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Review

Antibiotic resistance plasmids in wastewater treatment plants and their possible dissemination into the environment

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Antibiotic resistance plasmids found in wastewater treatment plants (WWTPs) may represent a threat to public health if they are readily disseminated into the environment and ultimately into pathogenic bacteria. The wastewater environments provide an ideal ecosystem for development and evolution of antibiotic resistance plasmids. Selective pressures for resistance to toxic compounds, high organic content and high bacterial diversity promotes gene exchange mechanisms involving interactions of conjugative plasmids with bacterial chromosomes, integrons and transposons resulting in the acquisition and accumulation of various antibiotic resistance genes into plasmids. Several studies have isolated plasmids from wastewater plants which carry resistance genes to almost all clinically relevant antibiotics. This review will discuss the possible release of these plasmids from WWTPs and their undesirable effects in the environment. Studies using advanced molecular detection tools and high throughput DNA sequencing technology help accurately quantify the prevalence and transmission of these plasmids in the environment. Ultimately assessing the significance of these plasmids as pollutants will help to determine the implications to public health.

Key words: Antibiotic resistance, plasmids, wastewater, treatment plants.

INTRODUCTION

The increase in antibiotic resistance within clinical bacterial isolates is undermining the efforts of antibiotic therapy in the treatment of infectious diseases. At the same time, there has been a significant increase in the level of organic and inorganic pollutants, including antibiotic residues, entering the environment (Moura et al., 2010). Intensive use of antibiotics in clinical and agricultural settings has been suggested to promote an increase in antibiotic resistance bacterial populations (Aminov, 2009; Martinez, 2009a, b). The recently developed Antibiotic Resistance Database (ARDB, http://ardb.cbcb.umd.edu/) estimates there are over 13000 antibiotic resistance genes (ARGs) identified in greater than 600 genomes of antibiotic resistance bacteria (ARB) (Liu and Pop, 2009). Notably, antibiotic resistance determinants found in potential pathogens comprised only a small portion of the total ARGs surveyed (Davies and Davies, 2010), which implies that the major reservoir for ARGs is in non-pathogenic environmental bacteria. This pool of ARGs was recently termed the environmental antibiotic resistome (Wright, 2007). In spite of the implications that this reservoir of resistance genes may spread to clinical pathogenic bacteria, the resistome has been relatively uncharacterized globally. A link between the environmental antibiotic resistome and the increasing antibiotic resistance problem in clinical pathogens seems plausible given the likely contact between

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Abbreviations: ARGs, Antibiotic resistance genes; **ARB,** antibiotic resistance bacteria; **ARDB,** antibiotic resistance database; **WWTPs,** wastewater treatment plants; **PCR,** polymerase chain reaction; **qPCR,** quantitative PCR; **BHR,** broad-host range; **MGEs,** mobile genetic elements.

clinical opportunistic pathogens, such as *Pseudomonas aeruginosa, Acinetobacter baumannii, Stenotrophomas maltophilia* and environmental microbes (Baquero et al., 2008; Martinez, 2009a, b). It is well established that ARB and ARGs existed prior to widespread antibiotic use (Hall and Barlow, 2004; Martinez, 2009a,b; Allen et al., 2010), however, the importance of the non-clinical environment in the increase of antibiotic resistance to clinical pathogens remains unclear (Martinez, 2009a; Davies and Davies, 2010). ARGs of clinical importance have been detected in various environmental non-pathogenic bacteria (Heuer et al., 2002; Riesenfeld et al., 2004; Martinez, 2009a, b) and from both soil and water ecosystems (Riesenfeld et al., 2004; Ansari et al., 2008; Baquero et al., 2008; Zhang, 2009). In several instances, the soil and water environments yielding significant populations of antibiotic resistant environmental isolates are from sites impacted by pollution with a variety of substances, including antibiotics, from human activities (Baquero et al., 2008; Martinez, 2009a, b; Allen et al., 2010).

