

Plasmid Donor Affects Host Range of Promiscuous IncP-1 β Plasmid pB10 in an Activated-Sludge Microbial Community

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Horizontal transfer of multiresistance plasmids in the environment contributes to the growing problem of drug-resistant pathogens. Even though the plasmid host cell is the primary environment in which the plasmid functions, possible effects of the plasmid donor on the range of bacteria to which plasmids spread in microbial communities have not been investigated. In this study we show that the host range of a broad-host-range plasmid within an activated-sludge microbial community was influenced by the donor strain and that various mating conditions and isolation strategies increased the diversity of transconjugants detected. To detect transconjugants, the plasmid pB10 was marked with *lacp-rfp*, while *rfp* expression was repressed in the donors by chromosomal *lacI*^L. The phylogeny of 306 transconjugants obtained was determined by analysis of partial 16S rRNA gene sequences. The transconjugants belonged to 15 genera of the α - and γ -Proteobacteria. The phylogenetic diversity of transconjugants obtained in separate matings with donors *Pseudomonas putida* SM1443, *Ralstonia eutropha* JMP228, and *Sinorhizobium meliloti* RM1021 was significantly different. For example, the transconjugants obtained after matings in sludge with *S. meliloti* RM1021 included eight genera that were not represented among the transconjugants obtained with the other two donors. Our results indicate that the spectrum of hosts to which a promiscuous plasmid transfers in a microbial community can be strongly influenced by the donor from which it transfers.

Broad-host-range (BHR) plasmids have contributed to the rapid spread of antibiotic resistance determinants among bacteria (7, 48), creating an “epidemic of microbial resistance” (15). This horizontal mobile gene pool becomes advantageous to a bacterial community when it is exposed to strong selective pressure from antibiotics, such as in hospitals and animal husbandry (7). Input of specialized wastewaters originating from these sources into a water treatment plant can introduce high amounts of antibiotics and antibiotic-resistant bacteria (39), in which resistance can be mediated by (multi)resistance plasmids (19). The spread of antibiotic resistance genes is greatly enhanced by conjugative transfer combined with increasing selective pressure for resistant organisms (47). Such plasmids can be directly transferred not only to pathogenic strains but also to human commensal gut bacteria that could later serve as a gene reservoir for invading pathogens (47).

The BHR plasmids of gram-negative bacteria are known to have the ability to promote conjugation and ensure vegetative replication in a wide variety of taxonomically distantly related hosts, belonging to the α -, β -, and γ -Proteobacteria (26, 48). To understand the spread of antibiotic resistance BHR plasmids in the environment, it is important to identify the extent to which antibiotic resistance can be transferred to different members of natural microbial communities. While several studies have identified the transconjugants formed in various

indigenous communities after uptake of BHR catabolic plasmids (3, 9, 12, 17, 18, 32, 35), little information (13, 36, 43) is available on the phylogenetic diversity of transconjugants that form after the introduction of a BHR antibiotic resistance plasmid in a microbial community. Moreover, no studies have explored the influence that phylogenetically distinct donors might have on the kinds of transconjugants obtained.

Although the process of conjugative plasmid transfer is largely determined by the plasmid-encoded transfer genes, it also relies in part on the characteristics of the host in which the plasmid resides. Nevertheless, very few studies have examined the possible effects of the plasmid host on plasmid transfer properties. It has been suggested that host factors may influence conjugative plasmid transfer by regulating the transcription of transfer genes and that the extent of this influence might be indicative of a broad or narrow host range (25). Alternatively, although little is known about recipient recognition and the nature of the conjugative pore (25, 27), it is possible that the plasmid donor has an effect on these processes through structural interactions with the recipient. Thus, although common wisdom is to consider plasmid host range as a purely plasmid-defined property, there is sufficient reason to investigate whether the plasmid donor could affect the host range of a BHR plasmid in a natural microbial community.

