). Molecular characterization of antibiotic resistance in *Pseudomonas* and *Aeromonas* isolates from catfish of the Mekong Delta, Vietnam. *Vet. Microbiol*. 171, 397–405. doi: 10.1016/j.vetmic.2014.01.028

Noonan, B., and Trust, T. J. (1997). The synthesis, secretion and role in virulence of the paracrystalline surface protein layers of *Aeromonas salmonicida* and *A. hydrophila*. *FEMS Microbiol. Lett*. 154, 1–7. doi: 10.1111/j.1574-6968.1997.tb12616.x

Nordmann, P., and Poirel, L. (2005). Emergence of plasmid-mediated resistance to quinolones in *Enterobacteriaceae*. *J. Antimicrob. Chemother*. 56, 463–469. doi: 10.1093/jac/dki245

Oliveira, P. H., Touchon, M., and Rocha, E. P. (2014). The interplay of restriction-modification systems with mobile genetic elements and their prokaryotic hosts. *Nucleic Acids Res*. 42, 10618–10631. doi: 10.1093/nar/gku734

Oliver, K. R., McComb, J. A., and Greene, W. K. (2013). Transposable elements: powerful contributors to angiosperm evolution and diversity. *Genome Biol Evol*. 5, 1886–1901. doi: 10.1093/gbe/evt141

Ottaviani, D., Parlani, C., Citterio, B., Masini, L., Leoni, F., Canonico, C., et al. (2011). Putative virulence properties of *Aeromonas* strains isolated from food, environmental and clinical sources in Italy: a comparative study. *Int. J. Food Microbiol*. 5, 538–545. doi: 10.1016/j.ijfoodmicro.2010.11.020

Palumbo, S. A., Maxino, F., Williams, A. C., Buchanan, R. L., and Thayer, D. T. W. (1985). Starch-ampicillin agar for the quantitative detection of *Aeromonas hydrophila*. *Appl. Environ. Microbiol*. 50, 1027–1030.

Pan, J. C., Ye, R., Wang, H. Q., Xiang, H. Q., Zhang, W., Yu, X. F., et al. (2008). *Vibrio cholerae* O139 multiple-drug resistance mediated by *Yersinia pestis* pIP1202-like conjugative plasmids. *Antimicrob. Agents Chemother*. 52, 3829–3836. doi: 10.1128/AAC.00375-08

Pasom, W., Chanawong, A., Lulitanond, A., Wilailuckana, C., Kenprom, S., and Puang-Ngern, P. (2013). Plasmid-mediated quinolone resistance genes, *aac(6′)-Ib-cr*, *qnrS*, *qnrB*, and *qnrA*, in urinary isolates of *Escherichia coli* and *Klebsiella pneumoniae* at a teaching hospital, Thailand. *Jpn. J. Infect. Dis*. 66, 428–432. doi: 10.7883/yoken.66.428

Pasquali, F., Kehrenberg, C., Manfreda, G., and Schwarz, S. (2005). Physical linkage of Tn*3* and part of Tn*1721* in a tetracycline and ampicillin resistance plasmid from *Salmonella Typhimurium*. *J. Antimicrob. Chemother*. 55, 562–565. doi: 10.1093/jac/dkh553

Pérez-Valdespino, A., Celestino-Mancera, M., Villegas-Rodríguez, V. L., and Curiel-Quesada, E. (2014). Characterization of mercury-resistant clinical *Aeromonas* species. *Braz. J. Microbiol*. 10, 1279–1283.

Picão, R. C., Cardoso, J. P., Campana, E. H., Nicoletti, A. G., Petrolini, F. V., Assis, D. M., et al. (2013). The route of antimicrobial resistance from the hospital effluent to the environment: focus on the occurrence of KPC-producing *Aeromonas* spp. and Enterobacteriaceae in sewage. *Diagn. Microbiol. Infect. Dis*. 76, 80–85. doi: 10.1016/j.diagmicrobio.2013.02.001

Picão, R. C., Poirel, L., Demarta, A., Silva, C. S., Corvaglia, A. R., Petrini, O., et al. (2008). Plasmid-mediated quinolone resistance in *Aeromonas allosaccharophila* recovered from a Swiss lake. *J. Antimicrob. Chemother*. 62, 948–950. doi: 10.1093/jac/dkn341

Piotrowska, M., and Popowska, M. (2014). The prevalence of antibiotic resistance genes among *Aeromonas* species in aquatic environments. *Ann. Microbiol*. 64, 921–934. doi: 10.1007/s13213-014-0911-2

Puah, S. M., Puthucheary, S. D., Liew, F. Y., and Chua, K. H. (2013). *Aeromonas aquariorum* clinical isolates: antimicrobial profiles, plasmids and genetic determinants. *Int. J. Antimicrob. Agents* 41, 281–284. doi: 10.1016/j.ijantimicag.2012.11.012

Puthucheary, S. D., Puah, S. M., and Chua, K. H. (2012). Molecular characterization of clinical isolates of *Aeromonas* species from Malaysia. *PLOS. ONE* 7:e30205. doi: 10.1371/journal.pone.0030205

Rahman, M., Colque-Navarro, P., Kühn, I., Huys, G., Swings, J., and Möllby, R. (2002). Identification and characterization of pathogenic *Aeromonas veronii* biovar *sobria* associated with epizootic ulcerative syndrome in fish in Bangladesh. *Appl. Environ. Microbiol*. 68, 650–655. doi: 10.1128/AEM.68.2.650-655.2002

Reith, M. E., Singh, R. K., Curtis, B., Boyd, J. M., Bouevitch, A., Kimball, J., et al. (2008). The genome of *Aeromonas salmonicida* subsp. *salmonicida* A449: insights into the evolution of a fish pathogen. *BMC Genomics* 9:427. doi: 10.1186/1471-2164-9-427

Rhodes, G., Huys, G., Swings, J., McGann, P., Hiney, M., Smith, P., et al. (2000). Distribution of oxytetracycline resistance plasmids between aeromonads in hospital and aquaculture environments: implication of Tn*1721* in dissemination of the tetracycline resistance determinant *tetA*. *Appl. Environ. Microbiol.* 66, 3883–3890. doi: 10.1128/AEM.66.9.3883-3890.2000

Rhodes, G., Parkhill, J., Bird, C., Ambrose, K., Jones, M. C., Huys, G., et al. (2004). Complete nucleotide sequence of the conjugative tetracycline resistance plasmid pFBAOT6, a member of a group of IncU plasmids with global ubiquity. *Appl. Environ. Microbiol*. 70, 7497–7510. doi: 10.1128/AEM.70.12.7497-7510.2004

Sánchez-Céspedes, J., Blasco, M. D., Marti, S., Alba, V., Alcaide, E., Esteve, C., and Vila, J. (2008). Plasmid-mediated QnrS2 determinant from a clinical *Aeromonas veronii* isolate. *Antimicrob. Agents Chemother*. 52, 2990–2991. doi: 10.1128/AAC.00287-08

Sandaa, R. A., and Enger, O. (1994). Transfer in marine sediments of the naturally occurring plasmid pRAS1 encoding multiple antibiotic resistance. *Appl. Environ. Microbiol*. 60, 4234–4238.

Sarria-Guzmán, Y., López-Ramírez, M. P., Chávez-Romero, Y., Ruiz-Romero, E., Dendooven, L., and Bello-López, J. M. (2014). Identification of antibiotic resistance cassettes in class 1 integrons in *Aeromonas* spp. strains isolated from fresh fish (*Cyprinus carpio L.*). *Curr. Microbiol*. 68, 581–586. doi: 10.1007/s00284-013-0511-6

Schmidt, A. S., Bruun, M. S., Dalsgaard, I., and Larsen, and, J. L. (2001). Incidence, distribution, and spread of tetracycline resistance determinants and integron-associated antibiotic resistance genes among motile aeromonads from a fish farming environment. *Appl. Environ. Microbiol.* 67, 5675–5682. doi: 10.1128/AEM.67.12.5675-5682.2001

Seetha, K. S., Jose, B. T., and Jasthi, A. (2004). Meningitis due to *Aeromonas hydrophila*. *Indian. J. Med. Microbiol.* 22, 191–192.

Shak, J. R., Whitaker, J. A., Ribner, B. S., and Burd, E. M. (2011). Aminoglycoside-resistant *Aeromonas hydrophila* as part of a polymicrobial infection following a traumatic fall into freshwater. *J. Clin. Microbiol.* 49, 1169–1170. doi: 10.1128/JCM.01949-10

Shen, C. J., Liu, Y. J., and Lu, C. P. (2012). Complete genome sequence of *Aeromonas hydrophila* phage CC2. *J. Virol.* 86:10900. doi: 10.1128/JVI.01882-12

Sørum, H., L'Abée-Lund, T. M., Solberg, A., and Wold, A. (2003). Integron-containing IncU R plasmids pRAS1 and pAr-32 from the fish pathogen *Aeromonas salmonicida*. *Antimicrob. Agents Chemother.* 47, 1285–1290. doi: 10.1128/AAC.47.4.1285-1290.2003

Stokes, H. W., and Hall, R. M. (1989). A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. *Mol. Microbiol.* 3, 1669–1683. doi: 10.1111/j.1365-2958.1989.tb00153.x

Stuber, K., Burr, S. E., Braun, M., Wahli, T., and Frey, J. (2003). Type III secretion genes in *Aeromonas salmonicida* subsp *salmonicida* are located on a large thermolabile virulence plasmid. *J. Clin. Microbiol.* 41, 3854–3856. doi: 10.1128/JCM.41.8.3854-3856.2003

Suarez, G., Sierra, J. C., Sha, J., Wang, S., Erova, T. E., Fadl, A. A., et al. (2008). Molecular characterization of a functional type VI secretion system from a clinical isolate of *Aeromonas hydrophila*. *Microb. Pathog.* 44, 344–361. doi: 10.1016/j.micpath.2007.10.005

Tanaka, K. H., Frenette, M., and Charette, S. J. (2013). IS-mediated loss of virulence by *Aeromonas salmonicida*: a tangible piece of an evolutionary puzzle. *Mob. Genet. Elements* 3:e23498. doi: 10.4161/mge.23498

Thomas, C. M., and Nielsen, K. M. (2005). Mechanisms of, and Barriers to, horizontal gene transfer between bacteria. *Nat. Rev. Microbiol.* 3, 711–721. doi: 10.1038/nrmicro1234

Trudel, M. V., Tanaka, K. H., Filion, G., Daher, R. K., Frenette, M., and Charette, S. J. (2013). Insertion sequence AS5 (IS AS5) is involved in the genomic plasticity of *Aeromonas salmonicida*. *Mob. Genet. Elements* 1:e25640. doi: 10.4161/mge.25640

Tsai, M. S., Kuo, C. Y., Wang, M. C., Wu, H. C., Chien, C. C., and Liu, J. W. (2006). Clinical features and risk factors for mortality in *Aeromonas* bacteremic adults with hematologic malignancies. *J. Microbiol. Immunol. Infect.* 39, 150–154.

Vally, H., Whittle, A., Cameron, S., Dowse, G. K., and Watson, T. (2004). Outbreak of *Aeromonas hydrophila* wound infections associated with mud football. *Clin. Infect. Dis.* 38, 1084–1089. doi: 10.1086/382876

Vanden Bergh, P., Heller, M., Braga-Lagache, S., and Frey, J. (2013). The *Aeromonas salmonicida* subsp. salmonicida exoproteome: determination of the complete repertoire of Type-Three Secretion System effectors and identification of other virulence factors. *Proteome Sci.* 11:42. doi: 10.1186/1477-5956-11-42

Vincent, A. T., Trudel, M. V., Paquet, V. E., Boyle, B., Tanaka, K. H., Dallaire-Dufresne, S., et al. (2014). Detection of variants of the pRAS3, pAB5S9, and pSN254 plasmids in *Aeromonas salmonicida* subsp. *salmonicida*: multidrug resistance, interspecies exchanges, and plasmid reshaping. *Antimicrob. Agents Chemother.* 58, 7367–7374. doi: 10.1128/AAC.03730-14

Winokur, P. L., Vonstein, D. L., Hoffman, L. J., Uhlenhopp, E. K., and Doern, G. V. (2001). Evidence for transfer of CMY-2 AmpC beta-lactamase plasmids between *Escherichia coli* and *Salmonella* isolates from food animals and humans. *Antimicrob. Agents Chemother.* 45, 2716–2722. doi: 10.1128/AAC.45.10.2716-2722.2001

Wu, C. J., Chen, P. L., Hsueh, P. R., Chang, M. C., Tsai, P. J., Shih, H. I., et al. (2015). Clinical implications of species identification in monomicrobial *Aeromonas* bacteremia. *PLoS ONE*10:e0117821. doi: 10.1371/journal.pone.0117821

Wu, C. J., Chuang, Y. C., Lee, M. F., Lee, C. C., Lee, H. C., Lee, N. Y., et al. (2011). Bacteremia due to extended-spectrum-β-lactamase-producing *Aeromonas* spp. at a medical center in Southern Taiwan. *Antimicrob. Agents Chemother.* 55, 5813–5818. doi: 10.1128/AAC.00634-11

Ye, Y., Xu, X. H., and Li, J. B. (2010). Emergence of CTX-M-3, TEM-1 and a new plasmid-mediated MOX-4 AmpC in a multiresistant *Aeromonas caviae* isolate from a patient with pneumonia. *J. Med. Microbiol.* 59, 843–847. doi: 10.1099/jmm.0.016337-0

Yi, S. W., Chung, T. H., Joh, S. J., Park, C., Park, B. Y., and Shin, G. W. (2014a). High Prevalence of bla_{CTX-M} group Genes in *Aeromonas dhakensis* Isolated from Aquaculture Fish Species in South Korea. *J. Vet. Med. Sci.* 2014:3. doi: 10.1292/jvms.14-0274

Yi, S. W., Kim, D. C., You, M. J., Kim, B. S., Kim, W. I., and Shin, G. W. (2014b). Antibiotic and heavy-metal resistance in motile *Aeromonas* strains isolated from fish. *Afr. J. Microbiol. Res.* 8, 1793–1797. doi: 10.5897/AJMR2013.6339

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 27 August 2015
doi: 10.3389/fmicb.2015.00864

The horizontal transfer of antibiotic resistance genes is enhanced by ionic liquid with different structure of varying alkyl chain length

Qing Wang[1,2], Qian Lu[2], Daqing Mao[1], Yuxiao Cui[2] and Yi Luo[2]**

[1] School of Environmental Science and Engineering, Tianjin University, Tianjin, China, [2] Ministry of Education Key Laboratory of Pollution Processes and Environmental Criteria, College of Environmental Science and Engineering, Nankai University, Tianjin, China

OPEN ACCESS

Edited by:
Amy Pruden,
Virginia Polytechnic Institute and State University, USA

Reviewed by:
Etinosa Igbinosa,
University of Benin, Nigeria
Pascal Saikaly,
King Abdullah University of Science and Technology, Saudi Arabia

***Correspondence:**
Daqing Mao,
School of Environmental Science and Engineering, Tianjin University, Tianjin 300072, China
mao@tju.edu.cn;
Yi Luo,
Ministry of Education Key Laboratory of Pollution Processes and Environmental Criteria, College of Environmental Science and Engineering, Nankai University, Tianjin 300071, China
luoy@nankai.edu.cn

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: 30 April 2015
Accepted: 07 August 2015
Published: 27 August 2015

Citation:
Wang Q, Lu Q, Mao D, Cui Y and Luo Y (2015) The horizontal transfer of antibiotic resistance genes is enhanced by ionic liquid with different structure of varying alkyl chain length. Front. Microbiol. 6:864.doi: 10.3389/fmicb.2015.00864

Antibiotic resistance genes (ARGs) have become a global health concern. In our previous study, an ionic liquid (IL) 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIm][PF6]) had been proven to facilitate the dissemination of ARGs in the environment. However, enhanced alkyl group chain length or the substitution of alkyl groups with the cation ring corresponded with increased antimicrobial effects. In this study, we investigated how different structures of ILs with 4, 6, and 8 C atoms in the longer alkyl chain on the imidazolium cations facilitated the dissemination of ARGs. The promotion of plasmid RP4 transfer frequency decreased with [CnMIM][BF4] increasing the alkyl chain length from 4 carbon atoms to 8 carbon atoms on the imidazolium cations, which is observed with [BMIM][BF4] ($n = 4$, 5.9 fold) > HMIM][BF4] ($n = 6$, 2.2 fold) > [OMIM][BF4] ($n = 8$, 1.7 fold). This illustrates that [CnMIM][BF4] with increasing the alkyl chain length exert decreasing ability in facilitating plasmid RP4 horizontal transfer, which is possibly related to IL-structure dependent toxicity. The IL-structure dependent plasmid RP4 transfer frequency was attributable to bacterial cell membrane permeability weaken with increasing alkyl chain length of [CnMIM][PF4], which was evidenced by flow cytometry. In freshwater microcosm, [CnMIm][BF4] promoted the relative abundance of the *sul*I and *int*I genes for 4.6 folds, *aph*A and *tra*F for 5.2 folds higher than the untreated groups, promoting the propagation of ARGs in the aquatic environment. This is the first report that ILs with different structure of varying alkyl chain length facilitate horizontal transfer of plasmid RP4 which is widely distributed in the environment, and thus add the adverse effects of the environmental risk of ILs.

