Integrons, transposons, IS elements promote diversification of multidrug resistance plasmids and adaptation of their hosts to antibiotic pollutants from pharmaceutical companies

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Abstract

Plasmids are important vehicles for the dissemination of antibiotic resistance genes (ARGs) among bacteria by conjugation. Here, we determined the complete nucleotide sequences of nine different plasmids previously obtained by exogenous plasmid isolation from river and creek sediments and wastewater from a pharmaceutical company. We identified six IncP/P-1ε plasmids and single members of IncL, IncN, and IncFII-like plasmids. Genetic structures of the accessory regions of the IncP/P-1ε plasmids obtained implied that multiple insertions and deletions had occurred, mediated by different transposons and class 1 integrons with various ARGs. Here we show that class 1 integrons, Tn*402*-like transposons, Tn*3*-like transposons, and/or IS*26* mediated the acquisition of ARGs by all plasmids. Our plasmid sequencing data provide new insights into how these mobile genetic elements could mediate the acquisition and spread of ARGs in environmental bacteria.

Originality-Significance Statement

In the present study, the complete nucleotide sequences of plasmids exogenously isolated from wastewater of pharmaceutical companies producing or formulating antibiotics were used to classify each plasmid, its ARGs and associated mobile genetic elements. Our data demonstrate the importance of integrons, transposons (Tn*402*-, Tn*3*-like) and IS elements within the flanking regions of ARGs for the genetic variation observed in the accessory regions of these closely related IncP/P-1ε plasmids. In addition, the captured IncN plasmid showed highly conserved genetic structures including their ARGs with IncN plasmids of different geographic and environmental origin. Our data confirm that conjugative plasmids with highly conserved genetic structure contribute to the spread of ARGs between environmental bacteria and clinical strains.

INTRODUCTION

Transferable antibiotic resistance (AR) evolved from the overuse and misuse of antibiotics is a serious threat to public health and of global concern. The dissemination and acquisition of antibiotic resistance genes (ARGs) by pathogenic bacteria of clinical importance has resulted in prolonged illness and death of infected patients in the last decades. However, ARGs found in pathogenic bacteria compromise only a small proportion of the total ARGs identified in genomes of antibiotic-resistant bacteria (ARB) (Davies and Davies, 2010; McArthur *et al.*, 2013), implying that the key reservoir for ARGs is in environmental non-

pathogenic bacteria. It has been suggested that terrestrial and aquatic environments, mainly if polluted with antibiotics either by industry or agriculture, harbor a high abundance and diversity of ARGs and can be regarded as potential sources, reservoirs and/or transmission routes for ARGs to pathogens (Bengtsson-Palme and Larsson, 2015). Therefore, such environments require more utmost attention to better understand their potential contribution to the global spread of clinically relevant AR.

Mobile genetic elements (MGEs) such as plasmids which can be rapidly transferred among bacterial populations by horizontal gene transfer, play an important role in the dissemination of ARGs and allow host bacteria to cope with antibiotic selective pressure (Heuer and Smalla, 2012).
Plasmids with a broad host range (BHR), such as those belonging to the incompatibility (Inc) groups including IncN, IncP/P-1, IncC/P-3, IncW, and IncQ are assumed to especially contribute to the spread of ARGs because of their efficient transfer and stable replication in phylogenetically distant bacterial lineages (Shintani *et al.*, 2014; Klümper *et al.*, 2015). Consequently plasmids have been considered as important for the transmission of multidrug resistance among bacteria in both clinical and environmental contexts (Chen *et al.*, 2014; Flach *et al.*, 2015; Blau *et al.*, 2018; González-Plaza *et al.*, 2019; Schweizer *et al.*, 2019). Plasmids typically consist of conserved backbone genes (replication, stability, and transfer genes) and accessory genes that vary among plasmids (antibiotic and heavy metal resistance genes, pathogenicity genes, mobile resistance components, etc.). These accessory genes are often proximal to MGEs inserted into the plasmid backbone, such as insertion sequence (IS) elements, integrons and transposons (Heuer and Smalla, 2012). Plasmids can be either conjugative (self-transmissible) as they code for their own set of conjugative transfer and mobilization genes (*tra*, *trb*, *vir*, *pil*, *fin*) or mobilizable as they lack a complete set of conjugative transfer genes and hence can only be mobilized by using another helper plasmid (Smillie *et al.*, 2010). A deep understanding of mobile plasmids carrying ARGs is important to fully understand their role in the spread and evolution of environmental determinants of AR.

Waste from companies manufacturing antibiotics is an important and often neglected source of antibiotics and ARB, which contaminate the environment more than well-known routes, i.e. discharge of municipal and hospital wastewater or manure (Šimatović and Udiković-Kolić, 2019). However, there are still no international standards to limit antibiotics in such waste. Release of excessively high concentrations of antibiotics as well as ARB carrying ARGs and MGEs into the surrounding environment via waste from companies manufacturing antibiotics has been reported previously (Kristiansson *et al.*, 2011; Rutgersson *et al.*, 2014; Bengtsson-Palme and Larsson, 2015; Bielen *et al.*, 2017; González-Plaza *et al.*, 2018, 2019; Milaković *et al.*, 20192020). In addition, increased relative abundance of plasmids carrying ARGs in such highly-polluted environments has also been reported (González-Plaza *et al.*, 2019). However, plasmids that carry ARGs from such antibiotic-polluted environments have been rarely studied in depth (Flach *et al.*, 2015), and complete sequences of plasmids from environments affected by pollutants from pharmaceutical companies are still underrepresented in most public databases.

Using exogenous plasmid isolation by the biparental approach, we previously obtained different plasmids from river and creek sediments into which wastewater from pharmaceutical companies were discharged, using erythromycin or tetracycline as selective markers (González-Plaza *et al.*, 2019). In addition, we characterized the AR profiles of *E. coli* transconjugants and analyzed the presence of ARGs (*tetA*, *qacE/qacEΔ1*, *sul1*, *sul2*) and plasmid replicon types by PCR. A large proportion of these plasmids conferred multidrug resistance and belonged to the IncP-1 group, whereas a smaller proportion could not be assigned to any of the plasmid groups tested. This study aimed to determine the complete nucleotide sequences of nine of these plasmids in order to obtain an accurate picture of their structure and, more importantly, of the ARGs and MGEs they carry and how they are linked. Details on the sites of their isolation and previously determined characteristics are given in Table 1.

EXPERIMENTAL PROCEDURES

Antibiotic abbreviations

SMX, sulfamethoxazole; SMZ, sulfamethazine; AM, ampicillin, AMX, amoxicillin; AZI, azithromycin, ERY, erythromycin; GEN, gentamicin; TET, tetracycline, D, doxycycline; TMP, trimethoprim; CHL, chloramphenicol.