We sought to explore the range of transconjugants that received the multiresistance plasmid pB10 after its introduction into an activated-sludge microbial community, and we specifically tested the hypothesis that the donor strain delivering the plasmid affects the plasmid host range. pB10 is a BHR, multiresistance IncP-1 β plasmid isolated from activated sludge

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TABLE 1. Strains and plasmids

Strain or plasmid	Species	Characteristics	Source or reference
Strains			
Mv1190	<i>Escherichia coli</i>	$\Delta(lac-proAB) thi supE (\lambda pir) \Delta(srl-recA)306::Tn10(F'::traD362pro$ $ABlacI^qZ\Delta M15)$	22
K-12	<i>Escherichia coli</i> MG1655	$F^- \lambda mbda^- ilvG^+ rfb^-50 rph^{-1}$	ATCC 47076
HB101	<i>Escherichia coli</i>	K-12/B hybrid; $Sm^r recA thi pro leu hsdRM^+$	4
UWC1	<i>Pseudomonas putida</i>	Rif ^r	30
SM1443	<i>Pseudomonas putida</i>	Rif ^r ; KT2442 with mini-Tn5- <i>lacI</i> ^q cassette inserted into the chromosome	5
JMP228	<i>Ralstonia eutropha</i>	Rif ^r	52
JMP228n	<i>Ralstonia eutropha</i>	Rif ^r , Nal ^r	52
JMP228:: <i>lacI</i> ^q	<i>Ralstonia eutropha</i>	Rif ^r ; JMP228 with mini-Tn5- <i>lacI</i> ^q cassette inserted into the chromosome	This study
AE815	<i>Ralstonia metallidurans</i>	Rif ^r	51
RM1021	<i>Sinorhizobium meliloti</i>	Rif ^r	8
RM1021:: <i>lacI</i> ^q	<i>Sinorhizobium meliloti</i>	Rif ^r ; RM1021 with mini-Tn5- <i>lacI</i> ^q cassette inserted into the chromosome	This study
EEZ23	<i>Delftia acidovorans</i>	Rif ^r Sm^r Km^r	38
Plasmids			
pSM1833		pUTKm $P_{A1-04/03}::rfp$ cassette	21
RK600		ColE1 oriV; RP4tra ⁺ RP4oriT; Cm^r ; helper in triparental matings	24
pSM1435		NotI fragment (<i>lacI</i> ^q) of pSM1431 <i>lacI</i> ^q fragment cloned into pUC18Not (Sca-SalI), inserted into pUTKm	5
pB10		Tc ^r Sm^r Amx^r Sul^r Hg ^r	44
pB10:: <i>rfp</i>		Tc ^r Sm^r Amx^r Sul^r Hg ^r ; mini-Tn5-Km- $P_{A1-04/03}::rfp$ cassette	This study

of a wastewater treatment plant (10, 44). Our data show that the spread of pB10 in an activated-sludge community was influenced by the donor strain from which the plasmid was transferred and that the diversity of transconjugants detected could be increased by using multiple mating conditions and isolation strategies.

(A preliminary report of this work was presented at the 2004 Plasmid Biology Meeting, Corfu, Greece, 15 to 21 September 2004 [7a].)

MATERIALS AND METHODS

Media. Luria-Bertani medium and M9 minimal medium were prepared according to the methods of Sambrook and Russell (41), but M9 medium was supplemented with 5 ml/liter stock E (11). M9 medium and DAB medium (11) were supplemented with succinate, acetate, and citrate (SAC), each at a concentration of 2 g/liter. For all solid media, 15 g/liter of agar was added. Difco R2A agar (BD, Franklin Lakes, NJ) was prepared according to the manufacturer's instructions. Cycloheximide (300 mg/liter) was used as an antifungal agent. Unless otherwise stated, antibiotics were used at the following concentrations: 10 mg/liter tetracycline (Tc), 50 mg/liter kanamycin (Km), 50 mg/liter streptomycin (Sm), 100 mg/liter rifampin (Rif), 100 mg/liter nalidixic acid (Nal).