Keywords: antibiotic resistance genes, ionic liquid, horizontal transfer, plasmid, environmental risk

Introduction

Antibiotic resistance genes (ARGs), as the emerging pollutants, have become a global health concern (Pruden et al., 2006, 2013; Mao et al., 2013; Yang et al., 2013). Horizontal gene transfer rather than the accumulation of point mutations play an important role in the propagation and proliferation of ARGs (Aminov, 2011; Dodd, 2012). The critical role played by plasmids in the horizontal transfer of ARGs has been widely recognized, and plasmid is the main vector for

horizontal transfer of ARGs between environmental bacteria (Sørensen et al., 2005; Zhang et al., 2011; Zhu et al., 2013). Plasmid-mediated horizontal transfer of ARGs to indigenous bacteria was previously reported in soil (Henschke and Schmidt, 1990), activated sludge (Merlin et al., 2011), and wastewater oxidation ponds (Mispagel and Gray, 2005). Dissemination and propagation of ARGs via horizontal transfer from antibiotic resistant bacteria to susceptible strains, either between different species or across genera, mainly occur via conjugation of plasmids (DeBruyn et al., 2011; Qiu et al., 2012). Particularly, *in situ* transfer of self-transmissible plasmid RP4 which is a 60-kb broad-host-range conjugative plasmid harbors genes for kanamycin resistance (Km^R, *aph*A gene), ampicillin resistance (Ap^R, *tnp*R gene), and tetracycline resistance (Tc^R, *tet*A gene; Soda et al., 2008).

Ionic liquids (ILs) which are characterized as a big family of compounds with various structures are being considered as "environmental friendly" solvents in modern industry with high polarity and ionic conductivity (Pham et al., 2010), a wide electrochemical window and excellent chemical and thermal stability (Rogers et al., 2002; Zhao et al., 2007). There has not yet been any report of ILs in the environment (Stepnowski, 2005; Markiewicz et al., 2013). However, due to the high water solubility, bioaccumulation, toxicity, and non-biodegradability (Zhu et al., 2009), the ILs have the great potential to increase the risk when they are entering the environment (Zhao et al., 2007; Pham et al., 2010), thus, recent efforts have been toward the potential ecological and environmental risks of ILs (Pham et al., 2010). Adverse effect of some ILs to bacteria are determined by cations (Ganske and Bornscheuer, 2006) and it was found that inhibition effects of *Escherichia coli* cell growth of various ILs change with the number of C atoms in the longer alkyl chain (Lee et al., 2005). Enhanced alkyl group chain length or the substitution of alkyl groups with the cation ring corresponded with increased antimicrobial effects (Docherty and Kulpa, 2005). Recent research also revealed that ILs showed higher antimicrobial activity with increasing alkyl chain length (Pernak et al., 2003; Ranke et al., 2004; Docherty and Kulpa, 2005). In our previous study, we demonstrated that the ILs are capable of facilitating the dissemination of ARGs via horizontal gene transfer (Luo et al., 2014). However, how different structures of ILs with various alkyl chain lengths influence on facilitating the dissemination of ARGs has remained unknown.

In this study, ILs 1-altyl-3-methyl imidazolium tetrafluoroborate ($[C_nMIM][BF_4]$, with 4, 6, and 8 C atoms in the longer alkyl chain (as shown in **Table 1**), with different structure of varying alkyl chain length were tested for their potential on horizontal transfer of ARGs mediated by plasmid RP4 from the donors (*E. coli* DH5α) to *Salmonella* (as recipients) in Luria-Bertani (LB) medium and to indigenous bacteria (as recipients) in freshwater microcosms. The effects of bacterial growth and plasmid RP4 horizontal transfer frequency with [CnMIM][BF4] influence of alkyl chain length was determined. Furthermore, alteration of cell membrane permeability with different structure of alkyl chain length by flow cytometry (FCM) was measured to explore a possible mechanism by how the ILs with different structures of alkyl chain length exert different effects on promoting horizontal transfer of plasmid RP4. To our best knowledge, this is the first study of ILs with different structure of varying alkyl chain length facilitating the dissemination of ARGs in both medium of LB and aquatic microcosm.

TABLE 1 | Carbon atoms and EC_{50} number of [CnMIM][BF4].

Name	Acronym	Carbon atoms in alkyl chain *(n)*
1-butyl-3-methyl-imidazolium tetrafluoroborate	$[BMIM][BF_4]$	4
1-hexyl-3-methyl-imidazolium tetrafluoroborate	$[HMIM][BF_4]$	6
1-octyl-3-methyl-imidazolium tetrafluoroborate	$[OMIM][BF_4]$	8

Materials and Methods

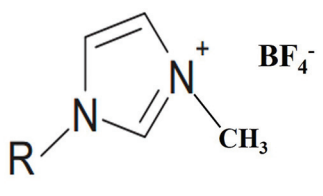
ILs Type

Cationic type of imidazolium (IM) is most widely used in modern industry (Cho et al., 2007; Romero et al., 2008; Zhu et al., 2009) and the 1-alkyl-3-methylimidazolium is one of the most often used IM cations which is attributed to its non-volatility, non-flammability, high thermal stability and an excellent solvent for a wide range of inorganic and organic materials (Gordon, 2001). Anions type of $BF4^-$were most widely used in ILs design (Romero et al., 2008).

The tested ILs in this study were purchased from Chinese Academy of Sciences (>99% pure). They consist of imidazolium cation and anion of BF_4^-, and cation have two alkyl substituents in positions R (**Figure 1**). The ILs 1-alkyl-3-methyl imidazolium tetrafluoroborate used in this study were abbreviated [CnMIM][BF4], where n represent the number of C atoms in the R alkyl chain.

Setup of Plasmid RP4 Horizontal Transfer in LB System

Plasmid RP4 horizontal transfer in LB system was used to determine the effects of ILs [CnMIM][BF4] on the transfer of plasmid RP4 from *E. coli* DH5α (*E. coli* DH5α, as the donor) to *Salmonella* (Str^R, as the recipient, across genera). The strain



$R=C_nH_{2n+1}$ (n=4, 6, 8)

Molecular structure of [1-R-3-methyl-imidazolium][BF_4]

FIGURE 1 | Molecular structure of 1-R-3-methyl-imidazolium tetrafluoroborate.

E. coli DH5α, harboring the plasmid RP4 carrying ampicillin, kanamycin, and tetracycline resistance genes (Ap^R, Km^R, and Tc^R) but no Str^R was used as the donor. The *Salmonella* carrying Str^R (in the genome) were used as the recipients and lacked Ap^R, Km^R, and Tc^R. The system was spiked with the ILs [CnMIM][BF4] of initial concentrations of 0, 0.00001, 0.0001, 0.001, 0.01, 0.1, 1.0, 2.5, 5.0, and 10.0 g/L. The IL concentrations in this study were comparable or lower than those in previous toxicity tests (Docherty and Kulpa, 2005; Song et al., 2011; Khudyakov et al., 2012). After mating for 12 h at 30°C, DNA was extracted and then *tra*F gene (indicator for plasmid RP4) and 16S rRNA gene were quantified. Transfer frequency (f) was calculated using the formula:

$$f_1 = \textit{tra}\text{F gene(copies/mL)}/\text{16S rRNA(copies/mL)} \quad (1)$$

Meanwhile, the number of transconjugants (Ap^R, Km^R, Tc^R, and Str^R) (N^T) were counted and the results were presented as colony forming units per milliliter culture (cfu/mL). The number of recipients (Str^R) (N_S) was determined by culturing the bacteria on LB agar plates containing 30 mg/L of streptomycin (cfu/mL). Transfer frequency (f) was also calculated using the formula:

$$f_2 = N_T\text{(cfu/mL)}/N_S\text{(cfu/mL)} \quad (2)$$

For details on horizontal transfer experiments, please refer to SI-1.

Setup of Plasmid RP4 Horizontal Transfer in Freshwater Microcosm

Setup of plasmid RP4 horizontal transfer in freshwater microcosms was based on the OECD 308 test (OECD, 2002) to test the effects of ILs [CnMIM][BF_4] (n = 4, 6, 8) on the plasmid RP4 horizontal transfer to indigenous bacteria in the microcosm. The freshwater sample was collected from the Water Parke (39°6′13″N, 117°9′21″E) in Tianjin, China on Sep 2013. Water properties are described in Supplementary Table S1. The strain of *E. coli* DH5α (plasmid RP4) was used as the RP4 donor strain and donor strain *E. coli* DH5α were tracked by specific primer (*E. coli*-FW: GTTCATGTGCCATCTGGTCTT; *E. coli*-RV: AAGTCTTCCTCGGTCGTGAT, Supplementary Table S2). The recipients were indigenous bacteria in freshwater microcosms. Meanwhile, in microcosms, donor isolates were negative in the PCR screening, *tra*F gene (indicator for RP4) and *aph*A gene (resistant gene to kanamycin on RP4) were negative also in the PCR screening. The microcosms were spiked with an IL [CnMIM][BF4] (n = 4, 6, 8) of initial concentrations of 0, 0.00001, 0.0001, 0.001, 0.01, 0.1, 1.0, 2.5, 5.0, and 10.0 g/L. Donor-free microcosms also used as controls (no horizontal transfer in donor-free microcosms in pre-experiment). Freshwater microcosms with no added donor and ILs also used as control to exclude mutation. Each concentration was set up in 500 mL of flask and the experiments lasted for 12 h at 30°C, as described in SI-2. Periodic sampling (10 mL) was collected and was used DNA extraction.

DNA Extraction and Quantitative Polymerase Chain Reaction (qPCR)

Freshwater microcosm samples (5 mL) were centrifuged and the total DNA was extracted using a bacterial DNA Kit according to manufacturer's instructions (OMEGA, USA). Quantitative polymerase chain reaction (qPCR) assays were used to quantify the 16S rRNA, *sul*1, *int*1, *aph*A, and *tra*F genes. Qualitative PCR assays were conducted in a Biometra T100 gradient thermal cycler (Biometra). The qPCR analyses were performed on a Bio-Rad iQ5 instrument to quantify bacteria 16S rRNA, *sul*1, *int*1, *aph*A, and *tra*F genes. Calibration standard curves for positive controls were generated as previously described. The establishment of negative and positive controls and qPCR reactions were described previously. The primers, amplification details, and standard curves of the 16S rRNA, *sul*1, *int*1, *aph*A, and *tra*F genes are listed in Supplementary Table S2, SI-3 and SI-4.

Change of Cell Membrane Permeability Measured by Flow Cytometry

To explore the change of cell membrane permeability induced by [CnMIM][BF4] and the relationship between cell membrane permeability and plasmid RP4 transfer frequency in LB system, FCM (BD FACSCalibur) was applied to differentiate and quantify the [CnMIM][BF4]-treated group and control group cells as a function of the percentage of positive cells based on the extent of cell membrane permeability. Propidium iodide (PI; 1 mg/mL; OMEGA, USA) was used to determine increased membrane permeability cells and control cells. Microcosm samples (2 mL) were centrifuged and washed 3x with phosphate buffer solution (PBS). Bacterial cells (the concentration was always less than 10^6 cells/mL) were then stained at concentrations of 10 μL PI to 1 mL of sample, and incubated in the dark for 8 min before measurement (Xue et al., 2012). The FCM was equipped with an excitation wavelength of 488 nm. All data were processed using CellQuest Pro software (USA).

Results and Discussion

Structure Dependent of ILs [CnMIM][BF4] Facilitate Plasmid RP4 Transfer

[CnMIM][BF_4] (n = 4, 6, 8) promoted the plasmid RP4 transfer with dose-effect in freshwater microcosms and LB medium (**Figure 2**). The plasmid RP4 transfer frequency enhanced with increasing [CnMIM][BF4] concentrations, however, decreased when concentration is higher than 1.0 g/L for [BMIM][BF4], 0.01 g/L for [HMIM][BF4], and 0.0001 g/L for [OMIM][BF4]. Meanwhile, the dose-effect dependent of ILs [CnMIM][BF4] facilitating plasmid RP4 transfer associated with ILs' effects on bacterial growth. In this study, the significant dose-effect inhibition was observed between the bacteria concentrations (the optical densities of OD_{600}) and the concentrations of each of the compound ([CnMIM][BF_4]; **Figure 3** and Supplementary Figure S2B).

Additionally, [CnMIM][BF4] promoted the plasmid RP4 transfer with significant structure-activity both in LB medium

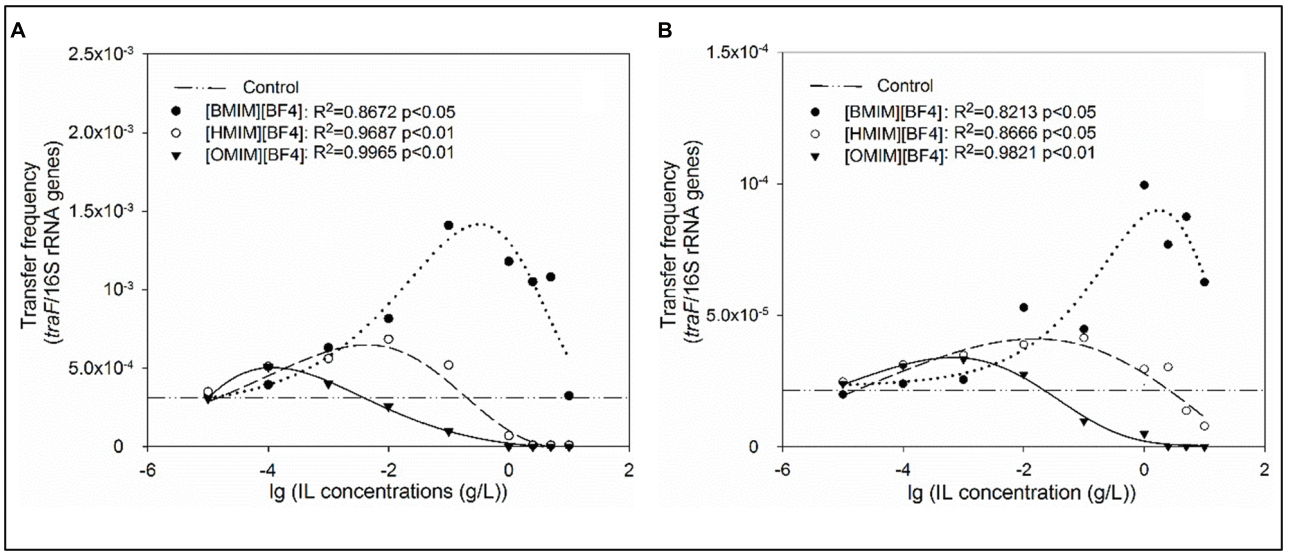


FIGURE 2 | Influence of alkyl chain length with different concentrations of ILs (0, 0.00001, 0.0001, 0.001, 0.01, 0.1, 1.0, 2.5, 5.0, and 10.0 g/L) on plasmid transfer frequency in freshwater microcosms (A) and in LB system (B) at mating for 12 h. The transfer frequency was based on formula (1), f_1 = *tra*F gene (copies/mL)/16S rRNA (copies/mL). ([BMIM][BF4], $n = 4$; [HMIM][BF4], $n = 6$; [OMIM][BF4], $n = 8$.)