Sample collection, exogenous plasmid capture and plasmid characterization

The sampling and procedures for capturing plasmids exogenously have been described previously (González-Plaza *et al.*, 2019). Briefly, the kanamycin- and rifampicin-resistant *Escherichia coli* CV601 *gfp*+ strain was used as the recipient strain for biparental exogenous plasmid capturing. The plasmid donors were bacteria from the sediments of the Sava River receiving wastewater from the AZI-producing pharmaceutical company (company 1), and from the wastewater of a pharmaceutical company formulating drugs (company 2) and from the sediments of the receiving creek. ERY (50 µg/mL) or TET (15 µg/mL) was used as a selective marker to capture resistance plasmids from Sava River sediments, and TET (15 µg/mL) was used as a selective marker for resistance plasmid isolation from creek sediments and wastewater from pharmaceutical company 2. After plasmid capture, antibiotic susceptibility of transconjugants was determined for eight antibiotic classes (β-lactams, sulfonamides, macrolides, aminoglycosides, fluoro/quinolones, tetracyclines, trimethoprim and chloramphenicol), and replicon typing of the plasmids and the presence of the *intI1*, *tetA*, *qacE/qacEΔ1*, *sul1*, *sul2*, and *merRTΔP* genes on them were analyzed by PCR (González-Plaza *et al.*, 2019). Based on the antibiotic susceptibility tests and plasmid replicon typing, nine plasmids were selected for further

investigation by sequencing: four plasmids captured from Sava River sediment bacteria, four plasmids from creek sediment bacteria and one plasmid from bacterial communities from wastewater of pharmaceutical company 2 (Table 1).

Plasmid DNA sequencing

Plasmid DNA was extracted, prepared, and sequenced on the PacBio Sequel 1 instrument (Pacific Biosciences, USA) using Single Molecule Real Time (SMRT) sequencing technology at Helmholtz München (Research Unit for Comparative Microbiome Analysis). Plasmid DNA was extracted from transconjugants using the Plasmid Midi Kit (Qiagen, Germany), following the manufacturer's instructions. The plasmid DNA quantity was assessed by fluorimetry (Qubit 3.0, Thermo Fisher Scientific, USA), while qualitative assessment was performed with the FragmentAnalyzer device (Advanced Analytical, USA). High molecular weight plasmid DNA was sheared with g-TUBE (Covaris, UK) aiming at DNA fragments of about 10 kb. 9-plex library was constructed with 200 ng of fragmented DNA as a starting material by using SMRTbell Barcoded Adapter Complete Prep Kit, SMRTbell DNA Damage Repair Kit, DNA/Polymerase Binding Kit, AMPure PB Kit, DNA Sequencing Kit and SMRT Cells for standard sequencing. The library was sequenced on a PacBio Sequel 1 device using the reagents Sequel sequencing plate 2.0., Sequel binding kit 2.0., and Sequel SMRT Cell V2. The loading concentration on the SMRT cell 1M v3 was 6pM. Data were collected with Stage Start with 600 minute movies. All materials used for preparing and sequencing the SMRTbell library were provided by PacBio.

Plasmid assembly and annotation

Two Sequel SMRT Cell V2 yielded 6.18 Gb and 6.52 Gb raw data with 4,657 and 4,085 Mb mean read length. Demultiplexing and assembly was done using SMRT Link v.4.0.0 (PacBio). All plasmid assemblies had +600 times coverage and assemblies from each individual SMRT Cell yielded 100 % identical plasmid sequences. Genes were predicted using Prodigal v2.6.1 (Hyatt *et al.*, 2010) and/or DFAST 1.2.18 (Tanizawa *et al.*, 2018). Functional annotation against PFAM v.28 was done using HMMER v3.2.1 (Finn *et al.*, 2011). The conserved genes in the IncP-1 plasmids were reannotated and named based on those in R751 (Thorsted *et al.*, 1998), except for *kfrB* (upf54.8 in R751) and *kfrC* (upf54.4 in R751). Accessory genes including putative metabolic genes and/or transporter genes were subjected to BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to find similar sequences. Genotypic screening of antibiotic resistance genes in these plasmids was performed by using the Comprehensive Antibiotic Resistance Database (CARD 3.2.5) and Resistance Gene Identifier (RGI 6.0.0) (Alcock *et al.*, 2020, 2023). Plasmid sequences were deposited in DDBJ/GenBank under accession numbers LC746219-LC746227.

Bioinformatical analysis

Comparative analyses of the plasmids were performed and visualized by Easyfig ver. 2.2.5 (Sullivan *et al.*, 2011). The nucleotide sequences of genes encoding replication initiation protein and relaxase of selected plasmids were analyzed by MEGA7 software (Kumar *et al.*, 2016). Multiple sequence alignments of nucleotide sequences and amino acid sequences were performed using ClustalW. All positions containing gaps and missing data were eliminated. The phylogenetic trees were inferred from the aligned sequences using the maximum likelihood method with the Tamura-Nei nucleotide substitution model and the JTT amino acid substitution model. Visualization of plasmid maps was performed using SnapGene (http://www.snapgene.com/).

RESULTS

Nine conjugative AR plasmids that were previously isolated from river and creek sediments and wastewater of pharmaceutical company 2 using an exogenous plasmid capture approach (González-Plaza *et al.*, 2019) were sequenced, assembled, closed and annotated. These plasmids (Table 1) varied in size from 51 to 146 kb. -As the previous study by Gonzalez-Plaza suggested, six of nine plasmids including pLBC54, pLBC56, pLBC62, pLBC70, pLBC75, and pLBC82 were classified as IncP/P-1 plasmids, as they harbored *trfA* (***t****rans*-acting **r**eplication **f**unction) which is essential to activate the origin of vegetative replication (*oriV*) on the IncP/P-1 plasmid (Pansegrau *et al.*, 1994). More detailed phylogenetic analyses based on the *trfA* and *traI* [encoding a relaxase involved in the cleavage of plasmid DNA in site-specific and single-stranded manner to initiate transfer of the single-strand plasmid DNA (Pansegrau *et al.*, 1994)] along with their gene products, TrfA and TraI showed that all six plasmids are members of the IncP/P-1ε subgroup (Figure S1). Notably, pLBC54 belonged to IncP/P-1ε-II while the other five were affiliated to IncP/P-1ε-I (Figure S1). The sequencing data of the other three plasmids were subjected to PlasmidFinder 2.1 (https://cge.food.dtu.dk/services/PlasmidFinder/) (Carattoli *et al.*, 2014). Although pLBC2 was predicted as an IncN plasmid in our previous study (#6) (González-Plaza *et al.*, 2019), sequence analysis revealed that the plasmid belongs to IncL. Indeed, its putative *repA* gene encoding replication initiation protein showed high identity (94.7% 1000/1056 bp) with that of R471, an archetype plasmid in the IncL group (Carattoli *et al.*, 2015) (Figure S2A). Plasmids pLBC4 (#27) and pLBC3_2 (#7), which had not previously been assigned to Inc groups, were identified as IncN and IncF groups, respectively, based on the complete plasmid sequence. The putative *rep* gene of pLBC4 showed 100% identity with that of R46, an archetype of IncN plasmid (Belogurov *et al.*, 1992) (Figure S2B). The result of PlasmidFinder 2.1 showed that pLBC3_2 (#7) was classified as IncFII group as it showed high identity with a part of the DNA region

