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Diverse Broad-Host-Range Plasmids from Freshwater Carry Few Accessory Genes

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Broad-host-range self-transferable plasmids are known to facilitate bacterial adaptation by spreading genes between phylogenetically distinct hosts. These plasmids typically have a conserved backbone region and a variable accessory region that encodes host-beneficial traits. We do not know, however, how well plasmids that do not encode accessory functions can survive in nature. The goal of this study was to characterize the backbone and accessory gene content of plasmids that were captured from freshwater sources without selecting for a particular phenotype or cultivating their host. To do this, triparental matings were used such that the only required phenotype was the plasmid's ability to mobilize a nonconjugative plasmid. Based on complete genome sequences of 10 plasmids, only 5 carried identifiable accessory gene regions, and none carried antibiotic resistance genes. The plasmids belong to four known incompatibility groups (IncN, IncP-1, IncU, and IncW) and two potentially new groups. Eight of the plasmids were shown to have a broad host range, being able to transfer into alpha-, beta-, and gammaproteobacteria. Because of the absence of antibiotic resistance genes, we resampled one of the sites and compared the proportion of captured plasmids that conferred antibiotic resistance to their hosts with the proportion of such plasmids captured from the effluent of a local wastewater treatment plant. Few of the captured plasmids from either site encoded antibiotic resistance. A high diversity of plasmids that encode no or unknown accessory functions is thus readily found in freshwater habitats. The question remains how the plasmids persist in these microbial communities.

Mobile genetic elements such as plasmids are thought to form communal gene pools that allow bacterial communities to rapidly adapt to changing environments (1). Plasmids that can transfer and replicate in phylogenetically diverse bacteria are called broad-host-range (BHR) plasmids (2, 3). BHR plasmids of Gram-negative bacteria with the widest host range belong to the incompatibility groups IncP (4), IncW (5), IncU (6), IncQ (7), and the recently defined PromA group (8). These plasmids are typically composed of two distinct regions: (i) the backbone region, which encodes genes responsible for plasmid replication, maintenance and gene regulation, as well as conjugative transfer, and (ii) the accessory region, which consists of genes that code for host-beneficial traits. BHR plasmids are thought to be particularly crucial in adaptation of bacterial communities to changing environments since they shuttle host-beneficial genes across taxonomic boundaries (9). Examples of exchanged traits are resistance to antibiotics or heavy metals and degradation of organic xenobiotics, like chlorinated aromatics.

A central question in plasmid ecology is whether or not plasmids can be maintained in bacterial communities without benefiting their hosts and thus be considered genetic parasites. Since in the absence of positive selection for plasmid-encoded traits plasmid carriage is assumed to impose a cost to the bacterial host, albeit sometimes small, plasmid-bearing bacteria are expected to be out-competed by their plasmid-free counterparts, which can arise spontaneously through incorrect plasmid segregation during cell division (10–14). Stewart and Levin (14) first argued that the conditions for plasmids to persist by infectious transfer alone, much like parasites, were very limited. However, later Lundquist and Levin (13) provided evidence through chemostat studies that naturally occurring plasmids may well be maintained by horizontal transfer due to high conjugation rates. We later showed that some BHR plasmids can invade bacterial populations grown on surfaces in the absence of any known selective pressure (15). The first description of completely sequenced self-transmissible plasmids that are devoid of identifiable accessory genes, e.g., IncP-1 plasmids pA1 (16) and pBP136 (17), supported the hypothesis that a parasitic existence through efficient horizontal transfer is possible for some BHR plasmids. However, while these plasmids are considered "cryptic" because they do not seem to confer any known benefits to their hosts, they still carry small open reading frames (ORFs) with unknown functions and represent a very small sample among the vast diversity of plasmids in various ecosystems. A better overview is needed of the natural occurrence of cryptic BHR plasmids.

The next logical question is in which environments such possibly cryptic plasmids are likely to be found. Most of our knowledge about accessory genes comes from plasmids that were isolated based on specific phenotypes that often match the environmental selection pressures. For example, plasmids isolated from bacteria found in soil contaminated with pesticides and

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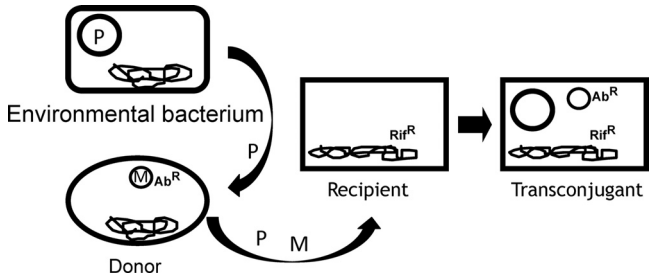

FIG 1 Illustration of the triparental mating method. Environmental bacteria containing plasmids (P) are mixed with a donor bacterium, which carries a mobilizable plasmid (M) with a specific marker, such as antibiotic resistance (Ab^R), and a recipient bacterium, which has a second selectable marker, such as rifampin resistance (Rif^R). When plated on both antibiotics, only recipient strains that carry a mobilizable plasmid can grow, and since only the environmental plasmid (P) can move the mobilizable plasmid into the recipient strain, the environmental plasmid is sometimes present too. One pathway by which transconjugants can be formed is shown by the arrows.

heavy metals encode degradation of, or resistance to, these xenobiotics (18). The same is true for wastewaters known to contain pollutants such as antibiotics and organic and inorganic xenobiotics (19–22). Plasmids carrying genes conferring resistance to all major classes of antibiotics have been found in wastewater treatment plants (WWTP) (23, 24). In contrast, nonpolluted or moderately polluted environments may not impose strong selection for these traits and therefore may sustain bacteria with plasmids devoid of the corresponding genes. Understanding plasmid diversity in these environments is thus crucial to our understanding of plasmid parasitism.

Various methods exist for isolating plasmids from environmental bacteria. In traditional so-called endogenous methods, bacteria are isolated in pure culture, and this is followed by plasmid DNA extraction. Exogenous isolation methods capture plasmids from microbial communities without culturing the plasmid hosts and include biparental and triparental matings (for a review, see reference 25). Biparental matings typically capture plasmids that transfer genes encoding a specific phenotype to a recipient strain, such as mercury or antibiotic resistance. The method involves mixing the indigenous bacterial community with a plasmid-free recipient strain, allowing conjugation to occur, and selecting for recipients that acquired the plasmid-borne trait. Triparental matings include one additional parental population, i.e., a donor strain carrying a nonconjugative, mobilizable plasmid (Fig. 1). Here, plasmids are captured based solely on their ability to transfer this mobilizable plasmid and themselves into the recipient and not for specific accessory phenotypes they encode (26, 27). This method thus provides an assessment of plasmid diversity that is not biased for plasmid-borne traits. Using this method, BHR plasmids of the PromA group were obtained (pIPO2 and pMOL98) whose putative accessory genes currently have unknown functions (8, 28).