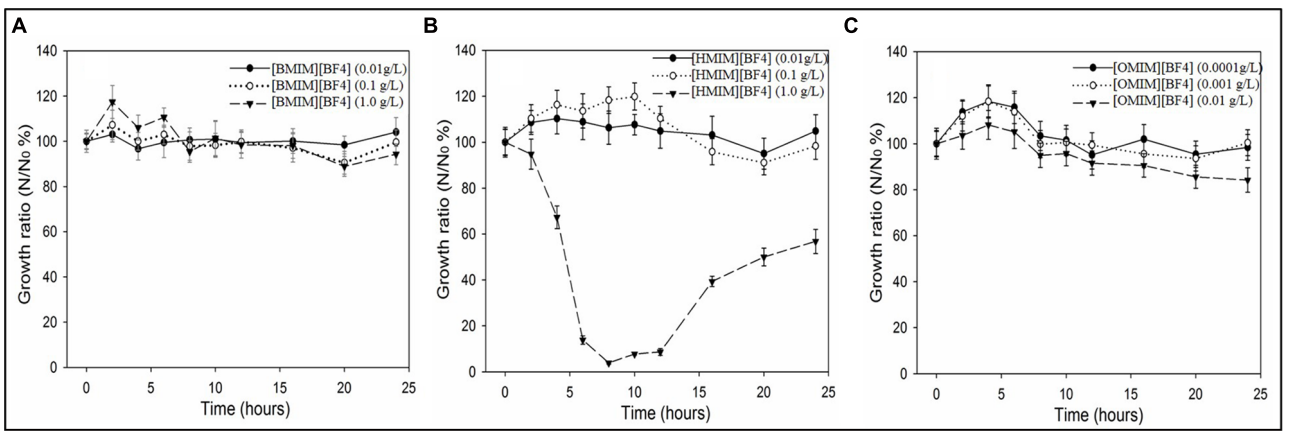


FIGURE 3 | Growth ratio (N/No, %) of *Escherichia coli* DH5α in LB system treated with [CnMIM][BF4]. (A) [BMIM][BF4] (concentration of 0.01, 0.1, 1.0 g/L); **(B)** [HMIM][BF4] (concentration of 0.01, 0.1, 1.0 g/L); **(C)** [OMIM][BF4] (concentration of 0.0001, 0.001, 0.01g/L). Control: untreated with [CnMIM][BF4]. ([BMIM][BF4], $n = 4$; [HMIM][BF4], $n = 6$; [OMIM][BF4], $n = 8$).

and freshwater microcosms (**Figure 2** and Supplementary Figure S1). The promotion of plasmid RP4 transfer frequency decreased with [CnMIM][BF4] increasing the alkyl chain length from 4 carbon atoms to 8 carbon atoms on the imidazolium cations, which is observed with [BMIM][BF4] ($n = 4$, 5.9 fold) > HMIM][BF4] ($n = 6$, 2.2 fold) > [OMIM][BF4] ($n = 8$, 1.7 fold). This illustrates that [CnMIM][BF4] with increasing the alkyl chain length exert decreasing ability in facilitating plasmid RP4 horizontal transfer, which is possibly related to their structure dependent toxicity differences. The size and charge distribution of the individual ions mainly affects the properties of an IL (Wilkes, 2004). Matsumoto et al. (2004) assessed the toxicity of [CnMIM][PF6] ($n = 4$, 6, 8) to the lactic acid-producing of microorganism *Lactobacillus* and found that the production activity of this microorganism decreased with increasing alkyl chain length on the imidazolium cations. Meanwhile, the structure-activity also associated with ILs effects on bacterial growth and enhanced inhibition of bacterial growth with increasing alkyl chain with [CnMIM][BF4] treated in LB system. In this study, the significant structure-activity between the microbial concentrations (the optical densities of OD_{600}) and [CnMIM][BF_4] alkyl chain length in position R were also found (**Figure 3**). The inhibition concentrations of growth of *E. coli*. DH5α for different structures of [CnMIM][BF4] is largely different, it is larger than 2.5 g/L for [BMIM][BF_4] ($n = 4$), 1.0 g/L for [HMIM][BF4] ($n = 6$), and 0.1 g/L for

[OMIM][BF4] (n = 8). Additionally, in this study, the ILs inhibition capability for bacterial growth was [BMIM][BF4] (n = 4) < [HMIM][BF4] (n = 6) < [OMIM][BF4] (n = 8) with the same concentration (e.g., 1.0 g/L), which attributed to more toxic of ILs with longer alkyl chains on the cations (Docherty and Kulpa, 2005; Ganske and Bornscheuer, 2006). A relationship between the length of alkyl chain and antimicrobial activities was also found and ILs with short alkyl chain substituent are not active against bacteria and fungi, in contrast ILs containing 10, 11, 12, and 14 carbon atoms in the alkyl chain show very high antimicrobial activities (Pernak et al., 2003; Nancharaiah et al., 2012).

Most interestingly, ILs [CnMIM][BF4] with concentrations of enhancing plasmid RP4 transfer have no influence on bacterial growth. In this study, growth of *E. coli*. DH5α were not influenced when [CnMIM][BF_4] concentrations are less than 1.0 g/L for [BMIM][BF4], 0.01 g/L for [HMIM][BF4], and 0.0001 g/L for [OMIM][BF4] (**Figure 3**). Consistently, the promotion effect on plasmid RP4 transfer is most obvious when the ILs concentrations are fall in the same range (less than 1.0 g/L for [BMIM][BF4], 0.01 g/L for [HMIM][BF4], and 0.0001 g/L for [OMIM][BF4]). Bacterial concentration plays a crucial role in the plasmid horizontal transfer. In this study, the observed highest values of plasmid transfer frequency were all based on the ILs concentration with no significant inhibition for bacterial growth. Therefore, special attention needs to be paid for ILs [CnMIM][BF4] with concentration especially below the sublethal concentration in the environment, this increase the risk of ILs facilitating the transmission and proliferation of multi-resistance media by plasmid RP4 through horizontal transfer among microorganisms in aquatic environment, and thus posing great risks to public health.

Structure Dependent of ILs [CnMIM][BF4] Increased Cell Membrane Permeability

A positive correlation (r^2 = 0.682, p < 0.05, **Figure 4H**) was found between cell membrane permeability and plasmid RP4 transfer frequency, indicating that enhanced plasmid RP4 transfer could be attributed to increased cell membrane permeability. Meanwhile, FCM assays of microorganisms showed that the percentage of PI-positive cells decreased with increasing alkyl chain length of [CnMIM][PF4], indicating the ability of ILs enhancing bacterial cell membrane permeability weaken with increasing alkyl chain length of [CnMIM][PF4] (**Figure 4**). In the concentration of highest plasmid RP4 transfer frequency, cell membrane permeability increased by 2-fold treated with [BMIM][BF_4] (n = 4; **Figure 4B**, 84.59%) compared with untreated bacteria (**Figure 4A**, 43.58%). The cell membrane permeability increased by 1.27 and 1.24 folds treated with [HMIM][BF_4] (n = 6; **Figure 4D**, 55.55%) and [OMIM][BF_4] (n = 8; **Figure 4F**, 54.28%). The capability of ILs enhancing cell membrane permeability follow the order of [BMIM][BF_4]

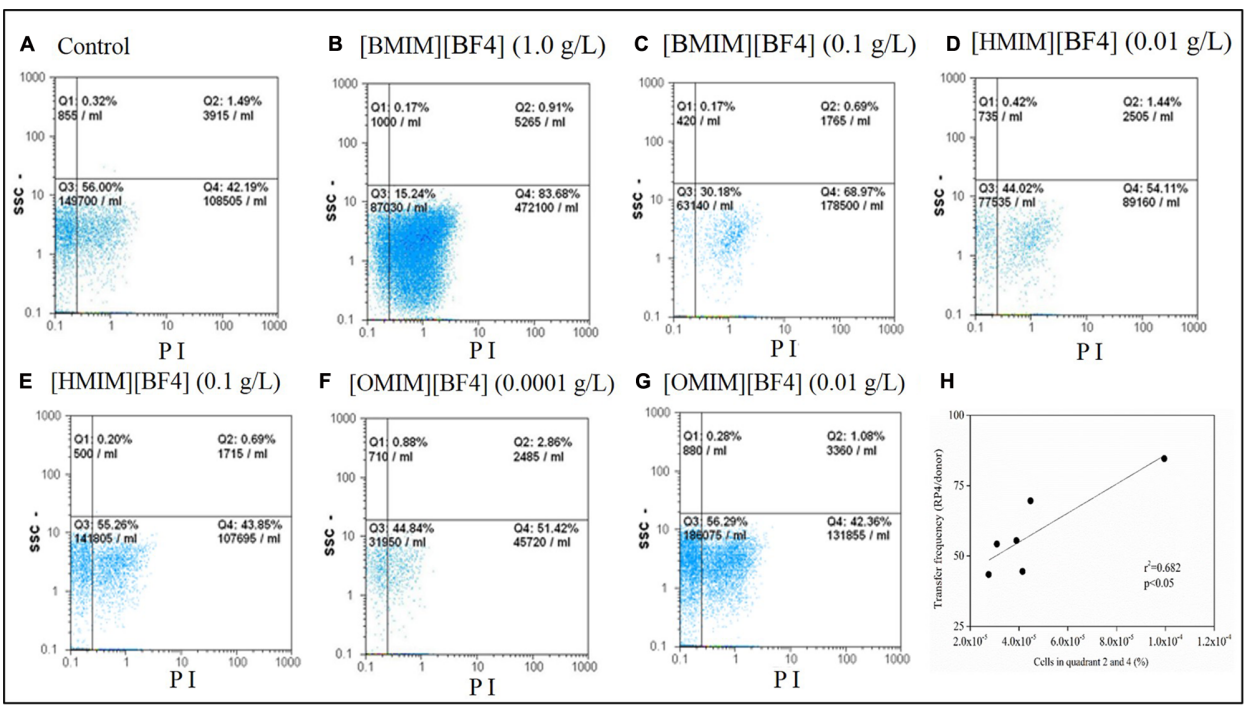


FIGURE 4 | Cell membrane permeability of microorganisms in LB system treated with [CnMIM][BF4] under the concentration that the highest plasmid RP4 transfer frequency quantified using flow cytometry. Flow cytometry data in dot plot: Quadrant 1 and 3: negative signal (normal cells); Quadrant 2 and 4: PI positive (cells with increased cell membrane permeability). **(A)** Control: untreated with [CnMIM][BF4]. **(B,C)**: [BMIM][BF4]; **(D,E)**: [HMIM][BF4]; **(F,G)**: [OMIM][BF4]. **(H)** Correlation between transfer frequency in LB system with percentage of cells with increased cell membrane permeability (PI positive) in the total cells treated with [CnMIM][BF4]. ([BMIM][BF4], n = 4; [HMIM][BF4], n = 6; [OMIM][BF4], n = 8.)

($n = 4$) > [HMIM][BF_4] ($n = 6$) > [OMIM][BF_4] ($n = 8$) which is the same as the order of ILs enhancing plasmid RP4 transfer frequency.

Cell membranes are selective semi-permeable membrane and constitute a barrier for horizontal transfer of genetic information among bacteria. Increased cell membrane permeability induced by ILs [CnMIM][PF4] could suppress cell membrane barrier and contribute to genetic information between cells (Thomas and Nielsen, 2005; Alekshun and Levy, 2007). ILs with imidazolium show similar structure to cationic surfactants (Markiewicz et al., 2013), which are known as amphiphilic compounds that contain ionic group and non-polar residues and can increase cell membrane permeability. In this study, the IL [OMIM][BF4] ($n = 8$) contribute to adsorb onto bacterial surface and damage the cell membrane, thereby assisting suppressed cell membrane barrier by increased cell membrane permeability (Thomas and Nielsen, 2005; Alekshun and Levy, 2007). In this study, the maximum horizontal transfer frequency facilitated by 1.0 g/L of [BMIm][BF4] was attributed to the highest cell membrane permeability.

ILs Facilitated Dissemination of ARGs in Freshwater Microcosms

In freshwater microcosms, [CnMIm][BF_4] ($n = 4, 6, 8$) promoted the relative abundance of the *sul*I (*sul*I gene/16S rRNA gene) and *int*I (*int*I gene/16S rRNA gene) genes for 4.6 folds versus that of the untreated controls (**Figure 5A**; $p < 0.05$, S–N–K test). Meanwhile, a positive correlation ($r^2 = 0.577$, $p < 0.05$) was found between relative abundance of the *sul*I and *int*I genes (**Figure 5B**), implying that the proliferation and propagation of *sul*I gene were primarily attributable to facilitation by class I integrons. The cassette consists of three parts: 5′ conserved segment (5′ CS), 3′ conserved segment (3′ CS) and the variable region between them (Rowe-Magnus et al., 2001). 3′ conserved segment includes two genes that encoding resistance, *sul*I (encoding sulfanilamide resistance) and qacE△1 (encoding quaternary ammonium salt compounds and ethidium bromine resistance). Variable region harbor different number of gene cassettes, which were used to encode drug-resistance (Sunde, 2005). The propagation of *sul*I, qacE△1, and ARGs in gene cassette were facilitated by class 1 integrons, which may exhibit

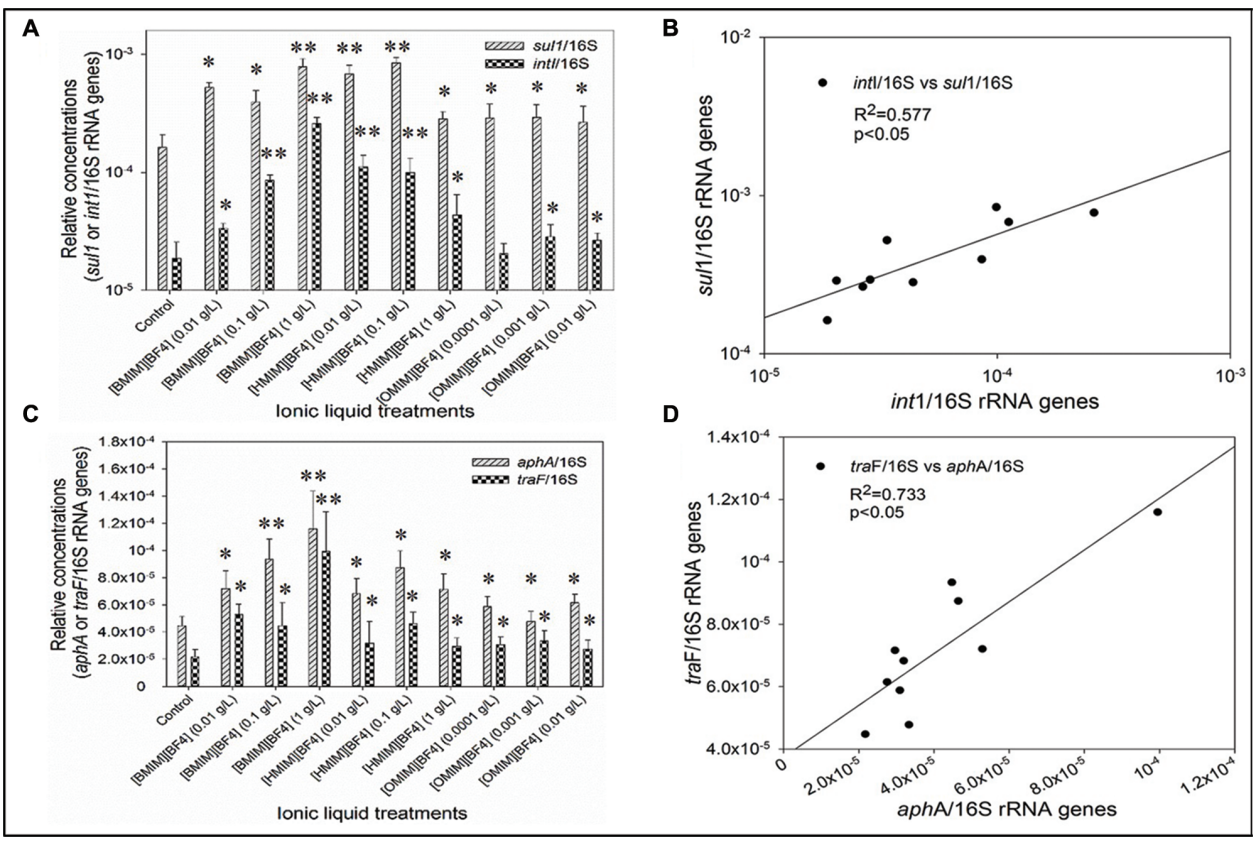


FIGURE 5 | The relative abundances of *sul*1 gene (*sul*1/16S rRNA) and *int*1 gene (*int*1/16S rRNA; A), and correlation between relative abundance of *sul1* gene (*sul*1/16S rRNA) versus relative abundance of *int*1 gene (*int*1/16S rRNA; B); the relative abundances of *aph*A gene (*aph*A/16S rRNA) and *tra*F gene (*tra*F/16S rRNA; C), and correlation between relative abundance of *aph*A gene (*aph*A/16S rRNA) versus *tra*F gene (*tra*F/16S rRNA; D) in the freshwater microcosms treated with [CnMIM][BF4] (0.01, 0.1, 1.0 g/L). ([BMIM][BF4], $n = 4$; [HMIM][BF4], $n = 6$; [OMIM][BF4], $n = 8$.) The concentration of [CnMIM][BF4] (0.01, 0.1, 1.0 g/L) had a significant effect on the dissemination of ARGs in freshwater microcosms (ANOVA, $p < 0.05$). Significant differences between each of the IL treated groups and the control (0 g/L of IL) were tested with the Student–Newman–Keuls (S–N–K) test, and showed with (*$p < 0.05$) and (**$p < 0.01$).

enhanced propagation characteristics compared to other mobile genetic elements. Additionally, microbial concentrations (16S-rRNA level) were not significantly change in the microcosm (**Figure 3**). This suggested that rather than bacterial growth, the enhanced the abundance of ARGs was attributable to the dissemination and propagation of ARGs within the cassette of class I integrons.