Bacterial strains and plasmids. The relevant characteristics of the bacterial strains and plasmids are listed in Table 1. The 64.5-kb plasmid pB10 was isolated from the bacterial community of a wastewater treatment plant in Germany (10) and has been completely sequenced and annotated (44). It has been identified as a self-transmissible, BHR IncP- β plasmid that mediates resistance against the antibiotics tetracycline, amoxicillin, sulfonamide, and streptomycin and against mercury ions.

The insertion of the mini-Tn5- $P_{A1-04/03}::rfp$ cassette into pB10 was performed in two steps (21). First, a triparental mating was performed in which the helper plasmid pRK600, present in *Escherichia coli* HB101, mobilized the delivery plasmid pSM1833 from the donor, *E. coli* Mv1190, into the recipient, *Pseudomonas putida* UWC1 (pB10). Selection in LB broth-RifKmTc resulted in *P. putida* UWC1 derivatives with the $P_{A1-04/03}::rfp$ cassette insertion either in the chromosome or in pB10. To obtain *rfp*-marked plasmids from this mixture, it was mated with *Ralstonia eutropha* JMP228n. Selection on LB-NalKmTc agar plates resulted in *R. eutropha* JMP228n clones carrying pB10 with the $P_{A1-04/03}::rfp$ cassette inserted. One clone, designated *R. eutropha* JMP228n(pB10::*rfp*), was

mated with *E. coli* K-12 to obtain *E. coli* K-12(pB10::*rfp*) after selection on LB-KmTc agar plates at 43°C. From this strain, the plasmid was extracted using the QIAprep Spin Miniprep kit according to the manufacturer's instructions (QIAGEN Inc., Valencia, CA) and digested with PstI (Invitrogen, Carlsbad, CA). Samples were loaded on a 0.8% agarose gel and run for approximately 16.5 h at 30 V. The place of the *rfp* cassette insertion was determined by sequencing the flanking plasmid region of the cassette with the primer Tn5out (5'-CTCGAATTCGGCCTAGGCGG-3'), which annealed to the cassette border, directed towards the flanking plasmid DNA region.

To insert the *lacI*^q gene into the chromosome of *Ralstonia eutropha* JMP228 and *Sinorhizobium meliloti* RM1021, a triparental mating was performed with the donor *E. coli* CC118 λ pir carrying the *lacI*^q delivery plasmid pSM1435 and HB101 carrying the helper plasmid pRK600 (5). After selection on M9-SAC supplemented with 300 mg/liter Km and 50 mg/liter Rif, one clone of each mating was picked and designated *R. eutropha* JMP228::*lacI*^q and *S. meliloti* RM1021::*lacI*^q, respectively. These strains, as well as *Pseudomonas putida* SM1443, which already harbors the *lacI*^q cassette (5), acquired pB10::*rfp* through mating with *E. coli* K-12(pB10::*rfp*) and selection on LB-KmTcRif (50 mg/liter) agar plates.

Transfer properties of pB10::*rfp*. To investigate the transferability of pB10::*rfp*, it was transferred to *R. eutropha* JMP228, *Ralstonia metallidurans* AE815, *P. putida* UWC1, *S. meliloti* RM1021, and *Delftia acidovorans* EEZ23, and *rfp* expression was monitored. Also, triplicate plate matings were performed with donors *E. coli* K-12(pB10) and *E. coli* K-12(pB10::*rfp*) and recipients *P. putida* SM1443, *R. eutropha* JMP228, and *S. meliloti* RM102, where the amounts of transconjugants, donors, and recipients were determined by plating onto LB-TcRif, LB-Tc, and LB-Rif agar plates, respectively.