(*tra*/*trb* genes) of pKPN3 (CP000648.1), an IncFII plasmid (Figure S2C). Although the putative *repA* gene of pLBC3_2 did not show high identity (24% identity at the nucleotide sequence level) with those of pKPN3 or R100, both archetype plasmids of the IncFII group (Datta and Kontomichalou, 1965; Cox and Schildbach, 2017), the genetic structure of pLBC3_2 showed similarities (Figure S2C). Therefore, pLBC3_2 was grouped as a/span>n IncFII-like plasmid.

IncP/P-1ε plasmids

Comparison of the backbones of IncP/P-1ε-I plasmids showed that they were highly conserved (red, yellow and green arrows in Figure 1): the nucleotide sequences of pLBC56 and pLBC82 and of pLBC62 and pLBC70 were completely identical (40563 and 40548 bp, respectively). The differences among them were that four nucleotides were inserted in *traG* (frame-shifted) in pLBC75 and 15 nucleotides were deleted in *trbL* genes in pLBC62 and pLBC70. Different accessory genes including ARGs were inserted into the two regions between *trfA* and *oriV*, and between *traC* and *parA* (Figure 2). Tn*3*-like transposons, Tn*402*-like transposons, and/or Tn*402*-class 1 integron-like elements associated with ARGs were found in the plasmids' genomes (Figures 1-3). Interestingly, IS*Pa17* contained genes for the MazEF-like toxin-antitoxin system (Haines *et al.*, 2005), whose inverted repeat sequences were similar to those of Tn*402*, which were found in all of IncP/P-1ε-I plasmids (Figures 1, 2). The nucleotide sequences of the accessory regions between *traC* and *parA* including the integrons in pLBC62 and pLBC70 were 100% identical (Figures 1, 3B).

Plasmid pLBC54 contained mercury resistance genes (*mer*) and two macrolide resistance genes *msrE* (encoding a ribosomal protection protein) and *mphE* (macrolide phosphotransferase) in a Tn*3*-like transposon (Figures 1-3). The Tn*3*-like transposon carried a Tn*402*-class 1 integron-like element with the well-conserved *qacEΔ-sul1-orf5* (Figures 1-3). In contrast, IncP/P-1ε-I plasmid pLBC82 from the polluted river sediment, possessed a Tn*402*-class 1 integron-like element carrying *tetRA*, *aadA-qacEΔ-sul1-orf5*, and *mphE-msrE* genes flanked by two IS*26* elements (Figures 1-3).

Another IncP/P-1ε-I plasmid pLBC56 from the upstream creek sediment carried a Tn*402*-class 1 integron carrying TET resistance genes, *tetRA*, and *dfrB1* (TMP resistance gene)-*qacEΔ-sul1-orf5* (Figures 1-3). Plasmids isolated from the wastewater of pharmaceutical company 2 (pLBC75) and creek sediment at the wastewater discharge site (pLBC62 and pLBC70) harbored more complex and additional ARG clusters with different MGEs. Interestingly, the bla_{OXA-2} gene encoding resistance to third generation cephalosporins was detected as a gene cassette in a class 1 integron on pLBC70 and pLBC62. IS*26*-related transposons with macrolide resistance genes including *mphE, msrE*, *mrxF*, *mphF*, *mphR* and a CHL resistance gene, *catB2*, were found in pLBC75 in the Tn*402*-class 1 integron-like element similar to those of pLBC62, pLBC70 and pLBC56 (Figure 2). A Tn*3*-like transposon with *tetAR* and Tn*402*-like element

with genes for ABC transporter were found in pLBC70 and pLBC62 (Figures 2 and 3B). pLBC62 had another Tn*3*-like transposon with aminoglycoside resistance genes, *aph(6)-Id* and *aph(3")-Ib* (Figures 1-3) .

IncL plasmid

The IncL plasmid (pLBC2) isolated from polluted river sediment at the discharge site, carried two Tn*3*-like transposons and a class 1 integron (Figure 3A). One of the Tn*3*-like transposons with 39-bp inverted repeats (IRs) and 5-bp direct repeats (DRs) contained no accessory genes, while the other one with 38-bp IRs but no DRs carried bla_{TEM-1} (Figure 3A). The class 1 integron carried *qacEΔ-sul1-orf5* genes with an insertion sequence (IS), IS*6100* with 14-bp IRs (Figure 3A, Table 1). The *msrE-mphE* genes were located between the Tn*3*-like transposon and the class 1 integron (Figures 3A, S2A).

IncN plasmid

The IncN plasmid (pLBC4) captured from antibiotic-polluted creek sediment downstream from the wastewater discharge carried a Tn*3*-family transposon with its 38-bp IRs but no DRs containing bla_{TEM-1}(Figure 3B). Plasmid pLBC4 also carried *sul2*, bla_{TEM-1}, *aph(6)-Id, aph(3")-Ib*, *tet*A, *dfrA14* (Figure S2B), a part of the DNA region was very similar to that of the inner region of Tn3-like element found in the IncL plasmid pLBC62 (Figure 3B). These ARGs with MGEs were inserted into variable region of IncN backbone (Figure S2B).

IncFII-like plasmid

The IncFII-like plasmid pLBC3_2, captured from polluted river sediment at the discharge site, carried six copies of IS*26* with 14-bp IRs and many genes for putative transposase (Figure 3A, S2C). Because IS*26* and IS*6100* belong to the same IS*6* family (Varani *et al.*, 2021), they showed identity with each other (Figure 3A). This plasmid carried a bla_{TEM-1} gene showing 99.4% identity with that of pLBC2 (Figures 3A). In addition, *tetDR*, *catII*, *dfrA18*, *sul2*, *aph(6)-Id*, *aph(3")-Ib, catI*, *aph(3')-Ia*, and *erm41* genes were also found (Figures 3A, S2C).