Meanwhile, [CnMIm][BF_4] ($n = 4, 6, 8$) facilitated the relative abundance of *aph*A (*aph*A/16S rRNA genes) and *tra*F (*tra*F/16S rRNA genes), and up to 5.2 folds higher than the untreated controls (**Figure 5C**; $p < 0.05$, S–N–K test). A significant correlation ($r^2 = 0.733$, $p < 0.05$; **Figure 5D**) was also found between the relative abundance of *aph*A and *tra*F genes, which implies that increased in ARGs were attributable to elevated plasmid RP4 horizontal transfer promoted by ILs [CnMIm][BF_4]. Meanwhile, [BMIm][PF6] exposure did not influence the donors ratio (Supplementary Figure S2A) and 16S rRNA *ratio* (microbial concentrations, Supplementary Figure S2B), implying that the [BMIm][PF6]-facilitated transfer of class I integrons or plasmid RP4 were not attributed to an increased total bacterial abundance. Plasmids play a critical role in horizontal gene transfer for the spread and dissemination of ARGs in the environment (Zatyka and Thomas, 1998; Sørensen et al., 2005). In this study, plasmid RP4 harbors multi-resistance genes of *aph*A gene (kanamycin resistance), *tnp*R gene (ampicillin resistance), and *tet*A gene (tetracycline resistance; Soda et al., 2008), thus, ILs [CnMIm][BF_4] exert a different enhanced selective pressure to increase levels of plasmid RP4 through horizontal transfer among microorganism, promoting the propagation of ARGs in the environment. Furthermore, excellent stability makes some ILs conceivable into wastewater treatment plants, and discharged into the aquatic environment (Pham et al., 2010). As consequent, ILs can represent a continuous selective pressure to promote the dissemination and propagation of ARGs.

Conclusion

In this study, ILs exerts a selective pressure to facilitate the spread of antibiotic resistance through plasmid RP4 horizontal transfer among bacteria, promoting increasing levels of the *sul*I, *int*I, *aph*A, *tra*F genes in aquatic environment. Meanwhile, the peak transfer frequency of promotion of plasmid RP4 transfer were decreased in different degrees with [CnMIM][BF4] ($n = 4, 6, 8$) increasing the alkyl chain length, which attributed to bacterial cell membrane permeability weaken with increasing alkyl chain length of [CnMIM][PF4]. Therefore, in a long period, residual [CnMIM][BF4] in aquatic environment has the great potential to promote the spread of antibiotic resistance. 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.

Acknowledgments

We express our sincerest thanks to Professor Junwen Li (Institute of Health and Environmental Medicine, Key Laboratory of Risk Assessment and Control for Environment & Food Safety, Tianjin in China) for provision of the RP4 plasmid. This work was financially supported by the National Natural Science Foundation of China (21277075, 31270542, 31470440), the State Environmental Protection Commonweal Project (201309031).

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00864

References

Alekshun, M. N., and Levy, S. B. (2007). Molecular mechanisms of antibacterial multidrug resistance. *Cell* 128, 1037–1050. doi: 10.1016/j.cell.2007.03.004

Aminov, R. I. (2011). Horizontal gene exchange in environmental microbiota. *Front. Microbiol.* 2:158. doi: 10.3389/fmicb.2011.00158

Cho, C. W., Pham, T. P., Jeon, Y. C., Vijayaraghavan, K., Choe, W. S., and Yun, Y. S. (2007). Toxicity of imidazolium salt with anion bromide to a phytoplankton *Selenastrum capricornutum*: effect of alkyl-chain length. *Chemosphere* 69, 1003–1007. doi: 10.1016/j.chemosphere.2007.06.023

DeBruyn, J. M., Mead, T. J., and Sayler, G. S. (2011). Horizontal transfer of PAH catabolism genes in *Mycobacterium*: evidence from comparative genomics and isolated pyrene-degrading bacteria. *Environ. Sci. Technol.* 46, 99–106. doi: 10.1021/es201607y

Docherty, K. M., and Kulpa, C. F. Jr. (2005). Toxicity and antimicrobial activity of imidazolium and pyridinium ionic liquids. *Green Chem.* 7, 185–189. doi: 10.1039/b419172b

Dodd, M. C. (2012). Potential impacts of disinfection processes on elimination and deactivation of antibiotic resistance genes during water and wastewater treatment. *J. Environ. Monit.* 14, 1754–1771. doi: 10.1039/c2em00006g

Ganske, F., and Bornscheuer, U. T. (2006). Growth of *Escherichia coli*, *Pichia pastoris* and *Bacillus cereus* in the presence of the ionic liquids [BMIM][BF4] and [BMIM][PF6] and organic solvents. *Biotechnol. Lett.* 28, 465–469. doi: 10.1007/s10529-006-0006-7

Gordon, C. M. (2001). New developments in catalysis using ionic liquids. *Appl. Catal. A Gen.* 222, 101–117. doi: 10.1016/S0926-860X(01)00834-1

Henschke, R. B., and Schmidt, F. R. J. (1990). Plasmid mobilization from genetically engineered bacteria to members of the indigenous soil microflora in situ. *Curr. Microbiol.* 20, 105–110. doi: 10.1007/BF02092881

Khudyakov, J. I., D'Haeseleer, P., Borglin, S. E., Deangelis, K. M., Woo, H., Lindquist, E. A., et al. (2012). Global transcriptome response to ionic liquid by a tropical rain forest soil bacterium, *Enterobacter lignolyticus*. *Proc. Natl. Acad. Sci. U.S.A.* 109, 2173–2182. doi: 10.1073/pnas.1112750109

Lee, S.-M., Chang, W.-J., Choi, A.-R., and Koo, Y. M. (2005). Influence of Ionic Liquids on the Growth of Escherichia coli (Short Communication). *Korean J. Chem. Eng.* 22, 687–690. doi: 10.1007/BF02705783

Luo, Y., Wang, Q., Lu, Q., Mu, Q., and Mao, D. (2014). An ionic liquid facilitates the proliferation of antibiotic resistance genes mediated by class I integrons. *Environ. Sci. Technol. Lett.* 1, 266–270. doi: 10.1021/ez500103v

Mao, D., Luo, Y., Mathieu, J., Wang, Q., Feng, L., Mu, Q. H., et al. (2013). Persistence of extracellular DNA in river sediment facilitates antibiotic resistance gene propagation. *Environ. Sci. Technol.* 48, 71–78. doi: 10.1021/es404280v

Markiewicz, M., Jungnickel, C., and Arp, H. P. H. (2013). Ionic liquid assisted dissolution of dissolved organic matter and PAHs from soil below the critical micelle concentration. *Environ. Sci. Technol.* 47, 6951–6958. doi: 10.1021/es304568w

Matsumoto, M., Mochiduki, K., and Kondo, K. (2004). Toxicity of ionic liquids and organic solvents to lactic acid-producing bacteria. *J. Biosci. Bioeng.* 98, 344–347. doi: 10.1263/jbb.98.344

Merlin, C., Bonot, S., Courtois, S., and Block, J. C. (2011). Persistence and dissemination of the multiple-antibiotic-resistance plasmid pB10 in the microbial communities of wastewater sludge microcosms. *Water Res.* 45, 2897–2905. doi: 10.1016/j.watres.2011.03.002

Mispagel, H., and Gray, J. T. (2005). Antibiotic resistance from wastewater oxidation ponds. *Water Environ. Res.* 77, 2996–3002. doi: 10.2175/106143005X73901

Nancharaiah, Y. V., Reddy, G. K. K., Lalithamanasa, P., and Venugopalan, V. P. (2012). The ionic liquid 1-alkyl-3-methylimidazolium demonstrates comparable antimicrobial and antibiofilm behavior to a cationic surfactant. *Biofouling* 28, 1141–1149. doi: 10.1080/08927014.2012.736966

OECD. (2002). *Aerobic and Anaerobic Transformation in Aquatic Sediment Systems, OECD Guidelines for the Testing of Chemicals, Section 3*. Paris: OECD Publishing.

Pernak, J., Sobaszkiewicz, K., and Mirska, I. (2003). Anti-microbial activities of ionic liquids. *Green Chem.* 5, 52–56. doi: 10.1039/b207543c

Pham, T. P., Cho, C. W., and Yun, Y. S. (2010). Environmental fate and toxicity of ionic liquids: a review. *Water Res.* 44, 352–372. doi: 10.1016/j.watres.2009.09.030

Pruden, A., Larsson, D. G., Amezquita, A., Collignon, P., Brandt, K. K., Graham, D. W., et al. (2013). Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. *Environ. Health Perspect.* 121, 878–885. doi: 10.1289/ehp.1206446

Pruden, A., Pei, R. T., Storteboom, H., and Carlson, K. H. (2006). Antibiotic resistance genes as emerging contaminants: studies in northern Colorado. *Environ. Sci. Technol.* 40, 7445–7450. doi: 10.1021/es0604131

Qiu, Z., Yu, Y., Chen, Z., Jin, M., Yang, D., Zhao, Z., et al. (2012). Nanoalumina promotes the horizontal transfer of multiresistance genes mediated by plasmids across genera. *Proc. Natl. Acad. Sci. U.S.A.* 109, 4944–4949. doi: 10.1073/pnas.1107254109

Ranke, J., Mölter, K., Stock, F., Bottin-Weber, U., Poczobutt, J., Hoffmann, J., et al. (2004). Biological effects of imidazolium ionic liquids with varying chain lengths in acute *Vibrio fischeri* and WST-1 cell viability assays. *Ecotoxicol. Environ. Saf.* 58, 396–404. doi: 10.1016/S0147-6513(03)00105-2

Rogers, R. D., Seddon, K. R., and Volkov, S. (2002). *Green Industrial Applications of Ionic Liquids: NATO Science Series II*. New York: Springer.

Romero, A., Santos, A., Tojo, J., and Rodriguez, A. (2008). Toxicity and biodegradability of imidazolium ionic liquids. *J. Hazard. Mater.* 151, 268–273. doi: 10.1016/j.jhazmat.2007.10.079

Rowe-Magnus, D. A., Guerout, A. M., Ploncard, P., Dychinco, B., Davies, J., and Mazel, D. (2001). The evolutionary history of chromosomal super-integrons provides an ancestry for multiresistant integrons. *Proc. Natl. Acad. Sci. U.S.A.* 98, 652–657. doi: 10.1073/pnas.98.2.652

Soda, S., Otsuki, H., Inoue, D., Tsutsui, H., Sei, K., and Ike, M. (2008). Transfer of antibiotic multiresistant plasmid RP4 from *Escherichia coli* to activated sludge bacteria. *J. Biosci. Bioeng.* 106, 292–296. doi: 10.1263/jbb.106.292

Song, X. L., Ye, S. Y., Xie, R., Yin, L., Shi, X., and Luo, S. C. (2011). Effects of bmim [PF6] treatments with different concentrations on microbial activity of Saccharomyces cerevisiae. *Korean J. Chem. Eng.* 28, 1902–1907. doi: 10.1007/s11814-011-0076-7

Sørensen, S. J., Bailey, M., Hansen, L. H., Kroer, N., and Wuertz, S. (2005). Studying plasmid horizontal transfer in situ: a critical review. *Nat. Rev. Microbiol.* 3, 700–710. doi: 10.1038/nrmicro1232

Stepnowski, P. (2005). Solid-phase extraction of room-temperature imidazolium ionic liquids from aqueous environmental samples. *Anal. Bioanal. Chem.* 381, 189–193. doi: 10.1007/s00216-004-2932-3

Sunde, M. (2005). Prevalence and characterization of class 1 and class 2 integrons in *Escherichia coli* isolated from meat and meat products of Norwegian origin. *J. Antimicrob. Chemother.* 56, 1019–1024. doi: 10.1093/jac/dki377

Thomas, C. M., and Nielsen, K. M. (2005). Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nat. Rev. Microbiol.* 3, 711–721. doi: 10.1038/nrmicro1234

Wilkes, J. S. (2004). Properties of ionic liquid solvents for catalysis. *J. Mol. Catal. A Chem.* 214, 11–17. doi: 10.1016/j.molcata.2003.11.029

Xue, Z., Sendamangalam, V. R., Gruden, C. L., and Seo, Y. (2012). Multiple roles of extracellular polymeric substances on resistance of biofilm and detached clusters. *Environ. Sci. Technol.* 46, 13212–13219. doi: 10.1021/es3031165

Yang, Y., Li, B., Ju, F., and Zhang, T. (2013). Exploring variation of antibiotic resistance genes in activated sludge over a four-year period through a metagenomic approach. *Environ. Sci. Technol.* 47, 10197–10205. doi: 10.1021/es4017365

Zatyka, M., and Thomas, C. M. (1998). Control of genes for conjugative transfer of plasmids and other mobile elements. *FEMS Microbiol. Rev.* 21, 291–319. doi: 10.1111/j.1574-6976.1998.tb00355.x

Zhang, T., Zhang, X. X., and Ye, L. (2011). Plasmid metagenome reveals high levels of antibiotic resistance genes and mobile genetic elements in activated sludge. *PLoS ONE* 6:e26041. doi: 10.1371/journal.pone.0026041

Zhao, D., Liao, Y., and Zhang, Z. (2007). Toxicity of ionic liquids. *Clean (Weinh.)* 35, 42–48. doi: 10.1002/clen.200600015

Zhu, S., Chen, R., Wu, Y., Chen, Q., Zhang, X., and Yu, Z. (2009). A mini-review on greenness of ionic liquids. *Chem. Biochem. Eng. Q.* 23, 207–211.

Zhu, Y. G., Johnson, T. A., Su, J. Q., Qiao, M., Guo, G. X., Stedtfeld, R. D., et al. (2013). Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proc. Natl. Acad. Sci. U.S.A.* 110, 3435–3440. doi: 10.1073/pnas.1222743110

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 15 January 2015
doi: 10.3389/fmicb.2014.00780

The impact of triclosan on the spread of antibiotic resistance in the environment

Daniel E. Carey and Patrick J. McNamara*

Department of Civil, Construction and Environmental Engineering, Marquette University, Milwaukee, WI, USA

Edited by:
Amy Pruden, Virginia Tech, USA

Reviewed by:
Julie Zilles, University of Illinois at Urbana–Champaign, USA
Eddie Cytryn, Agricultural Research Organization | Volcani Center, Israel

****Correspondence:***
Patrick J. McNamara, Department of Civil, Construction and Environmental Engineering, Marquette University, 1637 West Wisconsin Avenue, Milwaukee, WI 53233, USA
e-mail: patrick.mcnamara@mu.edu

Triclosan (TCS) is a commonly used antimicrobial agent that enters wastewater treatment plants (WWTPs) and the environment. An estimated 1.1×10^5 to 4.2×10^5 kg of TCS are discharged from these WWTPs per year in the United States. The abundance of TCS along with its antimicrobial properties have given rise to concern regarding its impact on antibiotic resistance in the environment. The objective of this review is to assess the state of knowledge regarding the impact of TCS on multidrug resistance in environmental settings, including engineered environments such as anaerobic digesters. Pure culture studies are reviewed in this paper to gain insight into the substantially smaller body of research surrounding the impacts of TCS on environmental microbial communities. Pure culture studies, mainly on pathogenic strains of bacteria, demonstrate that TCS is often associated with multidrug resistance. Research is lacking to quantify the current impacts of TCS discharge to the environment, but it is known that resistance to TCS and multidrug resistance *can* increase in environmental microbial communities exposed to TCS. Research plans are proposed to quantitatively define the conditions under which TCS selects for multidrug resistance in the environment.

Keywords: triclosan, antimicrobial resistance, antibiotic resistance, biosolids, wastewater

INTRODUCTION

The World Health Organization warns that we may enter a post-antibiotic era in the twenty-first century due to the spread of antibiotic resistance (World Health Organization [WHO], 2014). Antibiotic resistance is defined as the ability of bacteria to survive a concentration of antibiotics that typically inhibits growth of the majority of other bacteria (Russell, 2000). Antibiotics are extensively used in medicine to treat bacterial infections in humans and animals, and are widely used in agriculture to promote animal growth (Khachatourians, 1998; Kümmerer, 2004). Each year, in the United States (U.S.) alone, over two million people are infected by antibiotic resistant bacteria, leading to more than 25,000 deaths, and $50 billion spent managing antibiotic resistance (Centers for Disease Control and Prevention [CDC], U.S. Department of Health, and Human Services, 2013). The associated cost continues to increase as bacteria acquire mechanisms to fight against the antibiotics that are typically employed (Levy and Marshall, 2004).

In addition to antibiotics, synthetic antimicrobial agents are also pervasive in households and hospitals, mainly for disinfection and sanitation purposes. The term "antimicrobial" has been used to describe a broad range of compounds, including antibiotics that destroy or inhibit microorganisms (McDonnell and Russell, 1999; Kümmerer, 2004). For this paper, triclosan (TCS), which is not derived naturally, is referred to as an antimicrobial. Compounds produced or derived from microorganisms used *in vivo* to treat bacterial infections in eukaryotes (e.g., erythromycin, tetracycline, ciprofloxacin, etc.) will be referred to as antibiotics (even though antibiotics are a subset of antimicrobials).

Triclosan is widely used for personal hygiene and disinfection purposes; in fact, 350 tons were produced for commercial use in the European Union in 2002. Based on 1998 records from the Environmental Protection Agency, approximately 500–5000 tons were produced in the U.S., and the industry has reported growth (Singer et al., 2002; Heidler and Halden, 2007; Fang et al., 2010; Venkatesan and Halden, 2014). With these approximations, it is estimated that 1 kg of TCS is produced for every 3 kg of antibiotics produced (Food and Drug Administration [FDA], 2011; Department of Health and Human Services [DHHS], 2012). TCS is found in a wide range of consumer products including hand soap, toothpaste, deodorant, surgical scrubs, shower gel, hand lotion, hand cream, and mouthwash (Bhargava and Leonard, 1996; Jones et al., 2000).