Pollution is broadly defined as harmful inputs of chemical, biological and physical waste products into the environment from various sources such as hospitals, homes, urban and agricultural industries. Wastewater treatment plants (WWTPs) play a critical role in managing fecal wastes and removing toxic organic and inorganic contaminants contained in wastes originating from both municipal and agricultural activities. The goal of a waste-water treatment process is to reduce the concentration of dissolved organic carbon, nitrogen and phosphorous and eliminate viable pathogens from the liquid effluent (Zhang et al., 2010). Physical (e.g. filtration), chemical (e.g. disinfection) and microbial activities are used to effectively remediate waste entering the plant (Zhang et al., 2010). The diversity of microbial communities within a WWTP can be large depending on the bio-chemical ingredients received by the WWTP (Wagner and Loy, 2002; Zhao et al., 2008). DNA sequencing of 16s rRNA genes from WWTP samples has detected greater than twenty bacterial phyla. The major representatives include proteobacteria, chloroflexi, firmicutes, spirochaetes, and bacterioidetes (Wagner and Loy, 2002; Moura et al., 2007; Narihiro and Sekiguchi, 2007). These phyla represent a variety of gram positive and negative bacteria including some environmental opportunistic and clinically significant bacterial pathogens.

Plasmids are an important vehicle for carrying antibiotic resistance genes (Bennett et al., 2008) and the high organic load and large concentrations of diverse bacterial communities present in WWTP represents a unique opportunity for the evolution and transfer of antibiotic resistance genes. Recent technical advances in molecular microbiology such as, non-culture based techniques and high throughput DNA sequencing technology have advanced environmental microbiology research, including our abilities to characterize the antibiotic resistome in WWTPs. This paper will review the recent developments in investigating the diversity of antibiotic resistance plasmids found in WWTP bacterial communities and the possible dissemination of these plasmids into the environment. Proposals for monitoring the transmission of antibiotic resistance plasmids from WWTP effluents into the environment will also be reviewed.

PERSISTENCE AND EVOLUTION OF ANTIBIOTIC RESISTANCE PLASMIDS IN THE WASTEWATER ENVIRONMENT

WWTPs have been recognized as a reservoir for ARB and ARGs, including plasmids encoding resistance to antibiotics (Tennestedt et al., 2005; Schluter et al., 2008; Moura et al., 2010; Allen, 2010). Antibiotic resistance plasmids can harbour genes that confer resistance to most if not all clinically significant antibiotic classes such as macrolides, tetracyclines, cephalosporins, fluoroquinolines, aminoglycosides and β-lactams (Bennett, 2008; Martinez, 2009a; Szcepanowski et al., 2009). The accumulation of different antibiotic resistance genes on plasmids may be enhanced in the WWTP environment. The activated sludges and biofilms found in WWTPs are said to be rich in nutrients, have high load of organic and bacterial density, which is an ideal environ-ment for cell to cell contact and gene exchange (Dionisio et al., 2002; Sorensen et al., 2005; Haines et al., 2007, Zhang et al., 2009). The reservoir of antibiotic resistance mobile genetic elements (MGEs) in WWTPs include; conjugative transposable elements (transposons and insertion sequences) and integrative conjugative elements or integrons (Bennett et al., 2008; Slater et al., 2008; Allen et al., 2010). The combination of these elements with conjugative plasmids creates an environment where-by these plasmids can quickly acquire these MGEs via transposition or recombination and become mosaics of multiple resistance gene elements (Norman et al., 2009). Carattoli (2003) has reported this interaction as a factor for the rapid accumulation and spread of β-lactams resistance driven by related transmissible plasmids found in unrelated *Salmonella* strains.

Transposons often carry a variety of ARGs and are frequently incorporated into plasmids (Carattoli, 2003; Bennett, 2008). Non-replicative transposons and insertion sequences (IS) they encode a transposase enzyme that promotes the transposon carrying ARGs to transfer from one DNA replicon to another. These transposition events can result in acquisition of ARGs by a recipient plasmid. Different classes of transposons are widely distributed among bacterial populations conferring various antibiotic resistance phenotypes (Bennett, 2008). The Tn21 family of transposons represents one of the largest and first recognized groups involved in the accumulation and dissemination of ARGs, and they most often carry an