Class 1 integrons on IncP/P-1ε and IncL plasmids

In total, nine class 1 integrons were found on seven plasmids, all six IncP/P-1ε plasmids and the IncL plasmid pLBC2 (Figure 3). Five of these integrons contained ARG cassettes, including *aadA*, *dfrB1*, bla_{OXA-2}, and *catB2* on the five IncP/P-1ε plasmids (pLBC82, pLBC56, pLBC62, pLBC70, and pLBC75), whereas the other four on the IncP/P-1ε plasmids (pLBC54, pLBC82, and pLBC75) and the IncL plasmid pLBC2 contained only *sul1*, *qacEΔ,* and/or *orf5* (Figure 3). Notably, two IncP/P-1ε-I plasmids, pLBC75 and pLBC82, carried two copies of the integrons, one of each did not contain any ARGs (Figure 3). The four main variants in the cassette promoter region (Pc) of class 1 integron: PcW; PcH1; PcH2; and PcS are known to show different transcriptional strengths, PcW (ancestral and the weakest form), PcS (the strongest form), PcH1(stronger than PcW but weaker than PcH2), and PcH2 (between PcS and PcH1) (Jové *et al.*, 2010). PcW were found in the class 1 integrons without ARGs of pLBC2 and IncP/P-1ε-II plasmid pLBC54, while PcH1 were found in those of all other IncP/P-1ε-I plasmids, some of which contained ARGs (Figure 4).

DISCUSSION

In this study, nine conjugative plasmids captured from pharmaceutical wastewater and sediments of the receiving river/creek downstream and upstream from the wastewater discharge were characterized by in-depth sequence analysis. Previous studies have shown that these wastewater-impacted environments have increased levels of antibiotics released by pharmaceutical companies. For example, the macrolides AZI and ERY were found mainly in the wastewater from pharmaceutical company 1 and in the sediments of the receiving Sava River (Milaković *et al.*, 2019). In contrast, a mixture of antibiotics was detected in the wastewater from pharmaceutical company 2 and in the sediments of the receiving creek, with sulfonamides, TMP and/or TETs being the most common (González-Plaza *et al.*, 2019; Milaković *et al.*, 2019, 2020). Moreover, total macrolide levels measured in Sava River sediments (up to about 25 mg/kg) (Milaković et al., 2019) were comparable to antibiotic levels generally found in sediments receiving pharmaceutical wastewater in India (tens of mg/kg) (Kristiansson *et al.*, 2011; Gothwal and Shashidhar, 2017; Milaković *et al.*, 2019). In contrast, antibiotic levels found in creek sediments (up to 5 mg/kg) were much more modest (Milaković et al., 2020), but higher than those found in sediments exposed to wastewater from municipal wastewater treatment plants (up to 0.6 mg/kg total) (Marti *et al.*, 2014; Guan *et al.*, 2018; Li *et al.*, 2019). Therefore, ERY was mainly chosen as a selective marker for plasmid capture from river sediments and TET was chosen as a selective marker for plasmid capture from wastewater and creek sediments because the wastewater from company 2 was mainly polluted with TETs and sulfonamides.

ARGs conferring the antibiotic resistance phenotypes identified

The previously analyzed AR profiles of the plasmids studied in this work showed that all but one plasmid conferred a multidrug-resistance phenotype (Table 1). Complete sequencing of these plasmids revealed/span>the ARGs which conferred the previously determined resistance phenotypes, and their exact location on the plasmids.

Sulfonamide resistance was a common resistance phenotype conferred by all our plasmids. The most common sulfonamide resistance gene was *sul1*, which was found on all plasmids with class 1 integrons (IncP/P-1ε or IncL) (Figure 1) or by the *sul2* gene (IncFII and IncN). IncL and IncFII plasmid pLBC2 and pLBC3_2 from macrolide-polluted river sediment carried several ARGs conferring resistance to sulfonamides (*sul1* on IncL or *sul2* on IncFII), penicillins (bla_{TEM-1}), macrolides (*msrE-mphE) on IncL or erm41 on IncFII) (Table 1) or TETs (tetD on IncL) and TMP (dfrA18 on IncL). In contrast, the transconjugant with the IncFII plasmid pLBC3_2 was phenotypically susceptible to CHL, although the putative CHL resistance genes catI and catII were present, suggesting that these genes may not be functional or not expressed. However, there were four exceptions in which phenotypic resistance was previously detected but putative ARGs were not detected on the sequenced plasmids (resistance to TET and TMP/pLBC2, TMP/pLBC82 and CHL/pLBC4). This could be due to the acquisition of another ARG-carrying MGE integrated into the E. coli chromosome (Martinez et al, 2017). The IncN plasmid pLBC2* from polluted creek sediment also carried a diverse set of ARGs which explained the resistance to sulfonamides (*sul*2), penicillins (bla_{TEM-1}), aminoglycosides (*aph*(6)-Id, *aph*(3'')-Ib),TETs (*tet*A), and TMP (*dfr*A14) observed for the transconjugants. Similar phenotype and genotype profiles of IncN plasmids from the current study and those obtained from various environmental and clinical samples in previous studies (Figure S3) suggest that these plasmids have the potential to spread multidrug resistance among a variety of pathogens, posing risks to human and animal health.

Risk for co-selection of ARGs in polluted environments

Previous studies of river and creek sediments downstream from pharmaceutical wastewater discharges compared to upstream showed a significant increase in the relative abundance of several ARGs (e.g., *msrE*, *mphE*, *sul1*, *sul2*, *tetA*) and MGEs (classintegrons, IncP-1 plasmids) by quantitative PCR (qPCR) as well as in the transfer frequency of AR plasmids by biparental exogenous plasmid capturing (Milaković et al, 2019, 2020; Gonzalez-Plaza et al, 2019). This suggests that the adaptation of bacteria to the heavy antibiotic pollution in these environments was driven by the acquisition of plasmids carrying different ARGs. The results of the completely sequenced plasmids from these environments presented in this study show the co-occurrence of ARGs

conferring resistance to a range of antibiotic classes present on the same plasmid. Therefore, selection pressure from only one antibiotic in these polluted sediments may have contributed to the spread of resistance to several other antibiotic classes via co-selection. This is consistent with previous observations of elevated levels of macrolide ARGs (e.g., *msrE, mphE*) along with elevated levels of ARGs for sulfonamides (*sul1, sul2*), TETs (*tetA*) and biocides (*qacE*/*qacE*Δ1) in sediments of the Sava River polluted with macrolide antibiotics only (Milaković et al., 2019; Gonzalez-Plaza et al., 2019).