Because of its wide use, TCS is found in many natural and engineered environments, including surface water, wastewater, soil, drinking water, wastewater treatment plants (WWTPs), biosolids, landfills, and sediments (Singer et al., 2002; Miller and Heidler, 2008; Benotti et al., 2009; Kumar et al., 2010; Xia et al., 2010; Welsch and Gillock, 2011; Bedoux et al., 2012; Mavri et al., 2012). As TCS is commonly used in oral consumer products, it is widely found in human urine. In a survey of 181 pregnant women in an urban multiethnic population in Brooklyn, NY, TCS was found in 100% of urine samples (Pycke et al., 2014). In a geographically broader U.S. survey, 75% of people were found to have TCS in their urine (Calafat et al., 2008).

At application concentrations (0.1–0.3 w/v% or approximately 1,000–3,000 mg/L in hand soaps), TCS induces cell damage that causes cell contents to physically leak out of the membrane (Villalaín et al., 2001). At concentrations lower than 1 mg/L, TCS serves as an external pressure to select for TCS resistance as well as antibiotic resistance in many types of bacteria (Russell, 2000; Schweizer, 2001; Poole, 2002; Chapman, 2003; Yazdankhah et al., 2006; Birosová and Mikulásová, 2009; Saleh et al., 2011; Halden, 2014). At low concentrations, TCS interacts with physiological targets, and these interactions lead to numerous resistance mechanisms that are reviewed below (Chuanchuen et al., 2001; Bailey et al., 2008; Yu et al., 2010; Condell et al., 2012). In some cases, the mechanisms that convey resistance to TCS simultaneously confer resistance to more than one class of antibiotics (Poole, 2002; Alanis, 2005).

The wide use of TCS leads to concern about its potential to aid in the spread of antibiotic resistance (Russell, 2000; Kümmerer, 2004; Saleh et al., 2011). TCS exposure that leads to TCS resistance and antibiotic resistance has been widely reported, but the majority of these studies pertain to pure cultures of specific bacterial strains, and in most cases, pathogenic strains. This line of research is logical because antibiotic resistant pathogens are of greatest concern to public health. TCS might also impact the spread of resistance in environmental microbial communities as approximately 1.1×10^5 to 4.2×10^5 kg of TCS are distributed to the environment annually through WWTPs in the U.S. (Heidler and Halden, 2007). Studies on pure culture isolates provide insight into the *potential* impacts of TCS on antibiotic resistance in environmental bacterial communities. The important question then becomes: does TCS select for antibiotic resistance in these complex microbial communities?

Many engineered and natural processes are driven by microbes, and TCS is designed to impact microbes in homes and hospitals. Following discharge to the environment, the antimicrobial properties of TCS can impact complex microbial communities found in engineered and environmental systems. TCS has been linked to altering microbial community structure or function in wastewater operations, such as activated sludge and anaerobic digestion (Stasinakis et al., 2008; McNamara et al., 2014). Likewise, TCS can alter diversity and biofilm development in freshwater biofilms in receiving streams (Johnson et al., 2009; Proia et al., 2011; Lubarsky et al., 2012). In soils, TCS impacts respiration rates and denitrification, and enriches for species capable of dehalogenation (Butler et al., 2011; McNamara and Krzmarzick, 2013; Holzem et al., 2014). TCS induces responses in microbial communities, but the TCS concentrations that inhibit function are not often found in these complex microbial communities. At environmental concentrations, TCS is more likely to exert a stress that propagates resistance than to exert a stress that functionally inhibits complex microbial communities.

The purpose of this manuscript is to review the state of knowledge regarding the impact of TCS on antibiotic resistance in environmental systems and identify critical research questions that need to be addressed to better understand the impact of TCS-derived resistance in the environment on public health. This review describes TCS resistance and cross-resistance in pure cultures, and then considers the comparatively smaller amount of literature that addresses how TCS impacts antibiotic resistance in engineered environments containing complex microbial communities. Engineered environments are of prime interest because they contain TCS, bacteria, and resistance genes that can be subsequently dispersed to terrestrial soils and surface waters with the possibility of negative public health consequences (Pruden et al., 2006, 2012; Baquero et al., 2008; Cha and Cupples, 2009; Ghosh et al., 2009; Munir et al., 2010; LaPara et al., 2011; Ma et al., 2011; Burch et al., 2014; Yang et al., 2014).

GENETIC TARGETS OF TRICLOSAN

In 1998, TCS was first described by McMurry et al. (1998b) to have a specific target in *Escherichia coli*. At 1 mg/L, approximately 1000-fold lower than the application concentration, TCS inhibits FabI, an enoyl-acyl carrier protein reductase (ENR). The FabI protein catalyzes the elongation cycle in the synthesis of fatty acids, an essential process for cell viability (Bergler et al., 1996; Massengo-Tiassé and Cronan, 2008, 2009). Prior to McMurry et al.'s (1998b) report, low concentrations of TCS were assumed to have minimal effects on cell viability.

Up-regulation of *fabI* is a response mechanism which may overcome the effects of intracellular TCS (Condell et al., 2012; Yu et al., 2012; Sheridan et al., 2013). Bacteria can up-regulate and down-regulate many more genes in response to TCS, although it can be difficult to determine which expression changes are casual. No universal response has been observed; however, many bacteria respond to some degree with the up-regulation of transport proteins and membrane bound proteins (Bailey et al., 2008; Chuanchuen and Schweizer, 2012).

TCS RESISTANCE IN PURE CULTURES

The most common resistance mechanisms based on pure culture studies are target site modification, membrane resistance, and efflux. The following sections briefly review resistance mechanisms to TCS and describe their impact on cross-resistance; a comprehensive review of TCS resistance mechanisms can be found by Schweizer (2001).

FABI MODIFICATION OR REPLACEMENT

Target site modification is a resistance mechanism that involves a genetic alteration to the target site that reduces the effect of an inhibitory chemical (Hooper, 2005). Modification of TCS target site FabI is a common resistance mechanism observed in pure cultures. Mutation occurs whereby single or multiple amino acids are changed in the *fabI* gene, resulting in TCS-resistant FabI proteins (Brenwald and Fraise, 2003; Yu et al., 2010). Ciusa et al. (2012) suggested a resistance mechanism whereby an allele of a *fabI* gene is located on a mobile genetic element and transposed into *Staphylococcus aureus*. The presence of the *fabI* allele together with the intrinsic *fabI* gene increased the concentration of the FabI protein through heterologous duplication and increased bacterial tolerance to TCS. Alternatively, ENR isoenzymes, which perform similar functions to FabI, including FabL, FabK, and FabV, have been identified in TCS-resistant bacteria (Massengo-Tiassé and Cronan, 2009). These isoenzymes are naturally found in some strains of bacteria. In fact, FabV has been found to functionally replace FabI, rendering *Pseudomonas aeruginosa* 2,000 times more

resistant to TCS as seen by an increase in minimum inhibitory concentration (MIC; Zhu et al., 2010). Similarly, FabK replaces function for FabI in *Streptococcus pneumonia*, leading to increased tolerance to TCS (Heath et al., 2000), and FabL expression leads to increased resistance to TCS in *Bacillus subtilis* (Heath et al., 2000).

With respect to multidrug resistance, FabI alteration or replacement may specifically produce resistance to isoniazid, an important agent for the treatment of tuberculosis, which also targets FabI (Ciusa et al., 2012). However, FabI alterations are not generally known to cause resistance to other antibiotics. This type of resistance in environmental communities would not likely pose a threat to public health through increased multidrug resistance.

MEMBRANE ALTERATION

Modifications through changes to the outer membrane is a less-studied TCS resistance mechanism in bacteria. Champlin et al. (2005) concluded that outer membrane properties were responsible for low-level resistance to hydrophobic antimicrobials and antibiotics. The researchers compared *P. aeruginosa* strains that possessed highly refractory outer cell envelopes to strains that had highly permeable outer cell envelopes and discovered that the outer membrane properties conferred intrinsic resistance to TCS up to 256 mg/L. Tkachenko et al. (2007) suggested that TCS exposure could induce a genetic response which increases the concentration of branched chain fatty acids in the cell membrane in *S. aureus*; the membrane thereby sequesters the chemical agent and stops it from passing into the cell, preventing physiological disruption inside of the cell.

Outer membrane impermeability is a potential mechanism for cross-resistance to antibiotics. Particularly, non-specific rejection of hydrophobic chemicals could be a mechanism for resistance to TCS and other antibiotics that may be found in the environment.

EFFLUX PUMPS

Efflux pumps are often associated with multidrug resistance, which is a public health concern. Active efflux, whereby a bacterium physically removes a constituent from its intracellular space by pumping the constituent across the membrane and back into the environment, is an effective mechanism against a wide range of antimicrobials and antibiotics, including TCS (Kern et al., 2000; Levy, 2002). The AcrAB efflux pump is responsible for efflux of TCS in *E. coli* and *Salmonella enterica* (McMurry et al., 1998a; Webber et al., 2008). Non-specific multidrug efflux pumps (e.g., *mex* proteins) confer resistance to TCS as well as other antibiotics in *P. aeruginosa* and *Rhodospirillum rubrum* (Chuanchuen et al., 2001; Pycke et al., 2010a,b). Most non-specific efflux pumps are capable of expulsing antibiotics. Thus, in cases where bacteria acquire non-specific efflux pumps through horizontal gene transfer after exposure to TCS, the bacteria would likely acquire resistance to antibiotics as well. In some cases specific efflux pumps confer resistance to TCS. TriABC-OpmH is a TCS-specific efflux pump in *P. aeruginosa* that is not known to expel other compounds such as antibiotics (Mima et al., 2007).

TRICLOSAN AND CROSS-RESISTANCE TO ANTIBIOTICS

Resistance to TCS, incurred by exposure to TCS, can directly affect resistance to antibiotics. Cross-resistance has been tested for a wide range of antibiotics following exposure to TCS. Chloramphenicol and tetracycline are two antibiotics commonly included in antibiotic cross-resistance experiments. In studies done on *E. coli* and *P. aeruginosa*, resistance to chloramphenicol and tetracycline increased 10-fold following TCS exposure (**Figure 1**). Increased antibiotic resistance in *Stenotrophomonas maltophilia* and *Salmonella enterica* serovar Typhimurium following TCS exposure was also observed, but the increase was less severe. Cross resistance in *P. aeruginosa* (Chuanchuen et al., 2001), *Stenotrophomonas maltophilia* (Sanchez et al., 2005), and *Salmonella enterica* serovar Typhimurium (Karatzas et al., 2007) were attributed to efflux systems. Resistance mechanisms were not directly investigated in the studies on *E. coli* (Braoudaki and Hilton, 2004) and *Salmonella enterica* serovar Typhimurium (Birosová and Mikulásová, 2009), however *acrAB* genes, which encode for efflux, are known to confer resistance to TCS, chloramphenicol, and tetracycline in both of these species (Karatzas et al., 2007). These findings highlight a main concern regarding the widespread dissemination of TCS, i.e., that TCS exposure can spread multidrug resistance.

Triclosan resistance and antibiotic resistance have been found together in clinical isolates. In a survey of 732 clinical isolates of *Acinetobacter baumannii* from hospitals, 3% of isolates were found to have reduced susceptibility to TCS (MIC $>$ 1 mg/L; Chen et al., 2009). Those isolates which could tolerate higher than 4 mg/L also had increased tolerance to amikacin, tetracycline, levofloxacin and imipenem. Clinical isolates of *S. aureus*, which had MICs to TCS between 0.025 and 1 mg/L, were resistant to multiple antibiotics (Suller and Russell, 2000). Some, but not all, of the strains showed increased resistance to gentamicin, erythromycin, penicillin, rifampicin, fusidic acid, tetracycline, methicillin, mupirocin, and streptomycin. In some strains TCS resistance was stable when sub-culturing was performed in a TCS-free medium. In other strains TCS resistance was lost when the strain was propagated for 10 days in TCS-free media, indicating that the presence of TCS can select for resistance that is not regularly expressed. This finding implies that removing TCS from environmental systems through improved treatment processes or reduced consumer usage could lead to a decrease in TCS resistance. Research should be conducted to specifically test the impacts of removing TCS on TCS-derived resistance in complex microbial communities.

Conditions that perpetuate resistance to TCS frequently result in cross-resistance to antibiotics. TCS resistant *Salmonella enterica* serovar Typhimurium strains were selected by daily sub-culturing of TCS-exposed cultures and increasing TCS concentrations in media from 0.05 to 15 mg/L over 15 days (Karatzas et al., 2007). The TCS MIC in the resulting strains increased from 0.06 mg/L to as high as 128 mg/L, and the strains were also more resistant to ampicillin, tetracycline, and kanamycin. The authors concluded that the overexpression of the *acrAB* efflux pump was likely involved in the increased tolerance to TCS and antibiotics. In another study, TCS selected for ciprofloxacin resistant mutants in *Salmonella enterica* serovar Typhimurium when exposed to 0.5 mg/L of TCS (Birosová and Mikulásová, 2009). These studies, along with the concentrations of TCS found in the environment, imply that TCS could select for bacteria in environmental communities that have efflux pumps.

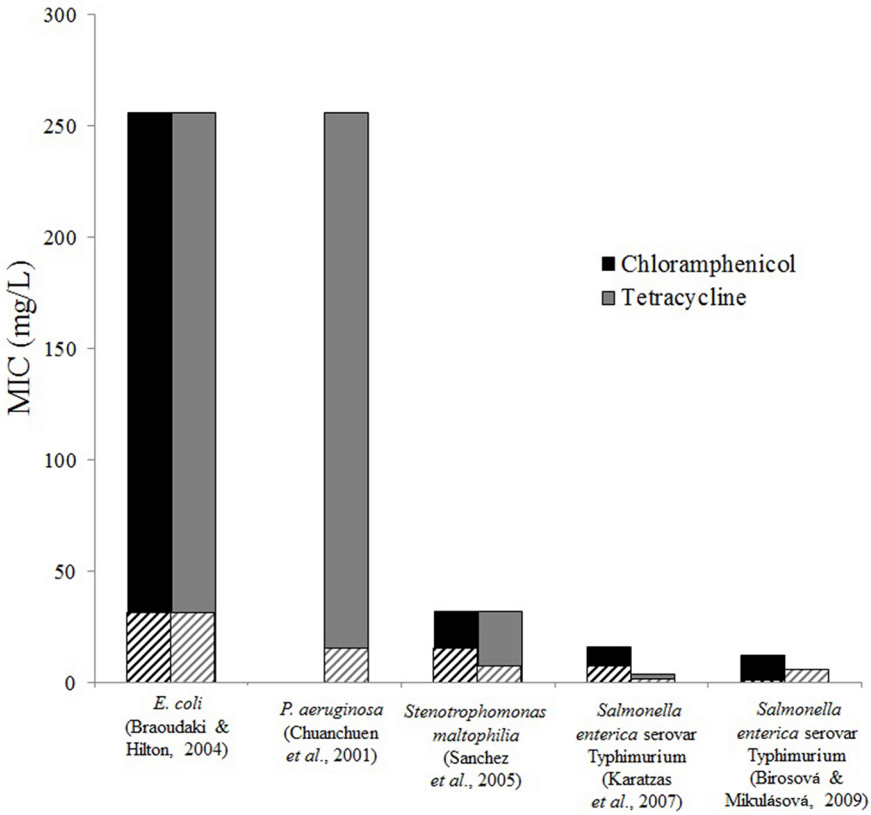


FIGURE 1 | Triclosan exposure increases resistance to antibiotics. Minimum inhibitory concentrations of chloramphenicol and tetracycline for control strains (striped bars) and TCS adapted strains (solid bars) are shown from various studies and bacteria. Differences were observed in most cases, however, no difference was found for tetracycline resistance for *Salmonella enterica* in the study by Birosová and Mikulásová (2009). Chloramphenicol resistance was not tested in *Pseudomonas aeruginosa* (Chuanchuen et al., 2001).

Efflux is a common method of resistance, but the specific efflux system used and the resulting cross-resistance profile can vary between species. In *P. aeruginosa*, MexAB-OprM, MexCD-OprJ, and MexEF-OprN, contribute to TCS resistance (Chuanchuen et al., 2001). Exposure to TCS selected for up-regulation of these efflux systems due to mutations in the regulatory gene, *nfxB*, which increased the tolerance to tetracycline, ciprofloxacin, trimethoprim, erythromycin, and gentamicin. In some cases the TCS resistant strains could tolerate up to 500-fold higher antibiotic concentrations than the non-TCS resistant strains. Strains which lacked these efflux systems showed increased sensitivity to antibiotics. In the opportunistic pathogen *Stenotrophomonas maltophilia*, TCS binds to the repressor SmeT, allowing expression of an efflux pump, SmeDEF (Hernández et al., 2011). Expression of this efflux pump following exposure to TCS resulted in increased resistance to the antibiotics ciprofloxacin, norfloxacin, nalidixic, and ofloxacin. Sanchez et al. (2005) also found that TCS-resistant mutants of *Stenotrophomonas maltophilia* (tolerant up to 64 μg/L of TCS) overexpress SmeDEF. These mutants had an increased tolerance to tetracycline, chloramphenicol, and ciprofloxacin. Even though SmeDEF is intrinsically contained in the genome of *Stenotrophomonas maltophilia,* TCS exposure selected for up-regulation of this efflux pump which increased antibiotic resistance.