integron (Liebert et al., 1999; Bennett, 2008). Integrons have been described as naturally occur-ring MGEs responsible for the recruitment and accumulation of ARGs into mobile cassettes (Carattoli, 2003; Boucher et al., 2007). These MGEs are commonly found on plasmids and in transposons (Liebert et al., 1999; Boucher et al., 2007; Bennett, 2008) and they use a recombination strategy to capture new ARGs. A site-specific recombinase enzyme (integrase), encoded by the integron facilitates capturing of exogenous genes (Carattoli, 2003; Bennett et al., 2008). Five main classes of the integrons system have been described (Carattoli, 2003; Schluter et al., 2007a) and over 100 gene cassettes carrying most of the known ARGs to major antibiotic classes have been identified (Recchia and Hall, 1995; Mazel, 2006). The class 1 integron was the first to be discovered and is the best studied system and commonly associated with clinical pathogens (Mazel, 2006; Bennett, 2008). It is frequently found in association with transpo-sons, which mobilize the integrons between plasmids and bacterial chromosomes by transposition mechanisms (Recchia and Hall, 1995; Carattoli, 2003). A typical example is Tn402-like transposition system, which is reported to help the integrons in their recruitment by plasmids and other transposons (Mazel, 2006; Stokes et al., 2006; Boucher et al., 2007; Schluter et al., 2007b).

Retrospective studies highlighted the role of integrons and their gene cassettes in genome evolution within bacterial populations (Mazel, 2006; Boucher et al., 2007; Gillings et al., 2008) and perhaps their association with conjugative transfer plasmids and transposons could play a major role in rapid antibiotic resistance development within plasmids in the WWTPs. It has also been suggested that wastewater environments favor the integration of chromosomaly encoded genes into plasmids (Cattoir et al., 2008; Picao et al., 2008; Martinez, 2009a, b). The published DNA sequences of plasmids carrying integrons and transposons isolated from WWTPs (Heuer et al., 2004; Schluter et al., 2003, 2005, 2007b; Tennstedt et al., 2003, 2005; Sczepanowski et al., 2007) suggest that these plasmids conjugate and acquire mobile elements via chromosomal integration, recombination and transposition. Antibiotic resistance plasmids from WWTP were all found to carry class 1 integrons and associated with the Tn402-like transposons (reviewed by Schluter et al., 2007a). In addition, these integrons were reported to contain up to six different antibiotic resistance determinants and resistance to quaternary ammonium compounds (Q.A.Cs) (Schluter et al., 2007a). Acquisition of these plasmids by WWTP bacterial communities may play an important role in microbial adaptation to wastewater environments (Schluter et al., 2008; Moura et al., 2010). For example, the accessory genes provided by these plasmids may not only confer resistance to antibiotics but to other toxic chemical compounds that are concentrated in the wastewater environment. This is particularly true for multidrug resistance efflux pumps that can be found on plasmids isolated in WWTPs (Tauch et al., 2003; Szczepanowski et al., 2004; Schluter et al., 2007a). A high selection pressure for resistance to toxic compounds in wastewater environments could imply that WWTPs represent hot spots for plasmid transfer and ultimately maintenance of plasmids conferring resistance to antibiotics and other toxins (Sorensen et al., 2005; Schluter et al., 2007a; Moura et al., 2010). Furthermore, phylogenetic analysis has revealed diverse antibiotic resistance plasmid populations within the WWTP as demonstrated in identification of the IncP-1 plasmid subgroups, grouped as α, β, γ, δ and the recently described ε subgroup in a Danish WWTP study (Bahl et al., 2009). This data provides evidence of persistence and evolution of antibiotic resistance plasmids in wastewater environments.

POSSIBLE DISSEMINATION OF ANTIBIOTIC RESISTANCE PLASMIDS INTO THE ENVIRONMENT

There has been a recent recognition that broad-host range (BHR) plasmids encoding antibiotic resistance genes and other accessory genes may escape the WWTP into the environment (Bonemann et al., 2006; Szczepanowski et al., 2005, 2007; Tennstedt et al., 2005; Schluter et al., 2007a,b; Bahl et al., 2009). The detection of 140 plasmid borne ARGs of clinical significance in Szczepanowski and colleagues (2009) lastest study provides insights on the plasmids' antibiotic resistance gene mosaics gene-rated in the WWTP. Some of these plasmids were isola-ted in the final effluent of the WWTP, implying possible dissemination of these plasmids into the environment. Several BHR plasmids have been isolated in the effluent from WWTPs across the world, including Germany (Heuer et al., 2004; Bonemann et al., 2006; Tennstedt et al., 2005; Schluter, 2007b; Szczepanowski et al., 2005, 2007, 2009), Denmark (Bahl et al., 2009) and Portugal (Moura et al., 2010). Additionally, our study has found similar antibiotic resistance plasmids in WWTP influent and effluent in Saskatchewan Canada (Rahube et al., unpublished). Taken together all these studies are evidence that antibiotic resistance plasmids are not eliminated during wastewater treatment process and can therefore potentially be disseminated downstream of the WWTP via the effluent and this could be a global issue. Moura and colleagues (2010) proposed that wastewater bacterial communities bring together BHR plasmids; these BHR plasmids can be self-transmissible and carry multiple ARGs on transposons and integrons. Plasmids belonging to the IncP-1 incompatability group are the best-characterized plasmids from WWTPs and are often considered the most promiscuous plasmids. Bahl et al. (2009) have reported isolation of antibiotic resistance IncP-1 plasmids similar to those in WWTPs from other