In this study, we isolated and sequenced plasmids from three freshwater sites around Moscow, ID, using the triparental mating method. A diverse set of plasmids was found, and several plasmids appear to carry only genes involved in core plasmid functions, suggesting that they persist in these environments without conferring an accessory phenotype to their hosts. Given the low incidence of plasmids with drug resistance genes, we then compared antibiotic resistance profiles of plasmids newly captured from one of the creeks with those captured from the effluent of the downstream Moscow, ID, WWTP. Although we expected the proportion of resistance plasmids to be higher in the WWTP than in the creek, equally low proportions were found at both sites.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. A list of the strains and plasmids used in this study is provided in Table 1. Luria-Bertani (LB) broth was generally used to culture the strains. Solid medium was prepared by addition of 1.5% agar. Difco R2A agar (Becton, Dickinson, and Co., Franklin Lakes, NJ) was used for the triparental matings. Difco Mueller-Hinton agar (Becton, Dickinson, and Co., Franklin Lakes, NJ) was used for the antibiotics sensitivity test. *Escherichia coli* strains were incubated at 37°C, while other bacterial species and mating mixtures were incubated at 30°C. When necessary for selection, antibiotics were added to the medium at the following concentrations: chloramphenicol (Cm), 25 μg/ml; tetracycline (Tc), 10 μg/ml; streptomycin (Sm), 50 μg/ml; rifampin (Rif), 100 μg/ml; nalidixic acid (Nal), 20 μg/ml; ampicillin (Ap), 50 μg/ml; zeocin (Zeo), 100 μg/ml; kanamycin (Km), 50 μg/ml; gentamicin (Gm), 10 μg/ml. Mercury chloride was used at 5 μg/ml for selection of pQKH54. Cycloheximide (300 μg/ml) was added to LB agar (LBA) to prevent growth of fungus during screening of transconjugants from mating mixtures containing freshwater samples.

A restriction system-free *E. coli* strain that expresses the enhanced yellow fluorescent protein (EYFP) was constructed to be used as a second recipient strain in the exogenous plasmid isolation experiments to compare creek water with WWTP effluent (see below). First, a mini-Tn*7* region containing a zeocin resistance gene and *eyfp* was moved from pUC18T-mini-Tn*7*T-*zeo*-$P_{A1/04/03}$-*ecfp* into an R6K replicon pHY835 (29). Then, the resulting plasmid, pHY956, and Tn*7* transposase expression plasmid pTNS2 were simultaneously introduced into *E. coli* EC100 by electroporation. Subsequent screening for zeocin-resistant clones gave rise to HY841. Mini-Tn*7* insertion into the specific site (*att*Tn*7*) was confirmed by PCR using primers EcoliglmS (CATGCACATCATCGAGAT GCC) and Tn7R109 (CAGCATAACTGGACTGATTTCAG). A spontaneous rifampin-resistant mutant of HY841 was designated HY842 and was used as the recipient strain.

Water samples. Water was sampled from three sources in and near Moscow, ID. The first site was a pond in the University of Idaho's Arboretum and Botanical Garden. This pond receives its water supply from ground water, precipitation, and irrigation runoff from the University of Idaho. The water used by the university to irrigate its lawns is chlorinated effluent from the Moscow, ID, WWTP, which is chlorinated for a second time before irrigation. The second site was Paradise Creek in downtown Moscow, ID. The source of this stream is the Palouse Range from which the stream meanders down the mountain, collecting agricultural and urban runoff. Typical crops grown in this area include wheat, barley, peas, and lentils. The third site was Idler's Rest Creek in the Palouse Range. The site of water collection was a few miles downstream from the source in the forests of the Palouse Range. A total of 500 ml of water was collected in sterile bottles for each mating experiment. To remove suspended solid particles from water, the samples were filtered through autoclaved, grade 1 Whatman filter paper (11 μm) and then through sterile Fisher P5 filter paper (5 μm) into sterile 1-liter bottles, using a sterile vacuum filtration system.

Triparental matings. To capture a wide range of self-transmissible mobilizing plasmids, two types of mobilizable plasmids were used: (i) pSU4814 (30), which has a CloDF13 mob region and has been shown to be mobilized efficiently by IncF, IncN, IncI, and IncW plasmids (31), and (ii) pBBR1MCS (32), derived from plasmid pBBR1 from *Bordetella bronchiseptica*, known to be mobilized by IncP-1 plasmid RK2 (33), and its derivative pBBR1MCS-5 (34). Triparental matings were carried out as shown in Fig. 1 and described here. First, *E. coli* DH5α harboring either of the mobilizable plasmids and a recipient *E. coli* strain CV601gfp were grown overnight. Then, 500 ml of filtered water was mixed with 500 μl of

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and relevant phenotype[a]	Reference or source
Strains (class)		
E. coli (*Gammaproteobacteria*)		
CV601gfp	Thr$^-$ Leu$^-$ Thi$^-$ chr::mini-Tn*5-gfp-aphA*; Rif^r Km^r	36
DH5α	λ$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*; Nal^r	35
EC100/DH10B	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 deoR endA1 thi-1 recA1 gyrA96 relA1 hsdR17 supE44* Δ(*ara-leu*)7697 *galU galK rpsL nupG*; Sm^r	Epicentre, Madison, WI
HY841	EC100 *att*Tn*7*::mini-Tn*7-zeo*-$P_{A1/04/03}$-*eyfp*; Sm^r Zeo^r	This study
HY842	Rif^r mutant of HY841; Sm^r Zeo^r Rif^r	This study
JM109	*endA1 recA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *relA1 supE44* Δ(*lac-proAB*) [F′ *traD36 proAB laq*I^{q}ZΔM15]	82
K12Rif	Rif^r mutant of MG1655	15
K12Nal	Nal^r mutant of MG1655	15
Pseudomonas putida (*Gammaproteobacteria*)		
UCW1gfp	UCW1 chr.::mini-Tn*5-gfp-aphA*; Rif^r Km^r	36
Cupriavidus pinatubonensis[b] (*Betaproteobacteria*)		
JMP228gfp	JMP228 chr.:: mini-Tn*5-gfp-aphA*; Rif^r Km^r	36
Plasmids		
pBBR1MCS	BHR mobilizable cloning vector; Cm^r	32
pBBR1MCS-5	BHR mobilizable cloning vector; Gm^r	34
pMS0705	p15A replicon carrying mini-Tn*21*OTc[c]; Cm^r Tc^r pMB1 *oriV*	M. Sota
pMT1247Tc	p15A replicon carrying mini-Tn*21*Tc; Cm^r Tc^r	M. Sota
pSU4814	p15A replicon carrying CloDF13 *oriT*; Cm^r	30
pQKH54	IncP-1γ replicon; Hg^r	46
pUC18T-mini-Tn7T-Zeo-eyfp	pUC replicon carrying mini-Tn*7*T-*zeo*-$P_{A1/04/03}$-*eyfp*; Ap^r Zeo^r	83
pHY835	R6K replicon carrying mini-Tn*7*	29
pHY956	pHY835::mini-Tn*7*T-*zeo*-$P_{A1/04/03}$-*eyfp*; Ap^r Zeo^r	This study
pTNS2	R6K replicon, Tn*7* transposase expression vector	84

[a] Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Nal, nalidixic acid; Rif, rifampin; Sm, streptomycin; Tc, tetracycline; Zeo, zeocin; Hg^r, mercury resistance.
[b] Previously named *Cupriavidus necator*.
[c] Mini-Tn*21*OTc carries *oriV* from pBR322 (85).

100-fold diluted overnight cultures of the two *E. coli* strains and filtered through a sterile Nalgene cellulose nitrate analytical filter (0.22-μm pore size) (Nalge Nunc International, Rochester, NY). The filters were transferred onto an R2A agar plate supplemented with 300 μg/ml of cycloheximide to prevent fungal growth and incubated at 30°C overnight. The mating mixtures from the filters were then resuspended in 2 ml of sterile saline (0.85% NaCl), and appropriate dilutions were plated onto LBA containing Rif and Cm (for pBBR1MCS and pSU4814) or on LBA containing Rif and Gm (for pBBR1MCS-5) to select transconjugants. The presence of captured mobilizing plasmids was verified as described below. At various stages, some of the plasmids that were initially captured were subsequently eliminated from the list of candidate plasmids, for example, because they were not self-transmissible or not successfully marked with the transposon. As far as we know, our procedure was not biased toward plasmids without known accessory genes. Because of this lack of quantitation, we do not draw any conclusions on the relative abundance of the captured plasmids in their natural habitats.