Transfer experiments in activated sludge. A fresh grab sample of activated sludge was collected from the aeration tank of the municipal wastewater treatment plant of Moscow, Idaho. The experimental protocol is depicted in Fig. 1. Overnight cultures (ca. 10^9 CFU/ml) of SM1443(pB10::*rfp*), JMP228::*lacI*^q(pB10::*rfp*), and RM1021::*lacI*^q(pB10::*rfp*), grown in LB broth-TcKmRif, were washed in saline and 100 μ l was separately added to 10 ml of activated sludge, in triplicate. The negative control consisted of sludge with 100 μ l sterile saline (in triplicate). After vortexing, 100 μ l from each "liquid mating" and negative control replicate was spotted onto DAB-SAC and R2A agar plates ("plate matings"). The liquid matings were incubated on a rotary shaker (200 rpm), and the plate matings were incubated stationary, both at 30°C for ca. 20 h.

From the liquid matings and the liquid negative controls, 1 ml of each replicate was transferred to an Eppendorf tube. The biomass from the plate matings and the plate negative controls was scraped off the plates, suspended in 1 ml of saline,

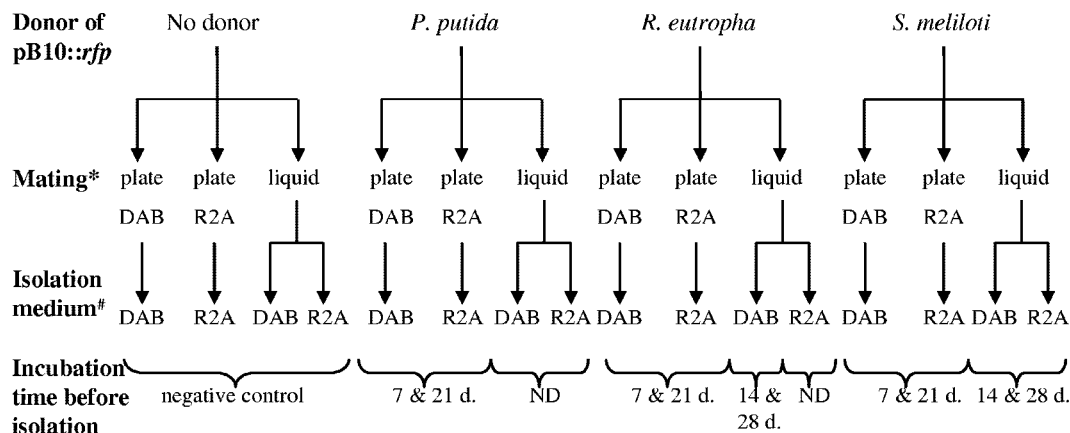


FIG. 1. Overview of the experimental conditions for obtaining transconjugants from the activated-sludge sample. *, all matings were performed in triplicate. #, all media were supplemented with 10 mg/liter tetracycline and 50 mg/liter streptomycin. ND, no transconjugants were detected.

and vortexed rigorously. These samples were subsequently homogenized by pulling up and down in a syringe needle (21 gauge, 1.5 in.). Several dilutions (10^0 to 10^{-2}) of the mating mixtures and negative controls were plated onto selective plates (R2A-TcSm and DAB-TcSm) (Fig. 1). All plates were incubated at 20°C for up to 28 days (Fig. 1).

Isolation of transconjugants. Transconjugant colonies were detected on the selective agar media based on a red fluorescent phenotype when exposed to blue light (400 to 500 nm) and viewed through an amber filter (Dark Reader; Clare Chemical Research, Denver, CO). At the first time point of isolation (7 days for plate matings and 14 days for liquid matings), a sector on the plate was marked wherein exactly 10 red fluorescent colonies were present (in some cases, plates contained less than 10 colonies), which were picked and their positions marked. Subsequently, at the second isolation (21 days for plate matings and 28 days for liquid matings), a maximum of five newly arisen colonies in that same sector were picked, which resulted in a maximum of 15 transconjugants per replicate. Colonies were purified by repeated restreaking on selective medium until pure red fluorescent colonies were obtained. From each plate, one colony was inoculated in liquid selective medium (DAB-SAC-TcSm or R2A-TcSm) and, after incubation at 30°C, an aliquot was archived at -80°C while the rest was centrifuged to obtain a cell pellet, which was stored at -20°C .