Of particular interest was the detection of clinically important bla_{OXA-2} gene on two IncP/P-1ε plasmids characterized in this study. Interestingly, in a previous study by Milaković et al. (2020), this gene was detected in the pharmaceutical company's wastewater and in the sediment of the receiving creek, well above background levels during the warm summer months by qPCR. This gene confers resistance to third generation cephalosporins by expressing the extended-spectrum β-lactamase (ESBL) enzyme OXA-2. However, OXA-2 has also been reported to possess carbapenem-hydrolyzing activity in certain bacterial hosts (Antunes *et al.*, 2014). Thus, this enzyme may complicate the treatment of bacterial infections with antibiotics because carbapenems are one of the last-resort antibiotics used to treat infections caused by ESBL-producing bacteria. The bla_{OXA-2} gene has been previously reported on conjugative plasmids of the IncF (Maurya *et al.*, 2015), IncA/C2 (Iovleva *et al.*, 2019) and IncP-1β groups (Schlüter *et al.*, 2003), but to our knowledge, this is the first report of bla_{OXA-2} on IncP/P-1ε plasmids. Thus, our results highlight the important role that IncP-1ε plasmids may play in the dissemination of clinically relevant ARGs.

Contribution of different MGEs for acquiring and spreading ARGs

The ε subgroup of IncP/P-1 plasmids was proposed in 2009 (Bahl *et al.*, 2009), and many IncP/P-1ε plasmids conferring antibiotic resistance or degradation pathway for 2,4-dichlorophenoxyacetic acid were isolated from manure, digestates of biogas plants, manure-treated soils, non-polluted rhizosphere, wastewater, wastewater treatment plants, and estuarine water (Sen *et al.*, 2011; Heuer *et al.*, 2012; Oliveira *et al.*, 2012, 2013; Wolters *et al.*, 2015; Hutinel *et al.*, 2021; Law *et al.*, 2021; Hayakawa *et al.*, 2022) (Table 2). Moreover, the study by González-Plaza *et al.* (2019) pointed to an important role of IncP-1 plasmids in the adaptation and survival of bacterial communities to antibiotics released into the environment by industrial wastewater as their abundance (based on *kor*B qPCR data) was increased in sediments downstream compared to upstream from wastewater discharges. Many class 1 integrons which seem to be the major vehicle for ARG dissemination, were predominantly found in the previously known IncP/P-1ε plasmids, including the six plasmids (22/29 plasmids) (Table 2). The variable integron regions of these six plasmids consisted of gene cassettes *aadA* (pLBC82), *catB2* (pLBC75), bla_{OXA-2} (pLBC62 and pLBC70), and *dfrB1*(pLBC56, pLBC62 and pLBC70) (Table 1). These genes and *sul1* were also found associated with the class 1 integrons in other

IncP/P-1ε plasmids, although the bla_{OXA} gene (probably bla_{OXA-20}) in pTE_C_2 showed 19.6% identity at the nucleotide sequence level with those wih bla_{OXA-2} (Table 2).

Currently, no IncP/P-1ε plasmids were isolated from clinical isolates but all of them were isolated from the environmental samples (Table 2). The class 1 integron of the IncP/P-1ε plasmids contained a mutated promoter PcH1 that did not change the IntI1 amino acid sequence (Figure 4). PcH1 showed higher expression levels of gene cassettes than the ancestral one (PcW) and was commonly found among clinical integrons and not in environmental samples (Jové *et al.*, 2010; Ghaly *et al.*, 2017). Indeed, the class 1 integrons in the IncP/P-1 plasmids isolated from environmental samples, including rhizosphere and wastewater treatment plants, possessed PcW (Shintani et al., 2020). Moreover, 15/25 of the class 1 integrons found in the IncP/P-1ε plasmids listed in Table 2 had PcW, and only three integrons on two plasmids, pTE_C_2 and pTE_T100_4, isolated from wastewater in Sweden (Hunlinel et al., 2021), had PcH1, in addition to the seven integrons found in the present study (Table 2). Thus, the presence of the promoter PcH1 on the class 1 integrons present on the plasmids from the present study might indicate high levels of antibiotic pollution from pharmaceutical companies. Potentially higher expression of ARGs carried by such plasmids could provide important benefits to environmental bacteria by enhancing their survival in hostile, antibiotic-polluted environments.

The genetic structures of the accessory regions on the obtained IncP/P-1ε plasmids implied multiple insertions and deletions mediated by different transposons, including Tn*402*-like transposons, Tn*3*-like transposons, and IS*26* (Figure 1). The insertion regions of these MGEs are usually between *trfA* and *oriV*, and between *traC* and *parA*, known as the two major 'hot spots' of IncP/P-1β plasmids (Sota *et al.*, 2007), and these hot spots also occurred in IncP/P-1ε plasmids. These MGEs with *tetA*, *msrE*, *mphE*, and/or *aph(3")-Ib* were also previously found on the IncP/P-1ε-I plasmids isolated from various environmental samples (Table 2), indicating that they were associated with ARGs in different environments as noted previously (Che *et al.*, 2021). The location of ARGs within transposons and/or IS elements on plasmids with a broad-host range under antibiotic selection pressure represents a favorable situation for the widespread dissemination of these ARGs in the river/creek sediments studied. IS*Pa17* with a putative toxin-antitoxin system was commonly found in many IncP/P-1ε plasmids in the same region (Table 2). The 25-bp inverted repeats of IS*Pa17* showed identity (92-100%) with those of Tn*402*-like transposon, implying that the same transposase could mediate the transpositions of these mobile elements. Our findings with IncP/P-1ε plasmids in addition to those already known plasmids imply the 'heteroplasmy' (Novick, 1987) in their ancestral hosts in nature. The IncP/P-1ε plasmids with identical nucleotide sequences of the MGEs and their backbone regions might have diversified in the same host.

IS*26* was found not only on the IncP/P-1ε plasmids pLBC75 and pLBC82, but also on the IncFII-like plasmid pLBC3_2, which was associated with different ARGs (Figure 3, Table 1). The IncL plasmid pLBC2 isolated from the polluted sediment of the Sava River had bla_{TEM-1} with a Tn*3*-family transposon, as had IncN plasmid pLBC4 isolated from the polluted sediment of the creek, whose nucleotide sequences were highly conserved (98.4% identity, 4870/4950 bp). It should be noted that Che *et al*. (2021) reported that IS*26* was extraordinarily abundant in plasmids deposited in public databases. They experimentally showed the IS*26* with ARG could be spread by a conjugative plasmid, RP4 (IncP/P-1α) (Che *et al.*, 2021). These facts suggest that these MGEs mediated the spread of ARGs independently of Inc groups and environments. These findings may help to understand one of the open questions in plasmid biology (Rodríguez-Beltrán *et al.*, 2021): “How are antibiotic resistance genes mobilized on plasmids?”.