In a second set of triparental matings to compare the proportion of antibiotic resistance plasmids from creek water and WWTP effluent, *E. coli* HY842 was used as recipient instead of CV601gfp.

General DNA manipulations. Plasmid presence in bacterial strains was confirmed by alkaline lysis extraction and agarose gel electrophoresis (35). Restriction fragment length polymorphisms of captured plasmids were determined by restriction digestion with enzymes from New England BioLabs (Ipswich, MA, USA), followed by agarose gel electrophoresis. Plasmid DNA to be used for sequence determination was obtained using a Plasmid Midi kit (Qiagen, Valencia, CA) according to the manufacturer's instructions for low-copy-number plasmids. PCR was carried out using Phusion DNA polymerase (New England BioLabs, Ipswich, MA, USA).

Plasmid marking with tetracycline resistance. Since none of the newly captured plasmids showed resistance to antibiotics or mercuric chloride, they were marked with tetracycline resistance transposons. To do this, mini-Tn*21* donor plasmids pMS0507 (kind gift from Masahiro Sota) and pMT1749Tc were introduced into *E. coli* CV601gfp carrying a captured plasmid (Table 1). The resulting strains were mated with *E. coli* K12Nal, and the transconjugants were selected for Tc and Nal resistance. This typically yielded K12Nal derivatives carrying a self-transmissible plasmid tagged with mini-Tn*21*OTc or mini-Tn*21*Tc, respectively. Mini-Tn*21*OTc on pMS0507 contains pBR322 *oriV*, which was used to increase the copy number of the tagged plasmids in *E. coli* and therefore improved the quantity and quality of purified plasmid DNA required for sequencing. Only plasmids that showed unique restriction fragment length patterns were sequenced. The tagged plasmids were designated pMBUI1T, pMBUI2T, pMBUI3T, pMBUI4T, pMBUI6T, pMBUI7T, pMBUI8T, pDS1T, pDS2T, and pDS3T.

Host range assays. The host range of the marked plasmids was evaluated by conjugative transfer to three bacterial strains: *Pseudomonas putida* UCW1gfp, *Cupriavidus pinatubonensis* JMP228gfp, and *Sinorhizobium meliloti* RM1021, belonging to the *Gamma-*, *Beta-*, and *Alphaproteobacteria*, respectively (36). The donor strain, *E. coli* K12Nal bearing the plasmid to be tested, was grown at 37°C in LB broth supplemented with 10 μg/ml tetracycline. All recipients were grown overnight in LB broth at 30°C. Biparental matings between the donor strain and each of the recipients

was carried out at 30°C as previously described (37) with selection for Rif and Tc resistance.

Incompatibility testing. To determine incompatibility with a known IncP-1 plasmid, pDS1T was first transferred by conjugation to *E. coli* DH10B with prototype IncP-1γ plasmid pQKH54 (37). Three transconjugants were grown overnight in 5 ml of LB containing Tc to select for pDS1T. Every 24 h for 10 days, 50 μl of culture was transferred to 5 ml of fresh medium containing Tc. Dilutions were plated on LBA every other day, followed by replica plating of 52 colonies onto LBA, LBA with Tc, and LBA with $HgCl_2$. The proportion of cells sensitive to $HgCl_2$ is a measure of the proportion that lost pQKH54. In parallel, *E. coli* DH10B bearing only pQKH54 was propagated in the absence of Tc for 10 days to determine its stability. Dilutions were plated on LBA, and colonies were replica plated on LBA and LBA with Hg. The same experiment was repeated for the IncP-1γ plasmid pMBUI1T as the positive control and for IncN plasmid pDS2T as a negative control.

Sequencing and annotation. The DNA sequences of the plasmids were determined by pyrosequencing at the Department of Energy (DOE) Joint Genome Institute (Walnut Creek, CA). The shotgun sequencing protocol using the Roche/454 platform has been described previously (38). The plasmids were sequenced with GS FLX Titanium Sequencing chemistry (Roche/454 Life Sciences, Branford, CT). Approximately 199.6 million bases of sequence information were generated from half of a sequencing run. The sequences were assembled using the Newbler software (Roche/454 Life Sciences, Branford, CT). Gaps between contigs were closed by PCR amplification and subsequent Sanger sequencing of the PCR product using BigDye Terminator, version 3.2, cycle sequencing (Applied Biosystems, Foster City, CA). The IGS Annotation Engine at the Institute for Genome Sciences, School of Medicine, University of Maryland (http://ae.igs.umaryland.edu/), provided automatic annotation of the plasmid sequences, which were further annotated manually by us. To reconstruct the genome sequences of the originally captured plasmids, the DNA sequences of the minitransposons used to tag the plasmids were removed from each plasmid sequence. The presence of repeated sequences at each side of the transposon was confirmed in each case, indicating that the transposition had not caused deletions of flanking regions.

Phylogenetic analysis of IncP-1 plasmids. The part of the *trfA* gene that is conserved among the IncP-1 plasmids was used to infer the phylogenetic relationship of pDS1 with the other IncP-1 subgroups. The complete sequences of *trfA* genes from 45 IncP-1 plasmids (AB237782, GQ495894, GQ983559, HQ891317, JF274988, JF274990, JN106164, JN106165, JN106166, JN106167, JN106168, JN106169, JN106170, JN106171, JN106172, JN106173, JN106174, JN106175, NC_001621, NC_003430, NC_004840, NC_004956, NC_005088, NC_005793, NC_006352, NC_006388, NC_007337, NC_007502, NC_007680, NC_008055, NC_008272, NC_008357, NC_008385, NC_008766, NC_010935, JX847411, NC_014911, NC_016968, NC_016978, JQ432563, NC_006830, NC_001735, EF107516, JQ432564, and KC170283) were aligned by first translating the genes to protein sequences, aligning the protein sequences using ClustalW (39), and then aligning the nucleotide sequences to the protein sequences using the transalign function of EMBOSS (40). Only the homologous 957 nucleotides (nt) of the 3′ end of the alignment were used in the phylogenetic analysis. This fragment coincides with the last 915 nt of pDS1. Sites that had gaps were excluded from the analysis, resulting in 843 aligned sites. A DNA sequence evolution model was chosen using DT-ModSel (41), and a phylogenetic tree was inferred using the maximum-likelihood method implemented in PAUP* (42).

Bioinformatics analyses and software. The IGS Annotation Engine (http://ae.igs.umaryland.edu/cgi/index.cgi) was used to automatically annotate plasmid sequences. Annotations were corrected manually using Manatee (http://ae.igs.umaryland.edu/cgi/manatee_intro.cgi). All unique hypothetical proteins were compared against the Interpro database using BLAST with a default E value of 10 (43). Interpro (http://www.ebi.ac.uk/interpro/) is a database of protein families, domains, regions, repeats, and sites in which identifiable features of known proteins can be used to infer functions for uncharacterized proteins based upon sequence similarity.

Comparison of antibiotic resistance profiles of plasmids captured from creek water and effluent. To compare resistance profiles of plasmids from the Moscow, ID, wastewater treatment plant to a new set of plasmids from Paradise Creek, triparental matings and disk diffusion assays were used. Plasmids from both locations were captured as described above, except that *E. coli* HY842 was used as a recipient instead of CV601gfp. HY842 has several advantages that CV601gfp does not have. First, this strain completely lacks genes for restriction enzymes, thus allowing establishment of more diverse plasmids. Second, this strain is easily and clearly distinguishable from environmental strains by colony PCR targeting the mini-Tn*7*–chromosome junction as well as by detection of the yellow fluorescence it encodes. The presence of captured plasmids was confirmed by plasmid DNA extraction, followed by gel electrophoresis. The recipient was confirmed to be *E. coli* HY842 by observing the fluorescence of the transconjugants under blue light of a fluorescence microscope. Plasmid DNA was digested with EcoRI, and only plasmids with distinct patterns were chosen for disk diffusion assays.