environmentally dissimilar locations. These plasmids are widespread in various soils and aquatic environments (Ansari et al., 2008; Binh et al., 2008; Malik et al., 2008; Bahl et al., 2009). This may suggest the possible spread of antibiotic resistance among different environmental reservoirs.

Wastewater effluent carrying antibiotic resistance plasmids is usually released into watersheds via rivers and creeks ultimately reaching geographically distant areas such as lakes and coastal waters (Zhang et al., 2009). Furthermore, antibiotic resistance plasmids may be introduced into the environment via application of WWTP effluent and activated sludges in agricultural soils (Chee-Sanford et al., 2009; Zhang, 2010). The introduction of antibiotic resistance plasmids to such environments may have detrimental consequences if they are acquired by human pathogens. These plasmids may be considered undesirable elements if they enter a pristine ecosystem and persist for a long time. An increase in the fraction of resistant microbes above the normal value, caused by introduction of ARGs in pristine, isolated or extreme environments could be described as evidence of pollution (Martinez, 2009a).

Dissemination of antibiotic resistance plasmids from WWTP bacteria to environmental microorganisms depends on the survival of these plasmids in the environments they are released into, that is, plasmids have to remain functional in order to be transferred into recipient bacteria. These antibiotic resistance plasmids do not always carry only ARGs, other accessory genes such as heavy metal resis-tance genes are vital to the bacterial host where such a phenotype is required, e.g. in mercury polluted environments (Tauch et al., 2003; Szczepanowski et al., 2005; Schluter et al., 2007a). Therefore, antibiotic resistance plasmids may be transferred and stably maintained even in environments with no antibiotic selection pressures (Sorensen et al., 2005; Schluter et al., 2007b; Allen et al., 2010). Although plasmid curing has been demonstrated *in vitro* (Sorensen et al., 2005), this may not be the case *in vivo* due to other environmental challenges the host bacteria may encounter and hence maintain the antibiotic resistance plasmid for other functions. Studies have shown the presence of some chromosomaly encoded ARGs from environmental bacteria in plasmids that can be transferred to human pathogens (D' Costa et al., 2006; Wright, 2007; Martinez, 2009a, b). There are also suggestions that MGEs carried by plasmids and host chromosomes may be re-shuffled in bacterial pathogens resulting in combined resistance mechanisms that are resistant to antibiotic therapy (Bahl et al., 2009). The development of multi-drug resistance in methicillin resistant *Staphylococcus aureus* (MRSA) has been associated with combined mechanisms of resistance (Walsh, 2000). Contact between the environmental microorganisms in WWTPs with human-associated microbiota may play a role in the emergence of multiple-resistance human pathogens (Baquero et al., 2008; Bahl et al., 2009; Martinez, 2009a,b).

MONITORING ANTIBIOTIC RESISTANCE PLASMIDS IN THE WWTP AND THE ENVIRONMENT

The study of the movement of resistance plasmids from WWTPs into the environment merits investigation, given the detrimental consequences of multiple antibiotic resistance plasmids being aquired by opportunistic human pathogens. Currently, the significance of antibiotic resistance plasmids entering the environment is not clear due to a paucity of monitoring data. There is a need for greater assessment of antibiotic resistance plasmid diversity and persistence in the environment following their introduction by anthropogenic activities such as release of wastewater effluent, or application of activated sludges as fertilizer. The current molecular advancements in detection of nucleic acids in the environment will greatly facilitate future studies. Metagenomics (or environmental genomics) (Handelsman, 2004) has emerged as powerful tool for environmental microbiology research. Total community DNA (or metagenome) is extracted directly from various environmental samples such as soil and water thereby bypassing the need for culture techniques (Handelsman, 2004; Reisenfeld et al., 2004). Plasmids can readily be isolated directly from environmental samples, plasmid metagenomes have been recently characterized from wastewater environments (Schluter et al., 2008; Sczepanowski et al., 2008, 2009). High-throughput DNA sequencing technologies, such as 454 sequencing (Jones, 2010) make it possible to obtain the whole genome sequence data of environmentally isolated plasmids in a short time, and at a reasonable cost. Complete sequencing of plasmids provide useful information of the plasmid-backbone sequences encoding for various genes e.g. plasmid core genes such as those involved in plasmid replication, conjugative transfer, and unique genes and accessory genes for resistance to antibiotics (Binh et al., 2009). This sequence data is made easily accessible in GenBank database (http://www.ncbi.nlm.nih.gov/) allowing classification and comparisons with other isolated plasmids from various sources.