Detailed information on the isolation of each transconjugant can be found on the website <http://www.sci.uidaho.edu/biosci/labs/top/research/index.html>.

Characterization of transconjugants. The cell pellet was used to obtain genomic DNA. After an enzymatic lysis (37), the DNA was purified with the MoBio UltraClean 15 kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's specifications and resuspended in 12 μl Tris-EDTA buffer. This DNA was used in a PCR (Peltier thermal cycler 200; MJ Research, Reno, NV) (3:30 min at 95°C ; 30 cycles of 1:10 min at 94°C , 40 s at 56°C , and 2:10 min at 72°C ; and 6 min at 72°C) to amplify the 16S rRNA gene, under the following conditions: 36.35 μl deionized-distilled H_2O (ddH $_2\text{O}$), 5 μl 10 $\times$ buffer B, 3 μl MgCl_2 (50 mM), and 0.25 μl *Taq* DNA polymerase (5 U/ μl) (all from Fisher, Fair Lawn, NJ); 2.5 μl dimethyl sulfoxide, 0.4 μl deoxynucleoside triphosphate (10 mM), 1 μl primer fD1, and 1 μl primer rD1 (10 μM) (55); and 0.5 μl of DNA template. All reactions were run on a 1% agarose gel to verify the presence of a ca. 1,500-bp PCR product. Of this product, 0.5 μl was used as template for the cycle sequencing reaction (5 min at 95°C and 25 cycles of 10 s at 96°C , 5 s at 50°C , and 4 min at 60°C), the mixture for which contained 7.5 μl ddH $_2\text{O}$, 2.0 μl BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA), and 0.5 μl of primer R926 (3.2 μM) (33), and which was performed in a 96-well reaction plate. The sequencing products were cleaned by ethanol-EDTA precipitation (37) and resuspended in 10 μl deionized Hi-Di formamide. After subjecting the samples to 95°C for 5 min and placing them on ice for 5 to 10 min, the samples were run on a 3730 DNA analyzer (Applied Biosystems).

The 16S rRNA gene sequences were analyzed in an automated way using an rRNA analysis pipeline (HiSTA; available at <http://www.ibest.uidaho.edu/tools>). This pipeline first identified high-quality sequences (less than 3% uncalled bases) greater than 500 bp. The sequences were then analyzed sequentially by BLAST (1) to search for the 25 most similar sequences, at least 1,200 bp long, among eubacterial type strains in the Ribosomal Database Project (RDP) (6). The RDP sequence for the closest relative of each input sequence was included in the

subsequent analyses. All of the input sequences used in the BLAST search, their closest relatives, and a set of 39 bacterial rRNA sequences representing a broad range of eubacterial sequences, plus a single *Archaea* sequence, were aligned using ClustalW (50). This alignment is available at the website <http://www.sci.uidaho.edu/biosci/labs/top/research/index.html>. At this point, the region of the alignment that was used to cluster the sequences was determined. Genetic distances for this region were calculated by the Jukes and Cantor method (23), and the sequences were clustered based upon these distances using the neighbor joining method (40) as implemented in the GCG (Accelrys Inc., San Diego, CA) programs "distances" and "growtree" to construct a phylogenetic tree. The resulting distance matrix was used to compute the following statistics on groups of sequences that had the same closest relative in the RDP type strain database: the mean and standard deviation of the sequence divergence within such a group and the mean and standard deviation of the sequence divergence of all sequences in that group compared to their common closest RDP type strain relative. The program that computes these statistics (Statgen) is available at <http://www.ibest.uidaho.edu/tools>.