Overnight cultures of transconjugants in LB broth were diluted 100 times in fresh LB broth and grown at 37°C until they reached an optical density at 600 nm (OD_{600}) of 0.4, corresponding to approximately 10^8 CFU/ml. Then 100 and 260 μl of this culture were spread onto 100-mm and 150-mm plates of Mueller-Hinton agar, respectively, with sterile cotton swabs. Negative controls were *E. coli* HY842 carrying only the mobilizable plasmids. Antibiotics were chosen from each of the main classes, and disks containing the following antibiotics were used (concentrations per disk in μg): kanamycin, 30; gentamicin, 10; spectinomycin, 100; polymyxin B, 300 IU; aztreonam, 30; cefoxitin, 30; imipenem, 10; ceftazidime, 30; ciprofloxacin, 5; amoxicillin and clavulanic acid, 30; trimethoprim, 5; sulfamethoxazole/trimethoprim, 23.75/1.25; tetracycline, 30; and chloramphenicol, 30 (BD BBL, USA). Plates were incubated at 37°C for 48 h, and transconjugants were classified as resistant or susceptible in comparison to the controls and in accordance with the guidelines provided by the Clinical and Laboratory Standards Institute (44).

Nucleotide sequence accession numbers. Complete nucleotide sequences of untagged plasmids were deposited in GenBank under accession numbers KC170278 to KC170285.

RESULTS

Capture of plasmids from freshwater sources. To capture self-transmissible plasmids from three freshwater sources in and near Moscow, ID, without selecting for a particular plasmid-encoded trait, multiple triparental matings were carried out with *E. coli* as the recipient. The first site was a pond in the university arboretum, which receives water from ground water, precipitation, and irrigation runoff of hyperchlorinated effluent from the municipal wastewater treatment plant (WWTP) (see Materials and Methods for details). The second sample was taken from Paradise Creek, which receives agricultural and urban runoff. The third was from Idler's Rest Creek in a sparsely inhabited forested area outside town, taken a few miles downstream from the spring. The genomes of 10 captured plasmids were completely sequenced. The plasmids were chosen based on their unique restriction profiles and because either they were shown to have a broad host range (Table 2) or their BHR nature was inferred from partial sequence information (from pDS1 and pDS2). None conferred resistance to any of the antibiotics tested. To facilitate host range and incompatibility assays, the plasmids were marked with a mini-Tn*21* transposon encoding tetracycline resistance (Table 1). Except for pDS1 and pDS2, all plasmids showed a broad host range as they transferred and replicated in alpha-, beta-, and gammaproteobacteria (Table 2).

TABLE 2 General features of completely sequenced plasmids

Plasmid	Size (bp)	Origin	Mobilizable plasmid	Host range[a]	BHR group	Accessory gene region(s)	GenBank accession no.
pMBUI1	44,304	Arboretum	pBBR1MCS	A, B, C	IncP-1γ	None	JQ432563
pMBUI2	37,976	Arboretum	pBBR1MCS	A, B, C	New	6.6 kb, 6 unique ORFs	KC170285
pMBUI3	33,736	Arboretum	pBBR1MCS	A, B, C	IncU	None	KC170281
pMBUI4	37,247	Arboretum	pSU4814	A, B, C	IncW-like	12 kb, 21 unique ORFs	KC170278
pMBUI6	47,999	Arboretum	pSU4814	A, B, C	PromA-like	14 kb, 10 unique ORFs	KC170282
pMBUI7	34,006	Paradise Creek	pBBR1MCS-5	A, B, C	IncU	None	KC170284
pMBUI8[b]	53,313	Paradise Creek	pBBR1MCS-5	A, B, C	IncP-1β	9.3 kb, 3 unique ORFs[c]	KC170279
pDS1	40,596	Idler's Rest	pBBR1MCS	C[d]	IncP-1η	None	KC170283
pDS2	56,683	Idler's Rest	pBBR1MCS	C[d]	IncN	15.6 kb[e]	KC170280
pDS3	40,806	Idler's Rest	pBBR1MCS	A, B, C	IncP-1β	None	JX469834

[a] The recipients used in matings to assess host range were *Pseudomonas putida* UCW1gfp (*Gammaproteobacteria*), *Cupriavidus pinatubonensis* JMP228gfp (*Betaproteobacteria*), and *Sinorhizobium meliloti* RM1021 (*Alphaproteobacteria*). A, *Alphaproteobacteria*; B, *Betaproteobacteria*; C, *Gammaproteobacteria*.
[b] Marked with mini-Tn*21*Tc; all others marked with mini-Tn*21*OTc (Table 1).
[c] Putative functions encoded by genes in accessory region of pMBUI8: resolvase, PepSY, phosphoesterase, histidine kinase, triphenylmethane reductase, dihydrolipoamide dehydrogenase, transcriptional regulator, transposases.
[d] Plasmids pDS1 and pDS2 were transferable into *E. coli* but could not be transferred to *P. putida*, *C. pinatubonensis*, or *S. meliloti*.
[e] Putative functions encoded by genes in the accessory region of pDS2: sodium-proton antiporter, multidrug ABC transporter, transposases, and insertion elements.

Plasmids from the arboretum pond. Five plasmids were captured from pond water at the university's arboretum: pMBUI1, pMBUI2, pMBUI3, pMBUI4, and pMBUI6. Each plasmid belonged to a different incompatibility group (Table 2), indicating high plasmid diversity in our sample. Two plasmids do not seem to encode accessory functions.

Only two of the five plasmid genomes showed high similarity to previously described BHR plasmids, i.e., IncP-1 plasmid pMBUI1 and IncU plasmid pMBUI3 (Fig. 2 and Table 3). They are also the only two plasmids from this habitat that seem to be devoid of known accessory genes. Plasmid pMBUI1 belongs to the IncP-1γ subgroup (45) and is closely related to the archetype pQKH54 (46). Its characteristics are described elsewhere (47). Plasmid pMBUI3 (Fig. 2b) is closely related to the completely sequenced IncU plasmids RA3 (48) and pFBAOT6 (6); therefore its annotation follows that of these two plasmids. Surprisingly, in contrast to these other two IncU plasmids, no known accessory genes were found on pMBUI3. Between the replication and transfer genes there are three ORFs of unknown function that are present on all IncU plasmids. Since expression of these and several plasmid backbone genes was recently shown to be regulated by the plasmid-encoded protein KorC, we infer that these ORFs are also backbone genes (49). We propose that pMBUI3 consists entirely of IncU plasmid backbone genes.

Plasmid pMBUI4 (Fig. 3) is an IncW-like plasmid that bears moderate gene synteny and sequence similarity in its replication (Table 3) and transfer regions to the IncW plasmid R388 (5). However, its establishment and stable inheritance and control regions are far less recognizable, and some of the genes in these regions on R388 are missing on pMBUI4 (5). The so-called establishment module of R388 is comprised of three genes, *ardC* (antirestriction activity), *ssb* (DNA metabolism), and *ardK* (transcriptional regulator of *ardC*). Of these, only *ardC* was found on pMBUI4 (Fig. 3), but its protein sequence was most similar to ArdC from pMOL98, a PromA plasmid (only 62% identity). The stable inheritance module of R388 is comprised of two operons. The first has *kfrA* (plasmid partitioning), *nuc1* (thermonuclease; a homologue of *parB* from RK2), *nuc2*, and *osa* (oncogenicity suppression). Of these, only *nuc1* could be identified on pMBUI4 (Fig. 3). The second operon of R388 is composed of genes *stbA*, *stbB*, and *stbC*, which are all present on pMBUI4. Their products have about 85% identity with the respective proteins in pIE321. Like R388, long direct repeats were found on pMBUI4, the functions of which are unknown. Of the six transcriptional regulators found on R388 (*ardK*, *klcB*, *stbA*, *trwA*, *korA*, and *korB*) (5), only the last four were identified on pMBUI4. Because of the many missing genes, we refer to pMBUI4 as an IncW-like plasmid (Table 2).