Research approaches for assessing and monitoring antibiotic resistance plasmids in the environment can be classified into two broad categories, the indirect and direct approach (Figure 1). The indirect approach involves amplification of the target sequences by polymerase chain reaction (PCR) or cloning. PCR approaches to detect ARGs in various water environments and soil ecosystems are widely utilized (Zhang et al., 2009; Reisenfeld et al., 2004). Quantitative PCR (qPCR) is a recently developed technique that has the advantage of

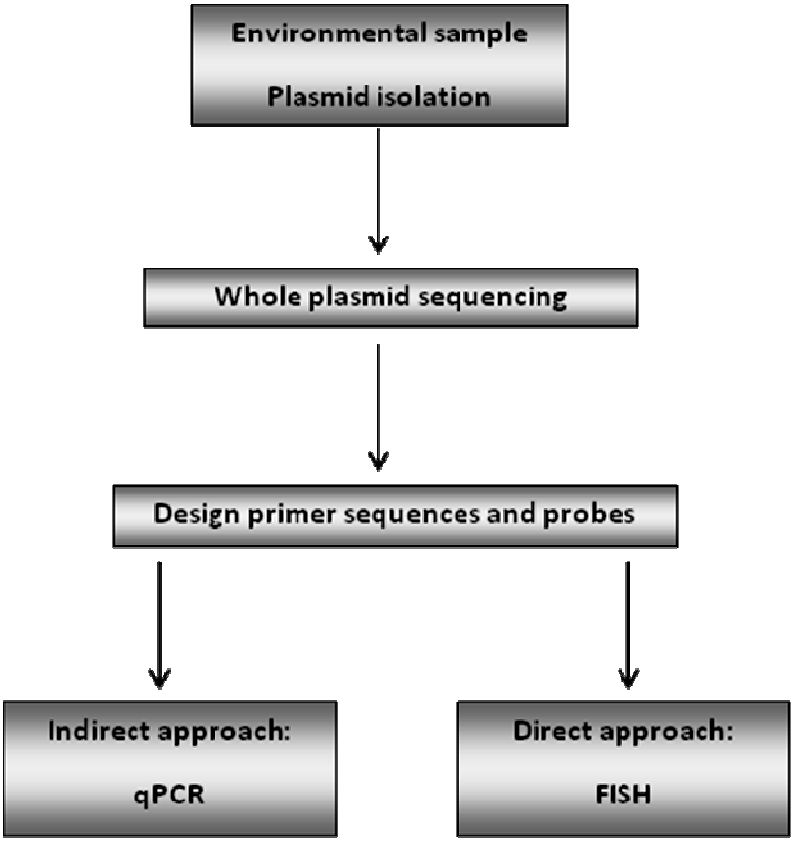

Figure 1. Diagram showing general approach for tracking antibiotic resistance plasmids in the environment. The illustration highlights the steps from isolating the plasmid, obtaining complete sequence data and designing primers and probes for detection plasmid-specific sequences in the environment by direct (FISH; Fluorescence *in situ* hybridization) and indirect (qPCR; Quantitative PCR) approaches.