IncN plasmids are known to transfer multidrug resistance and they have already been isolated from various environments such as lake sediments heavily polluted with antibiotics (fluoroquinolones) (Flach *et al.*, 2015). The IncN plasmid pLBC4 from sulfonamide-polluted creek sediment carried multiple ARGs, including the sulfonamide resistance gene *sul2*. In a previous study by Milaković *et al.* (2020), this gene was detected by qPCR in sediment at this site well above background levels, likely conferring a selection advantage to IncN plasmid group in sulfonamide-polluted sediment of the pharmaceutical company 2. Furthermore, the localization of *sul2* on wide-spread conjugative replicons such as IncN likely ensures that *sul2* can persist and be transferred among environmental bacteria in the environment. Interestingly, eight IncN plasmids carrying the exact same ARGs as pLBC4 in the exact same order were found in the public database (https://ccb-microbe.cs.uni-saarland.de/plsdb) (Figure S3). Three of them, pRHB27-C20_2, pRHB38-C24_3, and pRHB41-C14_5 were found in *E. coli* isolated from two different farms of livestock (pig) in the United Kingdom (AbuOun *et al.*, 2021), while the other five plasmids originated from different environments. Plasmid pRSB206 was obtained by exogenous plasmid capture method from the final effluent of a municipal wastewater treatment plant in Germany (Eikmeyer *et al.*, 2012). Further, the clinical *Klebsiella pneumoniae* strain 20_GR_12 isolated in Greece contained IncN plasmids (Pitt *et al.*, 2020), i.e., pGMI17-001_1 and pEQAS2016S1-1 detected in *Salmonella enterica* isolates from Germany and China, respectively, (deposited in NCBI as NZ_CP028173 and NZ_CP033359) or plasmid p15ODMR isolated from an *E. coli* pig isolate in Switzerland (Brilhante *et al.*, 2019) – all these plasmids showed highly conserved genetic structures including their ARGs (Figure S3), although they were isolated from geographically and environmentally different sites. This fact suggests that IncN plasmids including pLCB4 might contribute to the spread of ARGs between environmental bacteria and clinical strains. In contrast, no other IncL or IncFII plasmids with the same ARGs sets as pLBC2 (IncL) or pLBC3_2 (IncFII-like) were found in the public database.

Conclusion

New insights into the evolution of conjugative plasmids in various non-clinical environments polluted with antibiotics were gained from the in-depth analysis of the complete sequence of nine AR conferring plasmids captured from pharmaceutical wastewater and its receiving environment. The isolation of several multiple resistance conferring promiscuous plasmids such as IncP/P-1 and IncN provided evidence that anthropogenic activities such as pharmaceutical wastewater releases may contribute to their persistence in bacterial communities in antibiotic-polluted environments. These plasmids carried diverse and clinically relevant ARGs associated with transposable elements such as Tn*402*-, Tn*3*-like transposons and IS*26*. The abundance of bacterial populations carrying these plasmids and their spread through conjugation among bacteria is likely fostered by the diverse pollutants present. Our findings are based on nine plasmids captured using *E. coli* as recipient, and thus plasmid diversity and the ARGs carried are likely largely underestimated.

Figure legends

Figure 1. Alignment of IncP/P-1ε plasmids obtained in this study. Coding DNA regions, their directions, and their predicted functions are indicated as block arrows with colors, red for replication, green for conjugation, yellow for other genes in IncP-1 backbone, light blue for genes related to mobile genetic element, pink for genes related to antimicrobial resistance genes, and magenta for other accessory genes. Homologous regions are represented by frame areas. The key mobile genetic elements (Tn*402*-related transposons, Tn*402*-class 1 integron like element, Tn*3*-family transposons, and IS*Pa17*) are shown by colored rectangular. The plasmids isolated from Sava River are shown in red, while those from Creek are in blue.

Figure 2. Insertion sites of accessory genes including MGEs in the IncP/P-1ε plasmids. MGEs are shown with their inverted repeats (triangles), in green (Tn*402* with class 1integron-like element), red (Tn*3*-like transposon), blue (Tn*402*-like transposon), and brown (IS*Pa17*). Plasmids obtained from Sava River are in blue, while those from Creek are in red.

Figure 3. Alignment of accessory regions of the plasmids obtained in this study. Coding DNA regions, their directions, and their predicted functions are indicated as block arrows with colors, light blue for genes related to mobile genetic element, pink for genes related to antimicrobial resistance genes, and magenta for other accessory genes. Homologous regions are represented by frame areas. The key mobile genetic elements (Tn*402*-related transposons,

Tn*402*-class 1 integron like element, Tn*3*-family transposons, and IS*Pa17*) are shown by colored rectangular. Plasmids obtained from Sava River are shown in panel A, while those from Creek are in panel B.

Figure 4. Comparisons of promoters for gene cassettes in the class 1 integrons. Red boxes show –35 and –10 region of the gene cassette and +1 indicates transcription start point. Note that pLBC82 and pLBC75 have two class 1 integrons. The boxes with pink indicate PcH1, while those with light blue indicate PcW promoter. The integrons found in plasmids from Sava River are shown in blue, while those from Creek are in red.

Table 1. Profiles of the sequenced antibiotic-resistance plasmids studied in this work.

Plasmid name	Inc group	Size (bp)	Source	Cu/Zn mg/kg	Total antibiotics	Transfer frequency[a]	Antibiotic for selection[a]	ARGs	Phenotypic resistance[e]	MGEs
pLBC54 (#1)[a]	P/P-1εII	57553	RIVER sediment, Upstream/non polluted	11/**79**[b]	4.6 µg/kg [b]	$10^{-6.9}$	ERY	*sul1*, *msrE*, *mphE*	SMX, AZI, ERY	**Tn*3*-**like transposon (*mer* operon, *msrE*, *mphE*), **Tn*402*-class 1 integron**-like (*qacEΔ-sul1-orf*5)
pLBC2 (#6)[a]	L	76493	RIVER sediment Discharge/polluted	**41/148**[b]	10,176 µg/kg [b]	$10^{-4.2}$	TET	*sul1, bla*$_{TEM-1}$*, msrE, mphE*	SMX, AM, AMX, ERY, **TET**, **TMP**	**Tn*3*-**like transposon, **Tn*3*-**like transposon (*bla*$_{TEM-1}$), **class 1 integron**-like (*qacEΔ-sul1-orf5*-**IS*6100***)
pLBC3_2 (#7)[a]	FII-like	145941	RIVER sediment, Discharge/polluted	**41/148**[b]	10,176 µg/kg [b]	$10^{-5.5}$	ERY	*sul2, bla*$_{TEM-1}$*, aph(6)-Id, aph(3")-Ib, dfrA18, tetD, catI, catII, erm41*	SMX, AM, AMX, ERY, AZI, GEN, TET, TMP	**IS*26***-*bla*$_{TEM-1}$-*tetDR*-**IS*26***-*catII*-**IS*26***-*dfrA18 sul2, aph(6)-Id, aph(3")-Ib*, **IS*26***-*catI*-**IS*26***-*aph(3')-Ia*-**IS*26***
pLBC82 (#3)[a]	P/P-1εI	57862	RIVER sediment Discharge/polluted	**41/148**[b]	10,176 µg/kg [b]	$10^{-5.5}$	ERY	*sul1, msrE, mphE, tetA, aadA*	SMX, AZI, ERY, TET, **TMP**	**Tn*402*-class 1 integron**-like (*tetAR, orf5-sul1-qacEΔ-aadA-orf5*-**IS*26***-*mphE-msrE*-**IS*26***-*sul1*), **IS*Pa17***(*mazEF*)
pLBC56 (#17)[a]	P/P-1εI	51522	CREEK sediment Upstream/least polluted	**125/445**[c]	90 µg/kg [c]	$10^{-5.9}$	TET	*sul1*, *tetA, dfrB1*	SMX, TET, D, TMP	**Tn*402*-class 1 integron** like (*tetAR, orf5-sul1-qacEΔ-*