The cluster of hypothetical genes on pMBUI4 into which our artificial Tn*21* inserted does not have any homologues; even Interpro domains could not be detected. Of the genes with unknown functions, only *mpr*, which encodes a putative zinc metalloproteinase, has homologues in other plasmids.

Plasmid pMBUI2 is a member of a new group of BHR plasmids with similarities to pMATVIM-7 (50) from *Pseudomonas aeruginosa* (Fig. 3 and Table 3). The main difference between the backbone genes of these two plasmids is that pMBUI2 seems to have more transfer genes than pMATVIM-7. This new BHR group has several similarities to IncP-1 plasmids including the following: (i) the presence of transfer genes that show sequence similarity to those of IncP-1 plasmids and a similar gene arrangement to that of IncP-1 plasmid pNeutP1 from *Nitrosomonas eutropha* C91 (51, 52); (ii) a putative nick site, ATCTTG, between *traJ* and *traK*, with similarity to the nick site in the origin of transfer (*oriT*) on IncP-1 plasmids. Dissimilarities to IncP-1 plasmids include the following: (i) a replication initiation gene that bears no resemblance to *trfA*, (ii) the presence of a partitioning gene, and (iii) the absence of distinguishable iterons. In fact, most typical IncP-1-specific stable inheritance genes involved in partitioning, postsegregational killing, or multimer resolution were not identified on pMBUI2, with the exception of one encoding a putative fertility inhibition factor that has 37% identity to FiwA of IncP-1α plasmids. Finally, pMBUI2 has a cluster of hypothetical genes between *traB* and *fiwA* with no known homologues. Hidden Markov model (HMM)-based detection of Interpro domains (53) also did not help identify these gene products. Another putative *mpr* gene was found on this plasmid, whose protein product has 50% amino acid identity to its homologue on pMBUI4.

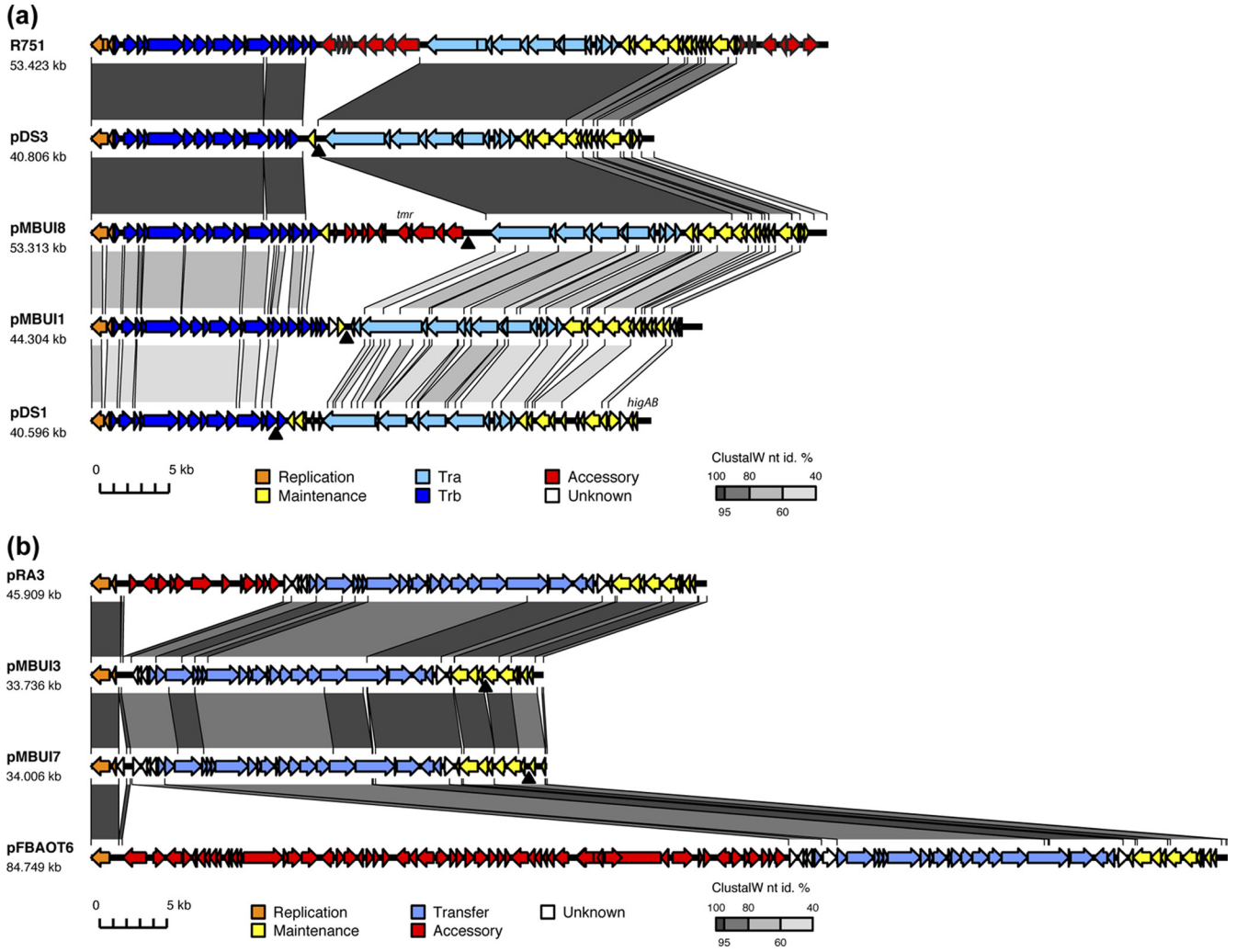

FIG 2 Alignment of newly sequenced plasmids showing their relationship to each other and to well-studied members of their incompatibility group. (a) IncP-1 plasmids. (b) IncU plasmids. Coding regions are shown as colored arrows; putative functions are indicated by the color key. The triphenylmethane reductase gene is labeled as *tmr* on pMBUI8. The degree of similarity between plasmids (percent nucleotide identity [nt id] of alignments performed using ClustalW) is indicated by gray scale-shaded regions; the darker the shading between two segments, the higher their similarity as shown in the heat key. Sites of mini-Tn*21* insertion in the tagged plasmids are marked with a filled black triangle.