being able to measure the level of specific resistance genes in an environmental sample. It has been used in detecting a wide range of clinically significant antibiotic resistance genes such as *tet, erm* (Morsczerk et al., 2004; Reinert et al., 2004), *van*A, *mec*A and *amp*C in a wastewater environment (Volkmann et al., 2004). Taqman probes and primers for qPCR can easily be obtained from the complete plasmid sequence of the antibiotic resistance plasmids. The nature of plasmids allows them to be easily manipulated *in vitro;* cloning results in amplification of antibiotic resistance plasmids allowing simultaneous detection and expression of functional resistance genes in heterogonous hosts such as *Escherichia coli*. Binh et al. (2009) used rifampicin and kanamycin-resistant *E. coli* tagged with green fluorescent protein (*gfp*) for capturing BHR antibiotic resistance plasmids in piggery manure. These plasmids were then characterized by PCR and dot-blot hybridization of PCR amplified plasmid-specific sequences with relevant probes. Conjugative potential of isolated plasmids can also be studied in a variety of bacterial hosts (Sorensen et al., 2005). Detection and expression of antibiotic resistance plasmids in broad range of bacterial hosts suggest potential dissemination of ARGs across bacterial species particularly pathogenic species.

A direct approach involves direct detection of the DNA without amplification of the target sequence, this could be a specific bacterial community, antibiotic resistance gene or a particular antibiotic resistance plasmid in a given environment. Fluorescence *in situ* hybridization (FISH) is a common technique originally developed in pathology for clinical diagnosis (Levsky and Singer, 2003). This approach applies the principle of hybridization involving the penetration of a fluorescent labeled sequence-specific nucleic acid probe into fixed cells, followed by specific binding to the complementary sequences of the target nucleic acid. It allows rapid simultaneous detection and visualization of target genes while they are structurally intact with the associated organism or particle (Bottarri et al., 2006; Ormerci and Linden, 2008). Examples of plasmid-specific

sequences recently targeted for BHR plasmids may include; *rep*A for IncN, *trf*A2 for IncP, *oriV* for IncQ and Inc W (Bahl et al., 2009; Binh et al., 2009). FISH may become a more accurate and sensitive approach in predicting the population of specific antibiotic resistance plasmids in contaminated environments. This method could become useful in environmental microbiology despite the technical challenges associated with processing environmental samples for FISH. According to Ormerci and Linden (2008), wastewater associated samples present challenges such as high background fluorescence caused by organic and inorganic particles. However, FISH has been used in the detection of ARGs in bacterial pathogens (Russmann et al., 2001; Werner et al., 2007; Laflamme et al., 2009). With reference to wastewater environments, FISH has been applied to detect and analyze bacterial communities in activated sludge and biofilm systems (Wagner and Loy, 2002; Atkan and Salih, 2006). The future usage of FISH in environmental microbiology is yet to be appreciated. However, there are improvements in overcoming technical limitations due to complex environmental matrices (Ormerci and Linden, 2008; Zhang et al., 2009), FISH could be a preferred method, particularly in monitoring the dissemination of antibiotic resistance plasmids from WWTPs. New innovative approaches inspired by current technology such as high-throughput DNA microarray (another DNA hybridization technique) are being developed specifically for the detection of ARB and plasmid mediated ARGs in clinical and environmental isolates (Frye et al., 2006; Chee-Sanford, 2009; Walsh et al., 2010). Advances in molecular-based genomics and *in situ* methods could lead to development of standard tracking tools for assessing the diversity of antibiotic resistance plasmids from WWTPs in complex environmental samples.

CONCLUSION

The dissemination of antibiotic resistance plasmids once they leave the WWTP into the environment is poorly understood. Recent reviews have detailed that ARGs are ubiquitous in the environment (Martinez, 2009a,b; Zhang, 2009; Davies and Davies, 2010), prompting the following questions: do WWTPs increase the diversity and abundance of these ARGs through dissemination of antibiotic resistance plasmids? Equally important, how stable and transferable are these plasmids once they enter the environment? Using advanced molecular and environmental microbiology methods and tools will help to address these questions concerning the fate and distribution of antibiotic resistance plasmids originating from the WWTP and entering the environment. Direct *in situ* studies of the development and dissemination of antibiotic resistance are under-appreciated. More studies on HGT of plasmids during residence in WWTPs and after leaving the WWTP could provide new insights into the evolution of these plasmids and spread to new environments. Sorensen et al. (2005) have provided a critical review for studying plasmid HGT *in situ*, and this could be applied to research on antibiotic resistance plasmids entering the environment. Perhaps recognizing antibiotic resistance plasmids as pollutants will lead to more research and further understanding of the role of WWTPs in the evolution of antibiotic resistance plasmids and the dissemination of plasmid-borne ARGs in the environment and ultimately into human pathogens.

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