Detection of pB10::rfp in transconjugants. To confirm the presence of the plasmid pB10::rfp in the transconjugants, a primer pair (F18836, 5'-TCATGGG TTCCTCCGGTCAAT-3'; R18900, 5'-CAATAACGACCCGGCCAAGA-3') was designed to amplify a pB10-specific 96-bp product by PCR (5 min at 94°C ; 35 cycles of 45 s at 94°C , 15 s at 55°C , and 30 s at 72°C ; 5 min at 72°C) in the following mixtures: 19.625 μl ddH $_2\text{O}$, 2.5 μl 10 $\times$ buffer B, 0.75 μl MgCl_2 (50 mM), and 0.125 μl *Taq* DNA polymerase (5 U/ μl) (all from Fisher, Fair Lawn, NJ), 0.5 μl deoxynucleoside triphosphate (10 mM), 0.75 μl of each primer (10 mM), and 1 μl of DNA template. The presence of an amplification product of the correct size (ca. 100 bp) was verified on a 2% agarose gel.

Statistical test to detect group differences. To examine the effects of different treatments on the diversity of observed transconjugants, a statistical test based on the previously described F_{ST} test (29) was employed. 16S rRNA gene sequences were aligned with Clustal X v1.83 (49) using default settings. A diversity index (θ) of a group of aligned sequences was calculated as the number of base pair differences between two sequences, divided by the number of base pairs considered, averaged over all unique pairs of sequences in that group. When either one of two sequences being compared had an ambiguous base or a gap at a particular base position, then that base position was ignored in the calculations. For each treatment (for example, donor) being tested, a test statistic F_{ST} was calculated as $(\theta_T - \theta_W)/\theta_W$, with θ_T being the diversity index for all sequences and θ_W the average of the diversity indices of the groups defined by the different levels of the treatment considered. Statistical significance of F_{ST} was evaluated by randomly assigning sequences across treatment groups (donor in the example) 1,000 times and recalculating F_{ST} each time. This way, the distribution of F_{ST} under the null hypothesis (in the example, no effect of donor on transconjugant 16S rRNA gene diversity) was determined, which allowed for the calculation of a two-sided P value by determining the position of our observed F_{ST} value in that distribution (16). When the observed F_{ST} value fell in one of the 0.5% tails of the distribution generated under the null hypothesis ($P \leq 0.01$), we rejected the null hypothesis and concluded that the treatment had an effect on transconjugant diversity.

We improved the original test (29) for application to our data set. When testing for one particular effect (for example, donor), we accounted for under-

lying effects of other experimental factors (for example, plate versus liquid mating) by introducing a restricted permutation scheme (blocking) (16). When permuting a list of sequences, those sequences that belonged to a particular treatment combination (for example *Pseudomonas* donor, liquid mating in DAB medium for 14 days incubation, replicate 1) were always kept together and reassigned as a group to the same treatment combination under the same or another level of the treatment being tested (for example, donor). Permutation was allowed to treatments that were performed in the experiment but did not generate transconjugants. Finally, we had observed that within a number of treatment combinations, the different replicate experiments resulted in a statistically different transconjugant diversity (a "jackpot" effect). To correct for this effect, the replicate labels assigned to each treatment combination were randomly shuffled (as blocks) before each permutation run. The programming code can be found at the website <http://www.sci.uidaho.edu/biosci/labs/top/research/index.html>.

Nucleotide sequence accession numbers. The partial 16S rRNA gene sequences of the transconjugants were deposited in GenBank under accession numbers AY972165 to AY972470.