Plasmid pMBUI6 is a PromA-like plasmid (Fig. 3). Some of its backbone genes bear close similarity to pXF51 from *Xylella fastidiosa* (54) and more distantly to BHR plasmids of the PromA group (Table 3), which includes pMOL98, pSB102, pIPO2, pTER331, and pMRAD02 (8). Important PromA-like features identified on pMBUI6 include (i) the location of the topoisomerase gene (*topA*) among genes of the mating pair formation complex, (ii) reverse orientation of the relaxase gene (*mobB*) with re-

TABLE 3 Sequence identity of replication initiation proteins from each plasmid with most similar protein sequence in GenBank

Plasmid	Most similar plasmid (Inc group)	Protein	GenBank accession no.	% Identity
pMBUI1	pQKH54 (IncP-1 γ)	TrfA	YP_619825	98
pMBUI2	pMATVIM-7 (unknown)	Rep	YP_001427363	72
pMBUI3	RA3 (IncU)	RepB	YP_001966818	99
pMBUI4	R7K (IncW)	Rep	YP_001874895	62
pMBUI6	pMOL98 (PromA)	Rep	CAC93881	39
pMBUI7	RA3 (IncU)	RepB	YP_001966818	99
pMBUI8	pB10 (IncP-1β)	TrfA	NP_858039	100
pDS1	pBS228 (IncP-1α)	TrfA2	YP_758655	72
pDS2	*Klebsiella pneumoniae* plasmid 9 (IncN)	RepA	YP_002286867	100
pDS3	pB10 (IncP-1β)	TrfA	NP_858039	100

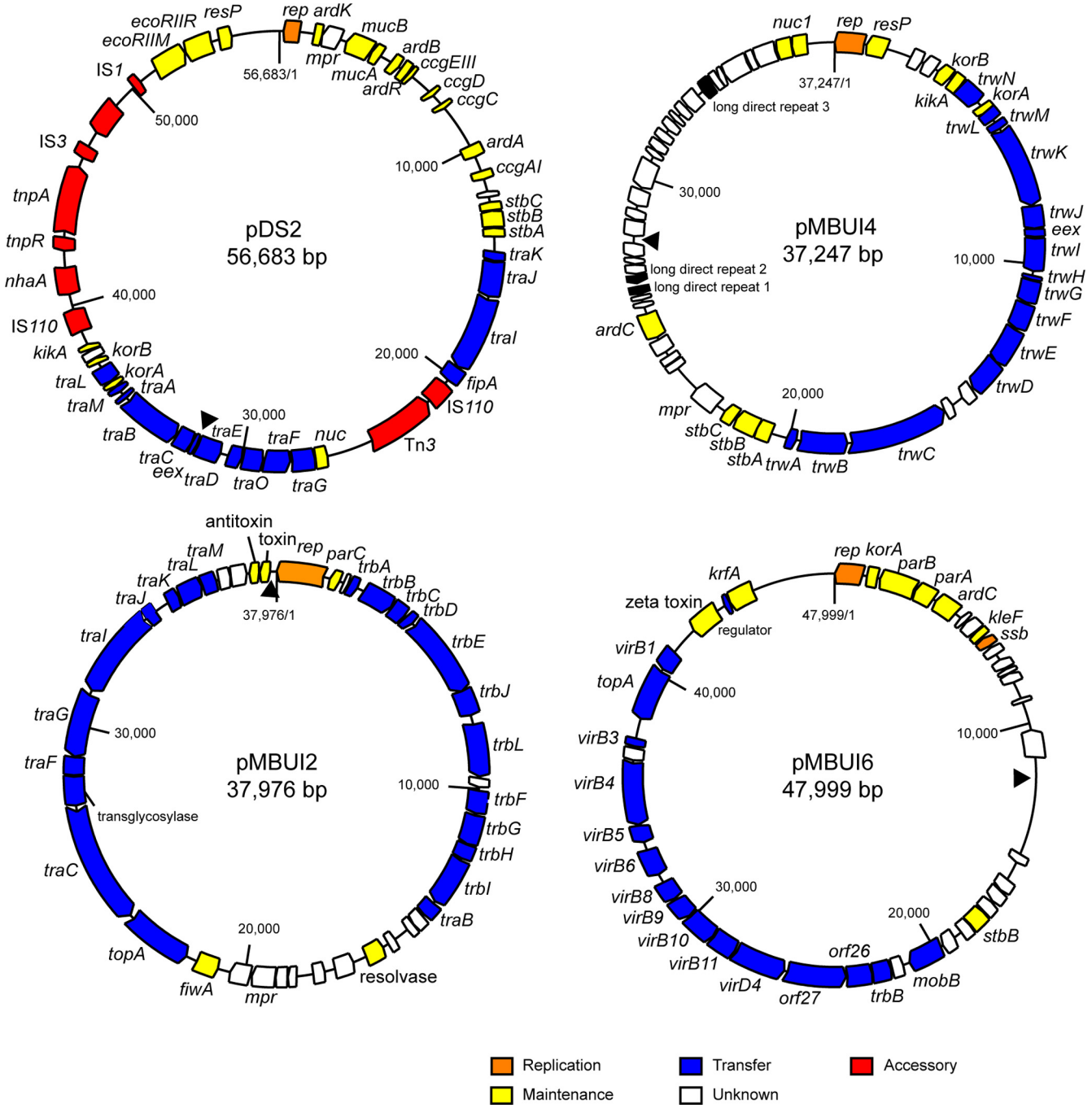

FIG 3 Genetic maps of 4 of the 10 plasmids isolated from Moscow, ID: the IncN plasmid pDS2, the IncW-like plasmid pMBUI4, and two plasmids whose Inc groups are unknown, pMBUI2 and pMBUI6. The gene modules are shown as colored arrows representing different functions (as indicated by the color key). In pMBUI6, the genes named "orf" are most closely related to transfer genes of different BHR plasmids. Genes with the same names on different plasmids are not necessarily homologous due to gene naming conventions for plasmids. Sites of mini-Tn*21* insertion in the tagged plasmids are marked with a filled black triangle.

spect to other transfer genes, and (iii) presence of long direct repeats in intergenic regions (at least 10 direct repeats of 168 bp downstream of *rep* and 4 more elsewhere) (Fig. 3). The primary difference between pMBUI6 and the PromA plasmids is the lack of synteny in the partitioning and regulatory region. Additionally, there is a total of 14 kb of sequence in which no known or conserved hypothetical genes are found; ORFs in these regions may encode host beneficial traits.

Plasmids from Paradise Creek. The two plasmids isolated from Paradise Creek, pMBUI7 and pMBUI8, have typical IncU and IncP-1β backbones, respectively. Plasmid pMBUI7 is similar to the IncU plasmid pMBUI3 from the arboretum pond and also has no known accessory genes (Fig. 2b). Plasmid pMBUI8 is a typical IncP-1β plasmid with accessory genes, one of which putatively encodes triphenylmethane reductase (*tmr*) (Fig. 2a). This gene has 99% identity with *tmr* genes previously found on the

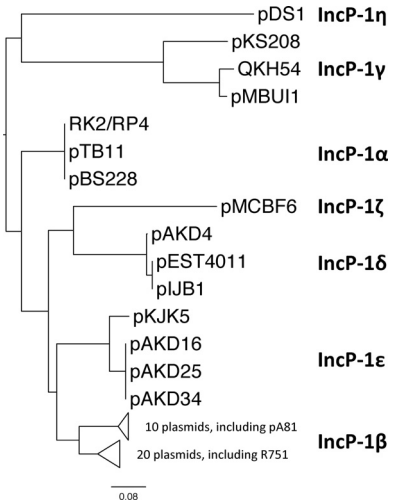

FIG 4 Phylogenetic relationship of pDS1 to other IncP-1 plasmid subgroups. The conserved 3′ end of the *trfA* gene was used to infer the phylogeny of 45 IncP-1 plasmids using the maximum-likelihood method (base frequencies, gamma shape parameter, proportion of invariant sites, and rate matrix [a b c c b a] were estimated from the data). The incompatibility subgroup of each clade is indicated on the right.

IncP-1β plasmids pGNB1, pKV11, pKV29, and pKV36, where it was shown to code for decolorization of crystal violet (55, 56).

Plasmids from Idler's Rest. Of the three plasmids isolated from Idler's Rest Creek, pDS1 and pDS3 are IncP-1 plasmids, and pDS2 belongs to the IncN group. Only pDS2 has identifiable accessory genes.