										dfrB1), **IS*Pa17***(*mazEF*)
pLBC62 (#24)[a]	P/P-1εI	77135	CREEK sediment Discharge/polluted	**43/186**[c]	970 µg/kg [c]	$10^{-3.2}$	TET	*sul1*, *bla*$_{OXA-2}$, *aph(6)-Id*, *aph(3")-Ib*, *tetA*, *dfrB1*	SMX, AM, AMX, GEN, TET, D, TMP	**Tn*3*-**like transposon (*aph(3")-Ib, aph(6)-Id*), **Tn*402*-class 1 integron**-like [(**Tn*3*-**like transposon (*tetAR*, [**Tn*402*-class 1 integron**-like (transporter)]), *orf5-sul1-qacEΔ-bla*$_{OXA2}$*-dfrB1*], **IS*Pa17***(*mazEF*)
pLBC70 (#25)[a]	P/P-1εI	71660	CREEK sediment Discharge/polluted	**43/186**[c]	970 µg/kg [c]	$10^{-3.2}$	TET	*sul1, bla*$_{OXA-2}$*, tetA, dfrB1*	SMX, AM, AMX, TE, D, TMP	**Tn*402*-class 1 integron**-like [(**Tn*3*-**like transposon (*tetAR*, [**Tn*402*-class 1 integron**-like (transporter)]), *orf5-sul1-qacEΔ-bla*$_{OXA2}$*-dfrB1*], **IS*Pa17***(*mazEF*)
pLBC4 (#27)[a]	N	50954	Creek sediment Downstream/polluted	**82/276**[c]	1,520 µg/kg [c]	$10^{-6.5}$	TET	*sul2, bla*$_{TEM-1}$*, aph(3")-Ib, aph(6)-Id, tetA, dfrA14*	SMZ[f], AM, AMX, GM, TE, D, TMP, **CHL**	**Tn*3*-**like transposon (*bla*$_{TEM-1}$)
pLBC75 (#33)[a]	P/P-1εI	63385	Effluent/ polluted	7.5/33.9[d]	150.6 µg/L [d]	$10^{-4.7}$	TET	*sul1, msrE, mphE, mphF, tetA, catB2, dfrB1*	SMX, ERY, TE, D	**Tn*402*-class 1 integron**-like (*tetAR, orf5-sul1-qacEΔ-catB2-orf5*-**IS*26***-*mrxF-mphF-mphR*-**IS*26***-*mphE-msrE*-**IS*26***-*sul1*), **IS*Pa17***(*mazEF*)

[a] previous plasmid designation (Gonzalez-Plaza *et al*., 2019)

[b] Sum of azithromycin and erythromycin (Milaković *et al*., 2019)

[c] Sum of sulfadiazine, sulfamethazine, trimethoprim, and azithromycin (Milaković *et al*., 2020)

[d] Sum of sulfadiazine, sulfamethazine, trimethoprim, enrofloxacin and oxytetracycline (Bielen *et al*., 2017)

[e] SMX, sulfamethoxazole; AM, ampicillin, AMX, amoxicillin; AZI, azithromycin, ERY, erythromycin; GEN, gentamicin; TET, tetracycline, D, doxycycline; TMP, trimethoprim; CHL, chloramphenicol. The antibiotics of which putative resistance gene(s) were not found on the plasmid are shown in bold.

[f] SMZ, sulfamethazine (resistance phenotype determined in this work)