In addition to variances between genera, cross-resistance varies within genera. TCS-adapted *E. coli* O157:H7 exhibited increased resistance to chloramphenicol, tetracycline, amoxicillin, amoxicillin/clavulanic acid, trimethoprim, benzalkonium chloride, and chlorhexidine, while TCS-adapted *E. coli* O55 exhibited resistance to only trimethoprim (Braoudaki and Hilton, 2004).

Although most evidence supports the notion that TCS increases resistance to antibiotics, this is not necessarily true for all classes of antibiotics. In one case, TCS-resistant mutants of *Salmonella enterica* were more (or no less) susceptible to antibiotics (Rensch et al., 2013). *Salmonella enterica* that were selected to have overexpression of *fabI* or a *fabI* mutation had increased susceptibility to the aminoglycoside antibiotics kanamycin and gentamicin.

The cross-resistance profiles vary among the bacteria surveyed in this review, and other types of bacteria yet to be studied are likely to have unique cross-resistance profiles. While resistance profiles vary, the overarching theme is the same: resistance to TCS can yield cross-resistance to multiple antibiotics. Given that TCS is not an antibiotic, resistance to TCS alone is not a public health threat. TCS-derived proliferation of multidrug resistant bacteria, however, could be a severe threat to public health. These pure culture studies indicate that TCS is likely to select for multidrug resistant bacteria above a critical concentration. In environmental

communities, such as anaerobic digesters or sediments, TCS is found at 2- to 1000-fold higher concentrations than any given antibiotic (McClellan and Halden, 2010). Is TCS selecting for resistant bacteria in the environment? The role of TCS on the selection of antibiotic resistance genes and multidrug resistance genes in the environment needs to be quantified to determine what steps, if any, are necessary for protecting public health.

TRICLOSAN-DERIVED RESISTANCE IN COMPLEX ENVIRONMENTAL COMMUNITIES

Environmental systems, including WWTPs and sediments, represent the most likely sites for TCS resistance to develop because of the high abundance of TCS and high density of bacteria. Wastewater treatment systems should be given special focus because they contain and discharge TCS and resistance genes to the environment. To understand the role of TCS and the remaining research gaps, the fate of TCS in the environment is summarized to highlight locations of prime interest, and the state of knowledge regarding TCS and resistance in complex microbial communities is assessed.

FATE OF TRICLOSAN

Triclosan is discharged into the environment with treated liquid and solid effluents from WWTPs. In the U.S. alone, WWTPs are estimated to receive approximately 100 tons of TCS each year, but the prevalence of TCS in treated effluent is not restricted to U.S. facilities. A survey of WWTPs in Germany found TCS in treated effluents at concentrations ranging from 1×10^{-5} to 6×10^{-4} mg/L (Bester, 2005). The concentrations of TCS and its aerobic degradation products in receiving waters were less than 3×10^{-6} mg/L. A study of eight WWTPs in Switzerland revealed that, on average, 6% of the influent TCS was found to discharge with the effluent water at concentrations of 4.2×10^{-5} to 2.13×10^{-4} mg/L (Singer et al., 2002); these receiving streams had concentrations at 1.1×10^{-5} to 9.8×10^{-5} mg/L. A more recent study found TCS in WWTP effluents at 9.7×10^{-5} mg/L, and in nearby sediments at 0.018 mg/kg (Blair et al., 2013). Several other studies have found TCS in surface water in concentrations ranging from $<2 \times 10^{-7}$ mg/L up to 0.022 mg/L (Bedoux et al., 2012).

Triclosan that is discharged with liquid effluent often partitions to sediments. Miller and Heidler (2008) found that TCS accumulated in sediments near WWTP outfalls for approximately 50 years, and similar results were found by other researchers (Buth et al., 2010; Anger et al., 2013). Sediment concentrations have been found at 53 mg/kg (Chalew and Halden, 2009). TCS is prevalent in liquid effluents and abundant in sediments, but this discharge route does not account for the majority of TCS that enters the environment. One study estimated that 0.24 kg/day of TCS are released with liquid effluent, but 5.37 kg/day are released with the treated residual solids from a midsized WWTP (Lozano et al., 2013).

Indeed, nearly half (or even higher) of the influent TCS load to WWTPs is captured by solids following sorption (Heidler and Halden, 2007; Lozano et al., 2013). The concentration of TCS in biosolids is often much higher than in aqueous systems because of the hydrophobic nature of TCS (Heidler and Halden, 2008). A nationwide U.S. survey of TCS in biosolids found the median concentration in treated biosolids to be 3.9 mg/kg and the maximum level was 133 mg/kg (United States Environmental Protection Agency [USEPA], 2009). The high levels found in biosolids can lead to high levels in soils when biosolids are land applied. TCS was found in biosolids-amended soils which had been receiving biosolids for 33 years (Xia et al., 2010). The concentrations in the soil ranged from approximately 1 mg/kg in the first 15 cm of soil to less than 0.1 mg/kg at a depth of 60–120 cm. The half-life of TCS in soil under aerobic conditions was 104 days, and TCS is even more persistent under anaerobic conditions (McAvoy et al., 2002; Ying et al., 2007). These fate data, along with the hydrophobic nature of TCS, indicate that TCS is most likely to impact microbial communities that contain high concentrations of organic matter, including anaerobic digesters, sediments, and soils, and these communities should receive special focus when investigating TCS-derived resistance in the environment.

The range of TCS concentrations found in the environment is depicted in **Figure 2** along with the MIC of TCS-acclimated and TCS-unacclimated pathogenic strains of bacteria. The concentrations in the biosolids and sediments are higher than the MICs of TCS-sensitive strains, indicating that TCS-sensitive strains would not thrive in these environments and TCS-resistant strains may be present. The MICs of TCS-acclimated strains, however, are higher than the current environmental TCS concentrations and could tolerate an increase in TCS concentrations. A future increase in TCS concentrations may select for resistance rather than functionally inhibit complex microbial communities. This figure indicates that biosolids and sediment environments with high TCS concentrations likely have TCS-resistant bacteria, but this figure does not indicate the level of TCS required to select or enrich for resistance in the environments with lower TCS concentrations. What happens when TCS is below the MIC? Certainly environments with very high levels of TCS will have TCS-resistant strains, but do environments with TCS concentrations below the MIC of acclimated strains select for resistance? What concentration of TCS is required to select for resistance in various environmental communities? These questions represent critical research gaps. By answering these questions with further research we can determine if and where TCS is selecting for resistance. Research plans are outlined in the final section to address these questions.

TRICLOSAN RESISTANCE IN COMPLEX MICROBIAL COMMUNITIES

Bacteria with resistance to TCS are found in the environment, and experiments have been performed to determine whether TCS could be the cause for resistance. Drury et al. (2013) constructed artificial streams to control for other selective pressures such as antibiotics. The artificial streams were inoculated with approximately 8 mg/L of TCS. Over 34 days, the relative abundance of benthic bacteria which were able to be cultivated in 16 mg/L of TCS in agar climbed from 0 to 14%. In a similar study, TCS was added to artificial stream mesocosms at 1×10^{-4}, 5×10^{-4}, 1×10^{-3}, 5×10^{-3}, and 1×10^{-2} mg/L, and resistance to TCS significantly increased in bacterial populations exposed to TCS concentrations over 5×10^{-4} mg/L (Nietch and Quinlan, 2013). This study was conducted at environmentally relevant

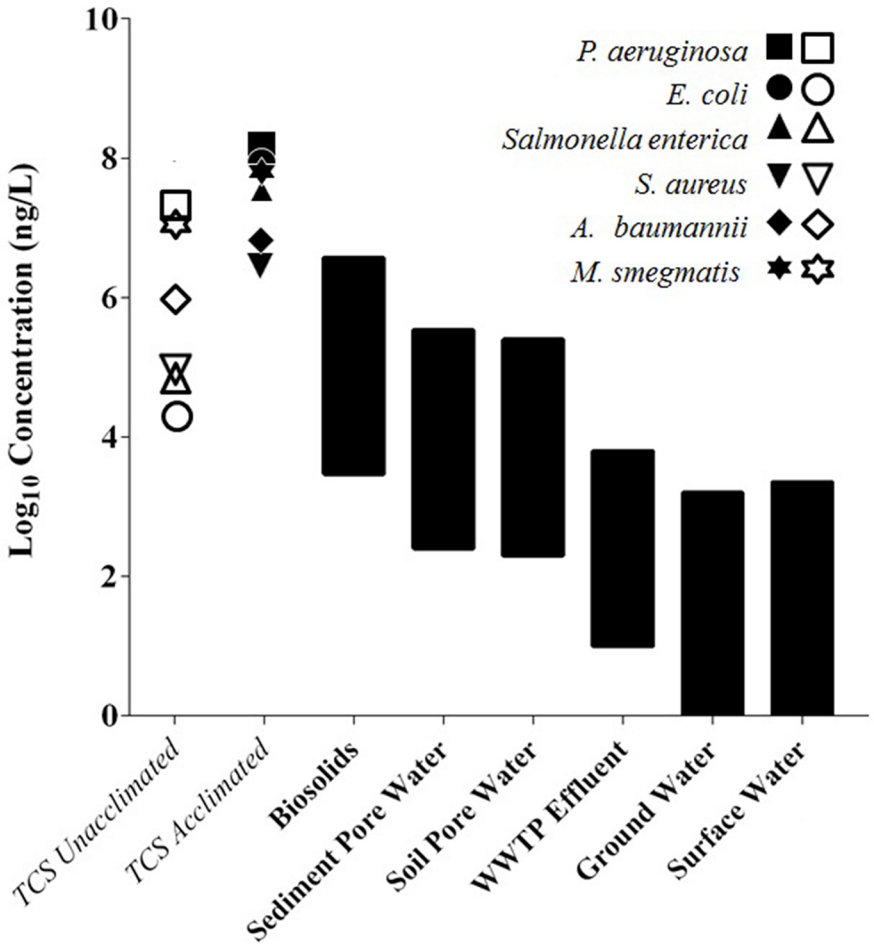


FIGURE 2 | The MIC of TCS-acclimated and TCS-unacclimated strains relative to environmental TCS concentrations. Open symbols represent the MIC for TCS sensitive strains, while closed symbols represent the MIC for TCS adapted strains. Black bars are ranges of TCS concentrations found in each environmental setting. Biosolids concentrations were converted from mg/kg to mg/L by assuming 3% total solids in reactors that produce biosolids (McMurry et al., 1998a, 1999; Chuanchuen et al., 2001; Slater-Radosti et al., 2001; Fan et al., 2002; Yazdankhah et al., 2006; Karatzas et al., 2007; Mima et al., 2007; Tkachenko et al., 2007; Bailey et al., 2008; Webber et al., 2008; Chalew and Halden, 2009; Chen et al., 2009; McClellan and Halden, 2010; Yu et al., 2010; Saleh et al., 2011; Bedoux et al., 2012).

concentrations, and suggested that TCS exposure leads to TCS-resistance. Middleton and Salierno (2013) discovered that TCS resistance was detected in 78.8% of fecal coliform samples from streams receiving wastewater, and 89.6% of these samples were resistant to four classes of antibiotics. *Escherichia*, *Enterobacter*, *Serratia*, and *Citrobacter* were also found in the stream with resistance to TCS and multiple antibiotics. This study investigated real-world surface water samples which are implicitly associated with many uncontrolled variables. Accordingly, it infers, but does not prove, that TCS may be an external stressor that results in increased abundance of resistance genes.

Studies on the impacts of TCS on anaerobic digesters, where TCS is of highest abundance, are lacking. Lab-scale studies revealed that TCS can affect multidrug resistance genes in anaerobic bioreactors. McNamara et al. (2014) found that TCS at 500 mg/kg selected for *mexB* in lab-scale anaerobic digesters inoculated with cow manure. In anaerobic digesters that were seeded with municipal biosolids, 500 mg/kg did not select for *mexB*, but methane production was inhibited. It is not yet known if anaerobic communities need to carry resistance genes in order to maintain function at these high TCS levels. The findings indicated that the microbial community structure, in addition to the concentration of TCS, influences the selection of resistance genes. Also, this research demonstrated that TCS can select for resistance, but does selection happen at environmental concentrations of TCS? Similarly, in activated sludge mesocosms, TCS selected for *tetQ* at 0.3 mg/L of TCS (Son et al., 2010). These two wastewater studies found a correlation between the presence of a resistance gene and TCS, but each study only investigated a single gene. A much more thorough research effort is required to determine the breadth of genes, with a special emphasis on multidrug resistance genes, that are selected for when environmental concentrations of TCS are applied to the complex microbial communities found in WWTPs.

It is also possible that TCS-resistant bacteria are formed in premise plumbing which can feed into municipal WWTPs. In a sink drain biofilm, TCS was shown to affect the bacterial

population structure when a 0.2% (∼2000 mg/L) solution of soap containing TCS was pumped over the biofilm (Mcbain et al., 2003). Overall bacterial diversity was reduced and several TCS-resistant bacteria related to *Achromobacter xylosoxidans* increased in abundance, while other species including aeromonads, bacilli, chryseobacteria, klebsiellae, stenotrophomonads, and *Microbacterium phyllosphaerae* were reduced. TCS in a drain following consumer usage may result in resistant bacteria which are then sent to WWTPs. Research is needed to determine if these bacteria survive in the sewer system and whether these resistant bacteria influence the resistance profile in WWTPs.

These studies show that TCS in the environment could select for resistance genes. It seems likely that TCS resistance coincides with TCS-derived cross-resistance to antibiotics in the environment, but further studies are required to validate this point.

RESEARCH GAPS AND CONCLUSIONS

It is noted that pathogenic bacteria, such as *S. epidermidis*, are less susceptible to TCS today than they were in the past (Skovgaard et al., 2013). Although resistance to TCS alone is not a threat to human health, antibiotic resistance is a major public health concern. TCS is widespread throughout the environment, but the direct role of TCS on antibiotic resistance in environmental systems is not yet defined. Four specific research questions, which are outlined below, need to be answered to identify the role of TCS on antibiotic resistance in environmental systems and ultimately determine the impact on human health.

IDENTIFY THE ROLE OF TRICLOSAN ON ANTIBIOTIC RESISTANCE IN ENVIRONMENTAL SYSTEMS

What is the threshold concentration of TCS that triggers resistance?

Triclosan is found at a wide range of concentrations in a wide range of environments (see **Figure 2**), and previous work found that TCS *can* select for a resistance gene in a complex microbial community (Son et al., 2010; McNamara et al., 2014). Moving forward it is important to determine the concentrations of TCS that trigger an increase in antibiotic resistance genes. Answering this question will also help address the question framed by the lack of data in **Figure 2**, i.e., what is the effect of TCS concentrations below the MIC? Do low levels of TCS select for resistance? Chronic exposure experiments using lab mesocosms should be performed at a range of steady-state TCS concentrations. In most real world cases, TCS levels will slowly increase, and lab experiments should be designed to reflect this slow loading rate. TCS levels should be slowly increased over time and held constant during steady-state operation of the mesocosm to determine the concentration of TCS that sustains changes in antibiotic resistance profiles. Metagenomics can be used with the Antibiotic Resistance Genes Database (Liu and Pop, 2009) to determine how the concentration of TCS impacts the relative abundance of antibiotic resistance genes. Additionally, qPCR can be employed to quantify changes in resistance gene abundance. After completion of these experiments we will have a better understanding about the concentrations of TCS that trigger increases in antibiotic resistance genes.

What is the role of the microbial community composition on TCS-derived antibiotic resistance?

Previous work revealed that the same concentration of TCS can lead to different impacts on the abundance of a resistant gene depending on the microbial community (McNamara et al., 2014). Experiments outlined in the question above should be performed on several different microbial communities. For example, communities found in river sediments, soils, and anaerobic digesters, should be investigated, and experiments should also be performed on several different communities from each type of environment. Wastewater communities can vary widely in their structure and so could the impact of TCS on resistance in these communities. Mesocosms should be inoculated with biosolids from several different cities to quantify how the same TCS concentrations impact the antibiotic resistance profiles of different communities. Is there a universal TCS concentration that is of concern in anaerobic digester communities, in sediments, or in soils? Illumina sequencing on 16S rRNA genes should be performed as well to determine if a link exists between certain microbes in a community and the TCS-impacted resistance profile.

What is the impact of TCS on resistance profiles in environments that are also perturbed by antibiotics?

Some resistance mechanisms, mainly efflux pumps, which are triggered by TCS are also triggered by antibiotics. In environments perturbed by TCS, antibiotics are also present (e.g., McClellan and Halden, 2010). Does the presence of TCS impact the acquisition of antibiotic resistance genes through horizontal gene transfer when antibiotics are already present? In other words, if TCS were not in these environments would the resistance profile look the same? To help answer this question, mesocosms could be inoculated with complex microbial communities from environments that are not heavily impacted by antibiotics or TCS. One set of mesocosms could be amended with antibiotics and another set would be amended with antibiotics and TCS. It is important to add TCS and antibiotics at ratios typically found in the environment. Granted, this question is difficult to answer because complex microbial communities from pristine environments will have inherent differences from the communities that are typically exposed to TCS and antibiotics. Another possibility would be to use a microbial community that has been widely exposed to antibiotics but not exposed to TCS; this type of community might be readily found in countries that have not adopted widespread use of TCS. Molecular techniques described above could be employed to determine the added impact of TCS on antibiotic resistance gene profiles.