The genetic organization of pDS1 (Fig. 2a) is typical of IncP-1 plasmids, but the sequence identity to known IncP-1 plasmids is low. IncP-1 plasmids are typically classified into different subgroups based on sequence similarity of backbone genes. For a recent thorough phylogenetic analysis of the five major IncP-1 subgroups, we refer to Sen et al. (45). A phylogeny inferred from the *trfA* gene showed that pDS1 is distinct from other known subgroups including the new IncP-1ζ plasmids isolated from marine biofilms (57) (Fig. 4). Surprisingly, unlike most other IncP-1 plasmids, pDS1 could not be transferred to *P. putida* or *C. pinatubonensis* (Table 2). To verify the placement of pDS1 in the IncP-1 group, an incompatibility assay was performed. The marked plasmid pDS1T was introduced into *E. coli* DH10B(pQKH54) by conjugation, and loss of pQKH54 was monitored when we selected for the incoming plasmid only. Nearly 80% of the clones tested had lost pQKH54 by day 1, in contrast to only a 16% loss of pQKH54 in a separate stability assay. This indicates that pDS1T is incompatible with IncP-1 plasmids and can be classified as an IncP-1 plasmid. We therefore propose the new subgroup IncP-1η for pDS1.

Although the gene content of pDS1 is conserved relative to other IncP-1 plasmids, some features stand out. Its putative *oriT* sequence is considerably divergent from that of other IncP-1 plasmids although a conserved nick site was found. The replication region consists of a replication initiation gene, *trfA*, and an origin of replication, *oriV*. The TrfA protein showed nearly 90% similarity to TrfA2 from IncP-1α plasmid pBS228 (Table 3) and is missing the N terminus found in many other TrfA proteins. The *oriV* has 10 direct repeats or iterons that differ in sequence from those of other IncP-1 plasmids. Like some other IncP-1 plasmids, pDS1 carries putative toxin-antitoxin genes (nt 39259 to 38951 and 39568 to 39269); they bear closest similarity to *higBA*, first identified on a *Proteus vulgaris* plasmid (58). No insertions of mobile elements or known accessory genes were detected on pDS1, suggesting that it is another "backbone-only" plasmid.

Plasmid pDS2 is a typical IncN plasmid (Fig. 3) with quite a narrow host range (Table 2), but it is the first IncN plasmid to be completely devoid of antibiotic resistance genes (59, 60). It has two accessory regions between *fipA* and *nuc* and between *kikA* and the EcoRII type II restriction modification gene complex. The first is composed of an IS*110* family insertion sequence, and the second contains another IS*110*-like element, a Na^+/H^+ antiporter gene (61), a Tn*3*-family transposon with only transposition genes, and an IS*3*-like transposon.

Finally, like pMBUI8 from Paradise Creek, plasmid pDS3 is a typical IncP-1β plasmid (45). Unlike pMBUI8, it has no accessory genes and is thus the fifth plasmid of the 10 examined that seems to exist in nature as a bare plasmid backbone (Fig. 2a).

Comparison of antibiotic resistance profiles of plasmids captured from Paradise Creek and WWTP effluent. The striking lack of antibiotic resistance genes on all 10 plasmids prompted us to perform a comparative analysis of antibiotic resistance profiles. Using the same plasmid capture protocol, a new set of plasmids was captured from Paradise Creek, upstream of the WWTP in Moscow, ID, and from the WWTP effluent. Twelve unique plasmids were chosen from each site based on distinct restriction profiles (Fig. 5). Transconjugants harboring these plasmids were screened for resistance to 14 antibiotics using the disk diffusion method. Only one plasmid from the creek conferred resistance, namely, to trimethoprim and sulfamethoxazole, and two plasmids from the effluent each showed resistance to six antibiotics: aztreonam, tetracycline, kanamycin, polymyxin B, spectinomycin, and ceftazidime. Thus, while the number of resistance determinants was higher among plasmids from the effluent, there was no clear difference in the proportions of resistance plasmids captured from the two sites. Strikingly, all three resistance plasmids were captured in matings with mobilizable plasmid pSU4814, and none was from matings with pBBR1MCS. We tested 16 additional transconjugants from the pSU4814 mating with WWTP effluent, but none conferred resistance. Thus, among the plasmids in raw effluent that can be captured in *E. coli* based on their mobilization potential, only a relatively small proportion (<10%) encoded common antibiotic resistance determinants.

DISCUSSION

One of the central questions in plasmid ecology and evolutionary biology is whether or not plasmids can be considered genetic parasites in their natural bacterial populations. Do they need to confer a benefit to their host to be successfully maintained in microbial communities, or can they survive by spreading horizontally by means of conjugation in spite of often imposing a cost to their hosts? As pointed out more than 35 years ago, for plasmids to persist in the absence of positive selection, high transfer rates must compensate for occasional plasmid loss and cost (14). One way to address this central question is to analyze the accessory gene content of extant pools of plasmids in various environments. Unfortunately, most studies on plasmid diversity have focused on plasmids that were isolated based on a trait of interest, such as virulence or resistance to or catabolism of antimicrobials or pollutants, and from habitats and bacteria that are under strong se-

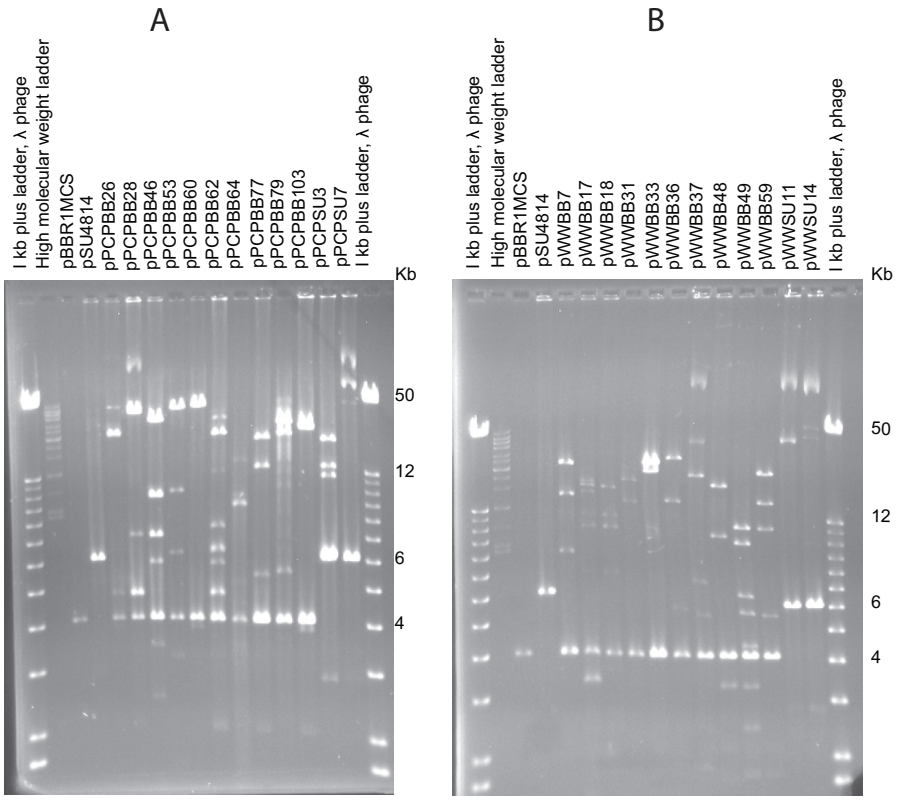

FIG 5 Unique plasmid restriction fragment patterns of captured plasmids generated by EcoRI digestion. (A) Plasmids isolated from Paradise Creek (PCP). (B) Plasmids isolated from Moscow, ID, WWTP (WW). All plasmid DNA samples were completely digested, as indicated by the linearized mobilizable plasmid at 4 kb (pBBR1MCS) or 6.2 kb (pSU4814), and were run on a 0.5% agarose gel for 18 h at 30 V. Plasmids captured using pBBR1MCS contain BB in their names while those captured with pSU4814 contain SU.