Table 2. IncP/P-1epsilon plasmids

Plasmids	Inc groups	Accession number	Size (bp)	Transposons	Integron structure[a]	Pc type of integron	Other accessory genes[a]	Source	References
pALTS27	ε-I	MN366356	58844	IS*Pa17*, IS*1326*, IS*26*	*ΔintI1-qacF5-**aadA5**-qacEΔ1-sul1-orf5*	**PcW**	***tetAR***, ***msrE***, ***mphE***	WWTP_Biosolids (USA)	Law *et al.*, 2021
pALTS32	ε-I	MN366360	57949	IS*Pa17*, IS*1326*, IS*26*	*ΔintI1-qacF5-**aadA5**-qacEΔ1-sul1-orf5*	**PcW**	***tetAR***, ***msrE***, ***mphE***	WWTP_Biosolids (USA)	Law *et al.*, 2021
pHH128	ε-I	JQ004406	56366	IS*Pa17*	*intI1-**dfrA1**-gcu39-**aadA1**-**catB2**-**catB2**-qacEΔ1-sul1-orf5*	**PcW**	***tetAR***	Arable field soil (with pig manure) (Germany)	Heuer *et al.*, 2012
pHH3408	ε-I	JQ004407	51230	IS*Pa17*, IS*1326*, IS*26*	*intI1-qacEΔ1-sul1-orf5*	**PcW**	***tetAR***, ***msrE***, ***mphE***	Arable field soil (with pig manure) (Germany)	Heuer *et al.*, 2012
pHH3414	ε-I	JQ004408	55424	IS*Pa17*, IS*1326*, IS*26*	*ΔintI1-**aadA1**-qacEΔ1-sul1-orf5*	**PcW**	***tetAR***, ***msrE***, ***mphE***	Arable field soil (with pig manure) (Germany)	Heuer *et al.*, 2012
pKJK172	ε-I	NZ_CP028340	53761	IS*Pa17*	*ΔintI1-**ereA**-qacEΔ1-sul1-orf5*	**PcW**	***tetAR***	Municipal sewage (Germany) *Thauera aromatica*	Tschech and Fuchs, 1987; Lo *et al.*, 2022
pKJK5	ε-I	AM261282	54383	IS*Pa17*, IS*1326*	*intI1-**dfrA1**-**aadA11b**-qacEΔ1-sul1-orf5*	**PcW**	***tetAR***	Soil (Denmark)	Bahl *et al.*, 2007
pKS77	ε-I	JQ004409	53419	IS*Pa17*, IS*1326*	*ΔintI1-**aadB**-qacEΔ1-sul1-orf5*	**PcW**	***tetAR***	Pig manure (Germany)	Heuer *et al.*, 2012
pMLUA1	ε-I	KC964605	58845	IS*Pa17*, IS*26*, IS*1326*	*ΔintI1-qacF5-**aadA5**-qacEΔ1-sul1-orf5*	**PcW**	***tetAR***, ***msrE***, ***mphE***	Estuarine water (Portugal)	Oliveira *et al.*, 2013
pMLUA3	ε-I	KC964606	57859	IS*Pa17*, IS*26*, IS*UnCu17*	*ΔintI1-qacF5-**aadA5**-qacEΔ1-sul1-orf5*	**PcW**	***tetAR, msrE***, ***mphE***	Estuarine water (Portugal)	Oliveira *et al.*, 2013
pMLUA4	ε-I	KC964607	55475	IS*Pa17*, IS*26*	*ΔintI1-qacF5-**aadA5**-qacEΔ1-sul1-orf5*	**PcW**	***tetAR***, ***msrE***, ***mphE***	Estuarine water (Portugal)	Oliveira *et al.*, 2013
pMNBL056	ε-I	LC623890	52432	IS*Pa17*, IS	*intI1-**dfrB1**-qacEΔ1-sul1-orf5*	**PcW**	***tetAR***	River sediment (Japan)	Hayakawa *et al.*, 2022
pTE_C_2	ε-I	NZ_MW574937	49709	IS*Pa17*	*intI1-**bla**$_{OXA}$-qacEΔ1-sul1-orf5*	**PcW**	not detected	Wastewater (Sweden)	Hutinel *et al.*, 2021

[a]

pTE_C_3	ε-I	NZ_MW574938	63269	IS*Pa17*, IS*26*	*intI1-**dfrB1**-qacEΔ1-sul1-orf5, ΔintI1-qacG2-**aadA6**-qacG2-qacEΔ1-sul1-orf5*	**PcH1, PcH1**	***tetAR***, ***msrE***, ***mphE***	Wastewater (Sweden)	Hutinel *et al.*, 2021
pTE_T100_2	ε-I	NZ_MW574941	51649	Tn*3* family transposon	*intI1-fabG-qacEΔ1-sul1-orf5*	**PcW**	*mazE*	Wastewater (Sweden)	Hutinel *et al.*, 2021
pTE_T100_4	ε-I	NZ_MW574943	51506	IS*Pa17*	*intI1-**dfrB1**-qacEΔ1-sul1-orf5*	**PcH1**	***tetAR***	Wastewater (Sweden)	Hutinel *et al.*, 2021
pYKBO007	ε-I	LC623922	42530	IS*Pa17*	not detected	-	no accessory	WWTP (Japan)	Hayakawa *et al.*, 2022
pLBC82	ε-I		57862	IS*Pa17*	*intI1-sul1, ΔintI1-**aadA**-qacEΔ1-sul1-orf5*	**PcH1, PcH1**	***tetAR***, ***msrE***, ***mphE***	River sediment (Croatia)	This study
pLBC75	ε-I		63385	IS*Pa17*	*intI1-sul1, ΔintI1-**catB2**-qacEΔ1-sul1-orf5*	**PcH1, PcH1**	***tetAR***, ***mrxF***, ***mphF***, ***mphR***, ***msrE***, ***mphE***	Creek sediment (Croatia)	This study
pLBC70	ε-I		71660	IS*Pa17*	*intI1-**dfrB1**-**bla**$_{OXA-2}$-qacEΔ1-sul1-orf5*	**PcH1**	***tetAR***, transporter	Creek sediment (Croatia)	This study
pLBC62	ε-I		77135	IS*Pa17*	*intI1-**dfrB1**-**bla**$_{OXA-2}$-qacEΔ1-sul1-orf5*	**PcH1**	***aph(3")-Ib***, ***aph(6)-Id***, ***tetAR***, transporter	Creek sediment (Croatia)	This study
pLBC56	ε-I		51522	IS*Pa17*	*intI1-**dfrB1**-qacEΔ1-sul1-orf5*	**PcH1**	***tetAR***	Creek sediment (Croatia)	This study
p712	ε-II	NC_019318	62798	IS*1071* (composite transposon)	not detected	-	*tfd* genes	Agricultural soils (Korea) (*Ralstonia pickettii*)	Kim *et al.*, 2013
pAKD16	ε-II	JN106167	74971	IS*1071*, ISPps1, ISCsp2, Tn*6048*	not detected	-	*mer*, putative dioxygenase	Agricultural soil (treated with mercury chloride) (Norway)	Drønen *et al.*, 1999; Sen *et al.*, 2011
pAKD25	ε-II	JN106170	75067	IS*1071*, IS*Pps1*, IS*Csp2*, Tn*402*-like transposon, Tn*501*-like transposon, IS*Pa17*	not detected	-	*mer*, *tfd* genes	Agricultural soil (treated with mercury chloride) (Norway)	Drønen *et al.*, 1999; Sen *et al.*, 2011

pAKD34	ε-II	JN106175	62798	IS*1071*, IS*Pps1*, IS*Csp2*, Tn*402*-like transposon, Tn*501*-like transposon, IS*Pa17*	not detected	-	*mer*, dichlorophenoxypropionic acid degradation genes	Agricultural soil (treated with mercury chloride) (Norway)	Drønen *et al.*, 1999; Sen *et al.*, 2011
pEMT3	ε-II	JX469827	63472	Tn*3* family transposon	not detected	-	*tfd* genes, ***aph(3')***	Agricultural soil (with 2,4-D)(USA)	Gstalder *et al.*, 2003; Sen *et al.*, 2013
pTL50	ε-II	MH392238	39671	not detected	not detected	-	no accessory	Non-polluted lettuce rhizosphere (Germany)	Shintani *et al.*, 2020
pLBC54	ε-II		57553	Tn*3*-like transposon, Tn*402*-like transposon	*intI1-qacEΔ1-sul1-orf5*	**PcW**	*mer,* ***msrE, mphE***	River sediment (Croatia)	This study

ARGs are shown in bold.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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