Will the abundance of resistance genes decrease if TCS concentrations decrease?

It is important to know the concentrations of TCS that select for resistance and the communities that are most vulnerable to resistance caused by TCS, but it is equally important to know if resistance caused by TCS is reversible. Mitigated use of TCS has been proposed in the U.S. in part because of the potential concerns over antibiotic resistance (Landau and Young, 2014). If there were to be a sudden decline in consumer usage, would TCS-resistance and associated multidrug

resistance decrease? Experiments should be performed where TCS is slowly increased to encourage TCS-resistance and the mesocosms should be operated at steady-state with a constant supply of TCS. After the resistance profiled is determined, TCS should be removed from the system while the mesocosms are maintained under TCS-free conditions. The resistance profile can then be quantified after TCS is washed out of the mesocosms to determine if TCS-derived resistance will decrease as TCS levels decrease. This set of experiments would help to determine the potential impacts of reducing TCS from environmental systems.

IDENTIFY THE IMPACT OF TRICLOSAN-DERIVED RESISTANCE IN THE ENVIRONMENT ON PUBLIC HEALTH

Complex microbial environments can be highly conducive for the transfer of resistance genes (Baquero et al., 2008). Locations with high densities of bacteria, such as WWTPs, produce conditions which are suitable for proliferation and exchange of resistance genes, and TCS may be serving as a selective pressure to increase the abundance of resistance genes in these communities. In a study focusing on plasmid genes found in activated sludge, a wide array of resistance genes, including genes that confer resistance to TCS in pure cultures (*mexB*, and other efflux pump homologues including *acrB* and *smeE*) were found on plasmids (Zhang et al., 2011). Research is needed to address the fate of environmentally derived resistance genes to understand how they impact human health.

The fate and transport of these resistance genes in the environment following discharge from WWTPs is not well defined. Transport of genes can occur through direct uptake of DNA (transformation), by viral infection (transduction), or by transfer of plasmids and other mobile genetic elements (conjugation); the resulting pathways for genetic transport are complicated to constrain for modeling (Baquero et al., 2008). Genetic tracking of resistance in the environment would require vast resources; using established models of viruses or bacteria may be an appropriate place to begin modeling resistance gene transport.

The rate of transfer of antibiotic resistance genes in the environment to humans is also under investigation (Viau et al., 2011; Ashbolt et al., 2013). Better understanding the threat of environmentally derived antibiotic resistance genes on human health is required to determine the role of TCS on public health. Employing quantitative microbial risk assessment for antibiotic resistance genes in environmental systems may be a useful avenue for pursuing this topic.

ACKNOWLEDGMENTS

Daniel E. Carey was supported by the Joseph A. and Dorothy C. Rutkauskas Scholarship. We would like to thank Tucker Burch and two anonymous reviewers for their comments on this manuscript.

REFERENCES

Alanis, A. J. (2005). Resistance to antibiotics: are we in the post-antibiotic era? *Arch. Med. Res.* 36, 697–705. doi: 10.1016/j.arcmed.2005.06.009

Anger, C. T., Sueper, C., Blumentritt, D. J., McNeill, K., Engstrom, D. R., and Arnold, W. A. (2013). Quantification of triclosan, chlorinated triclosan derivatives, and their dioxin photoproducts in lacustrine sediment cores. *Environ. Sci. Technol.* 47, 1833–1843. doi: 10.1021/es3045289

Ashbolt, N. J., Amézquita, A., Backhaus, T., Borriello, P., Brandt, K. K., Collignon, P., et al. (2013). Human health risk assessment (HHRA) for environmental development and transfer of antibiotic resistance. *Environ. Health Perspect.* 121, 993–1001. doi: 10.1289/ehp.1206316

Bailey, A. M., Paulsen, I. T., and Piddock, L. J. V. (2008). RamA confers multidrug resistance in *Salmonella enterica* via increased expression of *acrB*, which is inhibited by chlorpromazine. *Antimicrob. Agents Chemother.* 52, 3604–3611. doi: 10.1128/AAC.00661-08

Baquero, F., Martínez, J. L., and Cantón, R. (2008). Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol.* 19, 260–265. doi: 10.1016/j.copbio.2008.05.006

Bedoux, G., Roig, B., Thomas, O., Dupont, V., and Le Bot, B. (2012). Occurrence and toxicity of antimicrobial triclosan and by-products in the environment. *Environ. Sci. Pollut. Res. Int.* 19, 1044–1065. doi: 10.1007/s11356-011-0632-z

Benotti, M. J., Trenholm, R. A., Vanderford, B. J., Holady, J. C., Stanford, B. D., and Snyder, S. A. (2009). Pharmaceuticals and endocrine disrupting compounds in U.S. drinking water. *Environ. Sci. Technol.* 43, 597–603. doi: 10.1021/es801845a

Bergler, H., Fuchsbichler, S., Hogenauer, G., and Turnowsky, F. (1996). The enoyl-[acyl-carrier-protein] reductase (FabI) of *Escherichia coli*, which catalyzes a key regulatory step in fatty acid biosynthesis, accepts NADH and NADPH as cofactors and is inhibited by palmitoyl-CoA. *Eur. J. Biochem.* 242, 689–694. doi: 10.1111/j.1432-1033.1996.0689r.x

Bester, K. (2005). Fate of triclosan and triclosan-methyl in sewage treatment plants and surface waters. *Arch. Environ. Contam. Toxicol.* 49, 9–17. doi: 10.1007/s00244-004-0155-4

Bhargava, H., and Leonard, P. (1996). Triclosan: applications and safety. *Am. J. Infect. Control* 24, 209–218. doi: 10.1016/S0196-6553(96)90017-6

Birosová, L., and Mikulásová, M. (2009). Development of triclosan and antibiotic resistance in *Salmonella enterica* serovar Typhimurium. *J. Med. Microbiol.* 58, 436–441. doi: 10.1099/jmm.0.003657-0

Blair, B. D., Crago, J. P., Hedman, C. J., Treguer, R. J. F., Magruder, C., Royer, L. S., et al. (2013). Evaluation of a model for the removal of pharmaceuticals, personal care products, and hormones from wastewater. *Sci. Total Environ.* 444, 515–521. doi: 10.1016/j.scitotenv.2012.11.103

Braoudaki, M., and Hilton, A. C. (2004). Low level of cross-resistance between triclosan and antibiotics in *Escherichia coli* K-12 and *E. coli* O55 compared to *E. coli* O157. FEMS microbiology letters. 235, 305–309. doi: 10.1016/j.femsle.2004.04.049

Brenwald, N., and Fraise, A. (2003). Triclosan resistance in methicillin-resistant *Staphylococcus aureus* (MRSA). *J Hosp. Infect.* 55, 141–144. doi: 10.1016/S0195-6701(03)00222-6

Burch, T. R., Sadowsky, M. J., and Lapara, T. M. (2014). Fate of antibiotic resistance genes and class 1 integrons in soil microcosms following the application of treated residual municipal wastewater solids. *Environ. Sci. Technol.* 48, 5620–5627. doi: 10.1021/es501098g

Buth, J. M., Steen, P. O., Sueper, C., Blumentritt, D., Vikesland, P. J., Arnold, W. A., et al. (2010). Dioxin photoproducts of triclosan and its chlorinated derivatives in sediment cores. *Environ. Sci. Technol.* 44, 4545–4551. doi: 10.1021/es1001105

Butler, E., Whelan, M. J., Ritz, K., Sakrabani, R., and van Egmond, R. (2011). Effects of triclosan on soil microbial respiration. *Environ. Toxicol. Chem.* 30, 360–366. doi: 10.1002/etc.405

Calafat, A. M., Ye, X., Wong, L. Y., Reidy, J. A., and Needham, L. L. (2008). Urinary concentrations of triclosan in the U.S. population: 2003–2004. *Environ. Health Perspect.* 116, 303–307. doi: 10.1289/ehp.10768

Centers for Disease Control and Prevention (CDC), U.S. Department of Health, and Human Services. (2013). *REPORT—Antibiotic Resistance Threats in the United States*. Atlanta, GA: Centers for Disease Control and Prevention, 1–114.

Cha, J., and Cupples, A. M. (2009). Detection of the antimicrobials triclocarban and triclosan in agricultural soils following land application of municipal biosolids. *Water Res.* 43, 2522–2530. doi: 10.1016/j.watres.2009.03.004

Chalew, T. E., and Halden, R. U. (2009). Environmental exposure of aquatic and terrestrial biota to triclosan and triclocarban. *J. Am. Water Works Assoc.* 45, 4–13. doi: 10.1111/j.1752-1688.2008.00284.x

Champlin, F. R., Ellison, M. L., Bullard, J. W., and Conrad, R. S. (2005). Effect of outer membrane permeabilisation on intrinsic resistance to low triclosan levels in *Pseudomonas aeruginosa*. *Int. J. Antimicrob. Agents* 26, 159–164. doi: 10.1016/j.ijantimicag.2005.04.020

Chapman, J. S. (2003). Biocide resistance mechanisms. *Int. Biodeterior. Biodegradation* 51, 133–138. doi: 10.1016/S0964-8305(02)00097-5

Chen, Y., Pi, B., Zhou, H., Yu, Y., and Li, L. (2009). Triclosan resistance in clinical isolates of *Acinetobacter baumannii*. *J. Med. Microbiol.* 58, 1086–1091. doi: 10.1099/jmm.0.008524-0

Chuanchuen, R., Beinlich, K., Hoang, T. T., Becher, A., Karkhoff-Schweizer, R. R., and Schweizer, H. P. (2001). Cross-Resistance between triclosan and antibiotics in *Pseudomonas aeruginosa* is mediated by multidrug efflux pumps: exposure of a susceptible mutant strain to triclosan selects nfxB mutants overexpressing MexCD-OprJ. *Antimicrob. Agents Chemother.* 45, 428–432. doi: 10.1128/AAC.45.2.428-432.2001

Chuanchuen, R., and Schweizer, H. P. (2012). Global transcriptional responses to triclosan exposure in *Pseudomonas aeruginosa*. *Int. J. Antimicrob. Agents* 40, 114–122. doi: 10.1016/j.ijantimicag.2012.04.008

Ciusa, M. L., Furi, L., Knight, D., Decorosi, F., Fondi, M., Raggi, C., et al. (2012). A novel resistance mechanism to triclosan that suggests horizontal gene transfer and demonstrates a potential selective pressure for reduced biocide susceptibility in clinical strains of *Staphylococcus aureus*. *Int. J. Antimicrob. Agents* 40, 210–220. doi: 10.1016/j.ijantimicag.2012.04.021

Condell, O., Sheridan, Á., Power, K. A., Bonilla-Santiago, R., Sergeant, K., Renaut, J., et al. (2012). Comparative proteomic analysis of *Salmonella* tolerance to the biocide active agent triclosan. *J. Proteom.* 75, 4505–4519. doi: 10.1016/j.jprot.2012.04.044

Department of Health and Human Services (DHHS). (2012). *Drug Use Review*. Available at: http://www.fda.gov/downloads/Drugs/DrugSafety/InformationbyDrugClass/UCM319435.pdf [accessed October 8, 2014].

Drury, B., Scott, J., Rosi-Marshall, E. J., and Kelly, J. J. (2013). Triclosan exposure increases triclosan resistance and influences taxonomic composition of benthic bacterial communities. *Environ. Sci. Technol.* 47, 8923–8930. doi: 10.1021/es401919

Fan, F., Yan, K., and Wallis, N. (2002). Defining and combating the mechanisms of triclosan resistance in clinical isolates of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 46, 3343–3347. doi: 10.1128/AAC.46.11.3343-3347.2002

Fang, J. L., Stingley, R. L., Beland, F. A., Harrouk, W., Lumpkins, D. L., and Howard, P. (2010). Occurrence, efficacy, metabolism, and toxicity of triclosan. *J. Environ. Sci. Health C Environ. Carcinog. Ecotoxicol. Rev.* 28, 147–171. doi: 10.1080/10590501.2010.504978

Food and Drug Administration (FDA). (2011). *Summary Report On Antimicrobials Sold or Distributed for Use in Food-Producing Animals*. Available at: http://www.fda.gov/downloads/ForIndustry/UserFees/AnimalDrugUserFeeActADUFA/UCM338170.pdf [accessed October 8, 2014].

Ghosh, S., Ramsden, S. J., and LaPara, T. M. (2009). The role of anaerobic digestion in controlling the release of tetracycline resistance genes and class 1 integrons from municipal wastewater treatment plants. *Appl. Microbiol. Biotechnol.* 84, 791–796. doi: 10.1007/s00253-009-2125-2

Halden, R. U. (2014). On the need and speed of regulating triclosan and triclocarban in the United States. *Environ. Sci. Technol.* 48, 3603–3611. doi: 10.1021/es500495p

Heath, R. J., and Rock, C. O. (2000). A triclosan-resistant bacterial enzyme. *Nature* 406, 145–146. doi: 10.1038/35018162

Heath, R. J., Su, N., Murphy, C. K., and Rock, C. O. (2000). The enoyl-[acyl-carrier-protein] reductases FabI and FabL from *Bacillus subtilis*. *J. Biol. Chem.* 275, 40128–40133. doi: 10.1074/jbc.M005611200

Heidler, J., and Halden, R. U. (2007). Mass balance assessment of triclosan removal during conventional sewage treatment. *Chemosphere* 66, 362–369. doi: 10.1016/j.chemosphere.2006.04.066

Heidler, J., and Halden, R. U. (2008). Meta-analysis of mass balances examining chemical fate during wastewater treatment. *Environ. Sci. Technol.* 42, 6324–6332. doi: 10.1021/es703008y

Hernández, A., Ruiz, F. M., Romero, A., and Martínez, J. L. (2011). The binding of triclosan to SmeT, the repressor of the multidrug efflux pump SmeDEF, induces antibiotic resistance in *Stenotrophomonas maltophilia*. *PLoS Pathog.* 7:e1002103. doi: 10.1371/journal.ppat.1002103

Holzem, R., Stapleton, H., and Gunsch, C. K. (2014). Determining the ecological impacts of organic contaminants in biosolids using a high-throughput colorimetric denitrification assay: a case study with antimicrobial agents. *Environ. Sci. Technol.* 48, 1646–1655. doi: 10.1021/es404431k

Hooper, D. C. (2005). Efflux pumps and nosocomial antibiotic resistance: a primer for hospital epidemiologists. *Clin. Infect. Dis.* 40, 1811–1817. doi: 10.1086/430381

Johnson, D. R., Czechowska, K., Chèvre, N., and van der Meer, J. R. (2009). Toxicity of triclosan, penconazole and metalaxyl on *Caulobacter crescentus* and a freshwater microbial community as assessed by flow cytometry. *Environ. Microbiol.* 11, 1682–1691. doi: 10.1111/j.1462-2920.2009.01893.x

Jones, R. D., Jampani, H. B., Newman, J. L., and Lee, A. S. (2000). Triclosan: a review of effectiveness and safety in health care settings. *Am. J. Infect. Control* 28, 184–196. doi: 10.1067/mic.2000.102378

Karatzas, K. A. G., Webber, M. A., Jorgensen, F., Woodward, M. J., Piddock, L. J. V., and Humphrey, T. J. (2007). Prolonged treatment of *Salmonella enterica* serovar Typhimurium with commercial disinfectants selects for multiple antibiotic resistance, increased efflux and reduced invasiveness. *J. Antimicrob. Chemother.* 60, 947–955. doi: 10.1093/jac/dkm314

Kern, W. V., Oethinger, M., Jellen-Ritter, A., and Levy, S. B. (2000). Non-target gene mutations in the development of fluoroquinolone resistance in *Escherichia coli*. *Antimicrob. Agents Chemother.* 44, 814–820. doi: 10.1128/AAC.44.4.814-820.2000

Khachatourians, G. G. (1998). Agricultural use of antibiotics and the evolution and transfer of antibiotic-resistant bacteria. *Can. Med. Assoc. J.* 159, 1129–1136.