lection, such as polluted soils and water bodies, wastewater treatment plants, or known pathogens (18, 62–68). Not surprisingly, such plasmids contain accessory genes that encode the selected host-beneficial traits. Far fewer studies have explored plasmid diversity and gene content using plasmid isolation methods that are not based on a plasmid-encoded accessory phenotype and in environments not known to impose strong selection for accessory genes. While the relative abundance of plasmids or pollution-related accessory genes has been compared between polluted and clean control sites, no studies have so far compared gene contents of plasmids (64, 69–72). The recently developed metagenomics approaches to assess the "plasmidome" or "metamobilome" (73, 74) present one way to assess accessory gene content independent of what the plasmids encode, but obtaining reliable, closed genome sequences for large self-transmissible plasmids is still a challenge (75). Using the triparental mating approach, we showed the presence of diverse self-transmissible plasmids that do not carry known host-beneficial genes in natural environments. The few hypothetical ORFs detected in these plasmids may now become the focus of future research to determine whether they confer heretofore unknown host-beneficial traits.

In the first part of this study, two important observations were made. The first is the high diversity among the 10 plasmids captured from three sites: no less than six incompatibility groups were represented. Studies that selected for plasmids carrying specific traits appear to show noticeably lower plasmid diversity. For example, at least 14 of the 19 BHR plasmids captured from agricultural soils belonged to the IncP-1 group (76, 77). Of the 12 plasmids captured from activated sludge, 10 were members of the IncP-1 group (66). Older studies that characterized plasmids using probes only for known incompatibility groups were often not able to determine the diversity of plasmid groups since several plasmids would not hybridize to any of the probes (27, 46, 78). The mobilizable plasmids used in these studies may have selected for only certain Inc groups. Some plasmids are mobilized rarely by IncP-1 plasmids (31), while others like the IncQ plasmids used in previous studies (pD10 [26], pMOL155 [27], and pMOL187 [77]) may be preferentially mobilized by IncP-1 plasmids. By using different vectors known to be mobilized by different plasmids (30, 32), we captured 10 plasmids from no fewer than six divergent groups, as well as three different IncP-1 subgroups.

The second observation of interest is that in each of the three water bodies sampled around Moscow, ID, at least one self-transmissible plasmid was devoid of identifiable accessory gene regions (pDS1, pDS3, pMBUI1, pMBUI3, and pMBUI7). The two IncU plasmids captured in our study are the only plasmids of that group that lack known accessory genes (6, 48). Although the possibility exists that accessory genes were lost in the process of isolation, it seems unlikely that two such similar plasmids from two different locations would undergo the same loss of genetic material. Moreover, three of the four IncP-1 plasmids did not have any known accessory genes. This result is in stark contrast to the finding that only 2 of 65 previously sequenced IncP-1 plasmids encode no recognizable accessory functions (16, 17). These and other puta-

tive cryptic plasmids described in our study and other studies (8, 28) carry ORFs of unknown function that may, of course, provide unknown benefits to their hosts in their natural environment. The apparent success of these plasmids may also be due to known plasmid backbone genes that confer a host benefit, such as promotion of biofilm formation through the conjugation pilus (79), or to fluctuation selection through temporary benefits to the plasmid host provided by accessory genes that are frequently acquired and lost and not detected at the time of sampling. Whether these plasmids benefit their hosts in unknown ways or are simply persisting as parasitic elements due to efficient horizontal transfer is currently not known and should be investigated in future studies. A first step would be to determine if the ORFs of unknown functions are even expressed and what their role might be.

Five plasmids were identified as having accessory genes, but the functions encoded by these genes are currently mostly unknown. The large clusters of hypothetical genes found on pMBUI2, pMBUI4, and pMBUI6 (Fig. 3) may encode host-beneficial traits, and further work is needed to determine their function. The IncN plasmid, pDS2 has accessory genes but, surprisingly, none that encode antibiotic resistance. IncN plasmids are considered a health risk because they typically confer a variety of antibiotic resistances (59). As far as we know, pDS2 is the first IncN plasmid that is devoid of antibiotic resistance genes. The novel plasmid pMBUI2 and the IncW-like plasmid pMBUI4 encode homologous zinc metalloproteinases that are also encoded on pDS2, pMBUI3, and pMBUI7. These Mpr proteins share 50 to 60% sequence identity among the different Inc groups. They are unrelated to the Mpr proteins that are associated with bacterial pathogenesis (80) but are similar to those encoded by other plasmids such as IncU plasmid RA3 (48). Because the homologous *mpr* genes are consistently found near or within the control regions of diverse Inc groups, we hypothesize that they are backbone and not accessory genes.

The narrow host range of the IncP-1η plasmid pDS1 was unexpected, given that most IncP-1-like plasmids have a broad host range. A preliminary plasmid genomic signature analysis suggested a signature that is quite different from the other IncP-1 plasmids captured in this study (H. Suzuki, personal communication). As pDS1 transferred very well between *E. coli* strains, the plasmid's transfer system seems intact. Since the molecular basis of the apparent narrow host range of this IncP-1η plasmid is of great interest, a detailed host range investigation will be the subject of a future study, combining experimental host range testing with a plasmid genomic signature analysis to assess its potential host range (81).

Because of the predominance of antibiotic resistance plasmids shown in WWTPs around the world (23), we expected to find a large number of such plasmids in the Moscow, ID, WWTP. Indeed, using the biparental plasmid capture method and selection for antibiotic resistance, a plasmid with 11 genes encoding resistance to seven different antibiotics was isolated from this facility several years ago (data not shown). However, using the triparental plasmid capture method in the current study, less than 10% of the plasmids captured from both creek water and raw WWTP effluent conferred resistance to antibiotics. Such plasmids may, of course, occasionally act as vehicles for trafficking antibiotic resistance determinants and at other times persist as parasitic elements. Future studies should evaluate the potential role of these plasmids as *in situ* vectors for drug resistance spread.

Although triparental matings avoid the bias of selecting for specific accessory genes, they introduce other biases. For instance, this method selects for plasmids that efficiently transfer themselves under our specific mating conditions. Therefore, a bias might exist toward smaller conjugative plasmids with fewer accessory genes. However, there is no evidence for or against this conjecture. In previous studies, 50- to 80-kb plasmids with accessory genes were isolated (26, 27, 77). Given that matings were done overnight, it is unlikely that larger plasmids would be selected against because of the slightly longer time required for transfer or because of lower establishment efficiencies. Additionally, this study retrieved only plasmids that efficiently transferred the mobilizable plasmid pBBR1MCS or pSU4814 and stably replicated in the rapidly growing *E. coli* recipients. Strikingly, the only IncW-like plasmid isolated in this study was captured with pSU4814, a vector known to be mobilized well by the IncW transfer system (F. de la Cruz, personal communication). Thus, although the triparental mating approach cannot be used to infer relative abundance of cryptic plasmids or the plasmid richness in specific habitats because of these biases, our results clearly show the presence of diverse cryptic plasmids in natural freshwater communities.

Our study is a first attempt at assessing the occurrence of cryptic self-transmissible plasmids in various freshwater habitats. Future work will include whole-genome sequencing of much larger sets of plasmids captured in identical ways from different sites. Metagenomic approaches will also shed more light on the diversity of plasmids than triparental plasmid capture methods, but obtaining correctly closed genomes of large plasmids like the ones described in this study remains a challenge for complex communities. Thus, in the future a combination of different plasmid isolation and sequencing methods will be needed to improve our view of the diversity and accessory gene content of these important mobile genetic elements in the horizontal gene pool.

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