Kumar, K. S., Priya, S. M., Peck, A. M., and Sajwan, K. S. (2010). Mass loadings of triclosan and triclocarban from four wastewater treatment plants to three rivers and landfill in Savannah, Georgia, USA. *Arch. Environ. Contam. Toxicol.* 58, 275–285. doi: 10.1007/s00244-009-9383-y

Kümmerer, K. (2004). Resistance in the environment. *J. Antimicrob. Chemother.* 54, 311–320. doi: 10.1093/jac/dkh325

Landau, E., and Young, S. (2014). Minnesota issues ban on antibacterial ingredient. *CNN (online)*. Available at: http://www.cnn.com/2014/05/21/health/triclosan-ban-antibacterial/

LaPara, T. M., Burch, T. R., McNamara, P. J., Tan, D. T., Yan, M., and Eichmiller, J. J. (2011). Tertiary-treated municipal wastewater is a significant point source of antibiotic resistance genes into Duluth-Superior Harbor. *Environ. Sci. Technol.* 45, 9543–9549. doi: 10.1021/es202775r

Levy, S. B. (2002). Active efflux, a common mechanism for biocide and antibiotic resistance. *J. Appl. Microbiol.* 92, 65S–71S. doi: 10.1046/j.1365-2672.92.5s1.4.x

Levy, S. B., and Marshall, B. (2004). Antibacterial resistance worldwide: causes, challenges and responses. *Nat. Med.* 10, S122–S129. doi: 10.1038/nm1145

Liu, B., and Pop, M. (2009). ARDB—Antibiotic Resistance Genes Database. *Nucleic Acids Res.* 37, D443–D447. doi: 10.1093/nar/gkn656

Lozano, N., Rice, C. P., Ramirez, M., and Torrents, A. (2013). Fate of Triclocarban, Triclosan and Methyltriclosan during wastewater and biosolids treatment processes. *Water Res.* 47, 4519–4527. doi: 10.1016/j.watres.2013.05.015

Lubarsky, H. V., Gerbersdorf, S. U., Hubas, C., Behrens, S., Ricciardi, F., and Paterson, D. M. (2012). Impairment of the bacterial biofilm stability by triclosan. *PLoS ONE* 7:e31183. doi: 10.1371/journal.pone.0031183

Ma, Y., Wilson, C., Novak, J. T., Riffat, R., Aynur, S., Murthy, S., et al. (2011). Effect of various sludge digestion conditions on sulfonamide, macrolide, and tetracycline resistance genes and class I integrons. *Environ. Sci. Technol.* 45, 7855–7861. doi: 10.1021/es200827t

Massengo-Tiassé, R. P., and Cronan, J. E. (2008). *Vibrio cholerae* FabV defines a new class of enoyl-acyl carrier protein reductase. *J. Biol. Chem.* 283, 1308–1316. doi: 10.1074/jbc.M708171200

Massengo-Tiassé, R. P., and Cronan, J. E. (2009). Diversity in enoyl-acyl carrier protein reductases. *Cell. Mol. Life Sci.* 66, 1507–1517. doi: 10.1007/s00018-009-8704-7

Mavri, A., Kurin, M., and Smole, S. (2012). The prevalence of antibiotic and biocide resistance among *Campylobacter coli* and *Campylobacter jejuni* from different sources. *Food Technol. Biotechnol.* 50, 371–376.

McAvoy, D. C., Schatowitz, B., Jacob, M., Hauk, A., and Eckhoff, W. S. (2002). Environmental Chemistry Measurement of triclosan in wastewater treatment systems. *Environ. Toxicol. Chem.* 21, 1323–1329. doi: 10.1002/etc.5620210701

Mcbain, A. J., Bartolo, R. G., Catrenich, C. E., Charbonneau, D., Ledder, R. G., Price, B. B., et al. (2003). Exposure of sink drain microcosms to triclosan: population dynamics and antimicrobial susceptibility. *Appl. Environ. Microbiol.* 69, 5433–5442. doi: 10.1128/AEM.69.9.5433-5442.2003

McClellan, K., and Halden, R. U. (2010). Pharmaceuticals and personal care products in archived U.S. biosolids from the 2001 EPA National Sewage Sludge Survey. *Water Res.* 44, 658–668. doi: 10.1016/j.watres.2009.12.032

McDonnell, G., and Russell, D. A. (1999). Antiseptics and disinfectants: activity, action, and resistance. *Clin. Microbiol. Rev.* 12, 147–179.

McMurry, L. M., Mcdermott, P. F., and Levy, S. B. (1999). Genetic evidence that InhA of *Mycobacterium smegmatis* is a target for triclosan. *Antimicrob. Agents Chemother.* 43, 711–713.

McMurry, L. M., Oethinger, M., and Levy, S. B. (1998a). Overexpression of marA, soxS, or acrAB produces resistance to triclosan in laboratory and clinical strains of *Escherichia coli*. *FEMS Microbiol. Lett.* 166, 305–309. doi: 10.1111/j.1574-6968.1998.tb13905.x

McMurry, L. M., Oethinger, M., and Levy, S. B. (1998b). Triclosan targets lipid synthesis. *Nature* 394, 531–532. doi: 10.1038/28970

McNamara, P. J., and Krzmarzick, M. J. (2013). Triclosan enriches for *Dehalococcoides*-like Chloroflexi in anaerobic soil at environmentally relevant concentrations. *FEMS Microbiol. Lett.* 344, 48–52. doi: 10.1111/1574-6968.12153

McNamara, P. J., Lapara, T. M., and Novak, P. J. (2014). The impacts of triclosan on anaerobic community structures, function, and antimicrobial resistance. *Environ. Sci. Technol.* 48, 7393–7400. doi: 10.1021/es501388v

Middleton, J. H., and Salierno, J. D. (2013). Antibiotic resistance in triclosan tolerant fecal coliforms isolated from surface waters near wastewater treatment plant outflows (Morris County, NJ, USA). *Ecotoxicol. Environ. Saf.* 88, 79–88. doi: 10.1016/j.ecoenv.2012.10.025

Miller, T., and Heidler, J. (2008). Fate of triclosan and evidence for reductive dechlorination of triclocarban in estuarine sediments. *Environ. Sci. Technol.* 42, 4570–4576. doi: 10.1021/es702882g

Mima, T., Joshi, S., Gomez-Escalada, M., and Schweizer, H. P. (2007). Identification and characterization of TriABC-OpmH, a triclosan efflux pump of *Pseudomonas aeruginosa* requiring two membrane fusion proteins. *J. Bacteriol.* 189, 7600–7609. doi: 10.1128/JB.00850-07

Munir, M., Wong, K., and Xagoraraki, I. (2010). Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water Res.* 45, 681–693. doi: 10.1016/j.watres.2010.08.033

Nietch, C., and Quinlan, E. (2013). Effects of a chronic lower range of triclosan exposure on a stream mesocosm community. *Environ. Toxicol. Chem.* 32, 2874–2887. doi: 10.1002/etc.2385

Poole, K. (2002). Mechanisms of bacterial biocide and antibiotic resistance. *J. Appl. Microbiol. Symp. Suppl.* 92, 55S–64S. doi: 10.1046/j.1365-2672.92.5s1.8.x

Proia, L., Morin, S., Peipoch, M., Romaní, A. M., and Sabater, S. (2011). Resistance and recovery of river biofilms receiving short pulses of Triclosan and Diuron. *Sci. Total Environ.* 409, 3129–3137. doi: 10.1016/j.scitotenv.2011.05.013

Pruden, A., Arabi, M., and Storteboom, H. N. (2012). Correlation between upstream human activities and riverine antibiotic resistance genes. *Environ. Sci. Technol.* 46, 11541–11549. doi: 10.1021/es302657r

Pruden, A., Pei, R., Storteboom, H., and Carlson, K. H. (2006). Antibiotic resistance genes as emerging contaminants: studies in northern Colorado. *Environ. Sci. Technol.* 40, 7445–7450. doi: 10.1021/es060413l

Pycke, B. F. G., Crabbé, A., Verstraete, W., and Leys, N. (2010a). Characterization of triclosan-resistant mutants reveals multiple antimicrobial resistance mechanisms in *Rhodospirillum rubrum* S1H. *Appl. Environ. Microbiol.* 76, 3116–3123. doi: 10.1128/AEM.02757-09

Pycke, B. F. G., Vanermen, G., Monsieurs, P., De Wever, H., Mergeay, M., Verstraete, W., et al. (2010b). Toxicogenomic response of *Rhodospirillum rubrum* S1H to the micropollutant triclosan. *Appl. Environ. Microbiol.* 76, 3503–3513. doi: 10.1128/AEM.01254-09

Pycke, B. F. G., Geer, L. A., Dalloul, M., Abulafia, O., Jenck, A. M., and Halden, R. U. (2014). Human fetal exposure to triclosan and triclocarban in an urban population from Brooklyn, New York. *Environ. Sci. Technol.* 48, 8831–8838. doi: 10.1021/es501100w

Rensch, U., Klein, G., and Kehrenberg, C. (2013). Analysis of triclosan-selected *Salmonella enterica* mutants of eight serovars revealed increased aminoglycoside susceptibility and reduced growth rates. *PLoS ONE* 8:e78310. doi: 10.1371/journal.pone.0078310

Russell, A. D. (2000). Do biocides select for antibiotic resistance? *J. Pharm. Pharmacol.* 52, 227–233. doi: 10.1211/0022357001773742

Saleh, S., Haddadin, R. N. S., Baillie, S., and Collier, P. J. (2011). Triclosan—an update. *Lett. Appl. Microbiol.* 52, 87–95. doi: 10.1111/j.1472-765X.2010.02976.x

Sanchez, P., Moreno, E., and Martinez, J. L. (2005). The biocide triclosan selects *Stenotrophomonas maltophilia* mutants that overproduce the SmeDEF multidrug efflux pump. *Antimicrob. Agents Chemother.* 49, 781–783. doi: 10.1128/AAC.49.2.781-782.2005

Schweizer, H. P. (2001). Triclosan: a widely used biocide and its link to antibiotics. *FEMS Microbiol. Lett.* 202, 1–7. doi: 10.1111/j.1574-6968.2001.tb10772.x

Sheridan, A., Lenahan, M., Condell, O., Bonilla-Santiago, R., Sergeant, K., Renaut, J., et al. (2013). Proteomic and phenotypic analysis of triclosan tolerant verocytotoxigenic *Escherichia coli* O157:H19. *J. Proteom.* 80, 78–90. doi: 10.1016/j.jprot.2012.12.025

Singer, H., Müller, S., Tixier, C., and Pillonel, L. (2002). Triclosan: occurrence and fate of a widely used biocide in the aquatic environment: field measurements in wastewater treatment plants, surface waters, and lake sediments. *Environ. Sci. Technol.* 36, 4998–5004. doi: 10.1021/es025750i

Skovgaard, S., Nielsen, L. N., Larsen, M. H., Skov, R. L., Ingmer, H., and Westh, H. (2013). *Staphylococcus epidermidis* isolated in 1965 are more susceptible to triclosan than current isolates. *PLoS ONE* 8:e62197. doi: 10.1371/journal.pone.0062197

Slater-Radosti, C., Aller, G. V., Greenwood, R., Nicholas, R., Keller, P. M., DeWolf, W. E. Jr., et al. (2001). Biochemical and genetic characterization of the action of triclosan on *Staphylococcus aureus*. *J. Antimicrob. Chemother.* 48, 1–6. doi: 10.1093/jac/48.1.1

Son, A., Kennedy, I. M., Scow, K. M., and Hristova, K. R. (2010). Quantitative gene monitoring of microbial tetracycline resistance using magnetic luminescent nanoparticles. *J. Environ. Monit.* 12, 1362–1367. doi: 10.1039/c001974g

Stasinakis, A. S., Mamais, D., Thomaidis, N. S., Danika, E., Gatidou, G., and Lekkas, T. D. (2008). Inhibitory effect of triclosan and nonylphenol on respiration rates and ammonia removal in activated sludge systems. *Ecotoxicol. Environ. Saf.* 70, 199–206. doi: 10.1016/j.ecoenv.2007.12.011

Suller, M. T. E., and Russell, A. D. (2000). Triclosan and antibiotic resistance in *Staphylococcus aureus*. *J Antimicrob Chemother.* 46, 11–18. doi: 10.1093/jac/46.1.11

Tkachenko, O., Shepard, J., Aris, V. M., Joy, A., Bello, A., Londono, I., et al. (2007). A triclosan-ciprofloxacin cross-resistant mutant strain of *Staphylococcus aureus* displays an alteration in the expression of several cell membrane structural and functional genes. *Res. Microbiol.* 158, 651–658. doi: 10.1016/j.resmic.2007.09.003

United States Environmental Protection Agency (USEPA). (2009). *Targeted National Sewage Sludge Survey Statistical Analysis Report*. Report EPA-822-R-08-018. Washington, DC: USEPA.

Venkatesan, A. K., and Halden, R. U. (2014). Wastewater treatment plants as chemical observatories to forecast ecological and human health risks of manmade chemicals. *Sci. Rep.* 4, 1–11. doi: 10.1038/srep03731

Viau, E., Bibby, K., Paez-Rubio, T., and Peccia, J. (2011). Toward a consensus view on the infectious risks associated with land application of sewage sludge. *Environ. Sci. Technol.* 45, 5459–5469. doi: 10.1021/es200566f

Villalaín, J., Mateo, C. R., Aranda, F. J., Shapiro, S., and Micol, V. (2001). Membranotropic effects of the antibacterial agent Triclosan. *Arch. Biochem. Biophys.* 390, 128–136. doi: 10.1006/abbi.2001.2356

Webber, M. A., Randall, L. P., Cooles, S., Woodward, M. J., and Piddock, L. J. V. (2008). Triclosan resistance in *Salmonella enterica* serovar Typhimurium. *J. Antimicrob. Chemother.* 62, 83–91. doi: 10.1093/jac/dkn137

Welsch, T., and Gillock, E. (2011). Triclosan-resistant bacteria isolated from feedlot and residential soils. *J. Environ. Sci. Health A Tox. Hazard. Subst. Environ. Eng.* 46, 436–440. doi: 10.1080/10934529.2011.549407

World Health Organization (WHO). (2014). *Antimicrobial Resistance: Global Report on Surveillance*. Geneva: WHO. Available at: http://apps.who.int/iris/bitstream/10665/112642/1/9789241564748_eng.pdf?ua=1 [accessed September 15, 2014].

Xia, K., Hundal, L. S., Kumar, K., Armbrust, K., Cox, A. E., and Granato, T. C. (2010). Triclocarban, triclosan, polybrominated diphenyl ethers, and 4-nonylphenol in biosolids and in soil receiving 33-year biosolids application. *Environ. Toxicol. Chem.* 29, 597–605. doi: 10.1002/etc.66

Yang, Y., Li, B., Zou, S., Fang, H. H. P., and Zhang, T. (2014). Fate of antibiotic resistance genes in sewage treatment plant revealed by metagenomic approach. *Water Res.* 62C, 97–106. doi: 10.1016/j.watres.2014.05.019

Yazdankhah, S. P., Scheie, A. A., Høiby, E. A., Lunestad, B.-T., Heir, E., Fotland, T. Ø., et al. (2006). Triclosan and antimicrobial resistance in bacteria: an overview. *Microb. Drug Resist.* 12, 83–90. doi: 10.1089/mdr.2006.12.83

Ying, G.-G., Yu, X.-Y., and Kookana, R. S. (2007). Biological degradation of triclocarban and triclosan in a soil under aerobic and anaerobic conditions and comparison with environmental fate modelling. *Environ. Pollut.* 150, 300–305. doi: 10.1016/j.envpol.2007.02.013

Yu, B. J., Kim, J. A., Ju, H. M., Choi, S.-K., Hwang, S. J., Park, S., et al. (2012). Genome-wide enrichment screening reveals multiple targets and resistance genes for triclosan in *Escherichia coli*. *J. Microbiol.* 50, 785–791. doi: 10.1007/s12275-012-2439-0

Yu, B. J., Kim, J. A., and Pan, J.-G. (2010). Signature gene expression profile of triclosan-resistant *Escherichia coli*. *J. Antimicrob. Chemother.* 65, 1171–1177. doi: 10.1093/jac/dkq114

Zhang, T., Zhang, X.-X., and Ye, L. (2011). Plasmid metagenome reveals high levels of antibiotic resistance genes and mobile genetic elements in activated sludge. *PLoS ONE* 6:e26041. doi: 10.1371/journal.pone.0026041

Zhu, L., Lin, J., Ma, J., Cronan, J. E., and Wang, H. (2010). Triclosan resistance of *Pseudomonas aeruginosa* PAO1 is due to FabV, a triclosan-resistant enoyl-acyl carrier protein reductase. *Antimicrob. Agents Chemother.* 54, 689–698. doi: 10.1128/AAC.01152-09

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 18 October 2014; accepted: 19 December 2014; published online: 15 January 2015.

*Citation: Carey DE and McNamara PJ (2015) The impact of triclosan on the spread of antibiotic resistance in the environment. Front. Microbiol. **5**:780. doi: 10.3389/fmicb.2014.00780*

This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read, for greatest visibility

COLLABORATIVE PEER-REVIEW
Designed to be rigorous – yet also collaborative, fair and constructive

FAST PUBLICATION
Average 85 days from submission to publication (across all journals)

COPYRIGHT TO AUTHORS
No limit to article distribution and re-use

TRANSPARENT
Editors and reviewers acknowledged by name on published articles

SUPPORT
By our Swiss-based editorial team

IMPACT METRICS
Advanced metrics track your article's impact

GLOBAL SPREAD
5'100'000+ monthly article views and downloads

LOOP RESEARCH NETWORK
Our network increases readership for your article

Frontiers
EPFL Innovation Park, Building I • 1015 Lausanne • Switzerland
Tel +41 21 510 17 00 • Fax +41 21 510 17 01 • info@frontiersin.org
www.frontiersin.org

Find us on