RESULTS

Construction of plasmid and donor strains. To monitor the transfer of the plasmid pB10 to indigenous sludge bacteria, the *rfp* gene under the control of a strong *lac* promoter was inserted into pB10. The *rfp* gene was repressed in the donor strains that harbored the *lacI^q* repressor and became derepressed after transfer to recipient strains. The PstI restriction pattern of pB10:*rfp* was not visibly different from that of the original plasmid pB10. The location of the *rfp* cassette insertion was determined to be at position 21154 of the annotated plasmid map (44), located within *traC*, which is not necessary for transfer to most recipients (26). Expression of the *rfp* gene on pB10:*rfp* was confirmed in *P. putida* UWC1, *R. metalludurans* AE815, *R. eutropha* JMP228, *D. acidovorans* EEZ23, and *S. meliloti* RM1021. As expected, *P. putida* SM1443(pB10:*rfp*), *R. eutropha* JMP228:*lacI^q*(pB10:*rfp*), and *S. meliloti* RM1021:*lacI^q*(pB10:*rfp*) did not exhibit red fluorescence, while the presence of the plasmid and functionality of the *rfp* marker were confirmed by transferring the plasmid to *E. coli* K-12 and observing red fluorescence.

To investigate a possible effect of the *rfp* insertion on the transferability of pB10, plate matings were performed with *E. coli* K-12 donors harboring either pB10 or pB10:*rfp* and *P. putida* SM1443, *R. eutropha* JMP228, and *S. meliloti* RM1021 recipients. No significant differences in plasmid transfer frequencies (expressed as transconjugants/recipients) were detected for transfer to JMP228 and RM1021. The transfer frequency of pB10:*rfp* to SM1443 was slightly but significantly lower than for pB10 (7.8×10^{-3} versus 5.7×10^{-1} ; $P = 0.0038$; *t* test). Because the insertion of *rfp* in *traC* had a small to negligible effect on the transfer frequencies using these recipients, this marked plasmid was retained for this study.

Transfer of pB10:*rfp* in activated-sludge samples. To investigate the effect of the donor strain on the host range of plasmid pB10:*rfp* in an activated-sludge community, *P. putida* SM1443(pB10:*rfp*), *R. eutropha* JMP228:*lacI^q*(pB10:*rfp*), and *S. meliloti* RM1021:*lacI^q*(pB10:*rfp*) were introduced separately into a fresh sludge sample. The *rfp*-positive transconjugant colonies were easily recognized through their red fluorescence against the background growth of the donor strain and indigenous resistant sludge bacteria (ca. 10^2 CFU for liquid matings to 10^4 CFU for plate matings on 10^{-1} dilution). No *rfp*-positive colonies were detected in the negative controls,

where ca. 2×10^3 to 3×10^4 CFU/ml sludge bacteria grew on the selective media. To determine whether mating and transconjugant isolation conditions could influence the kinds of transconjugants detected, the matings were done by overnight incubation of conjugation mixtures on either R2A or DAB-SAC agar plates (plate matings) or in shaking activated-sludge samples (liquid matings) (Fig. 1), and transconjugant colonies were isolated after different time intervals. A total of 334 transconjugants were isolated from the entire set of mating and isolation procedures with the three donor strains, as outlined in Fig. 1. With two exceptions (Fig. 1, ND), all procedures yielded transconjugants.

General diversity of transconjugants. A total of 306 transconjugants were classified based on ca. 600-bp single-strand reads of their 16S rRNA gene sequences. Twenty-eight clones could not be identified, mostly due to clone purification or DNA isolation problems. Table 2 shows the list of closest relatives among the type strains in the RDP database, the number of transconjugant clones related to each RDP sequence, the sequence variation, and the source of the transconjugants. The transconjugants belonged to either the γ -*Proteobacteria* *Pseudomonas* or *Stenotrophomonas* or to 1 of 13 genera of the α -*Proteobacteria*. For each genus, the presence of pB10:*rfp* was confirmed in one representative clone by a pB10-specific PCR assay. Comparison of the 16S rRNA sequences of the transconjugants only to RDP type strains, rather than to all sequences available, allowed a more reliable transconjugant characterization, with 88% of the isolates exhibiting a sequence similarity of at least 97% with one of the type strains. To our knowledge, the transconjugants related to *Phenylobacterium*, *Aminobacter*, *Mesorhizobium*, *Defluviobacter*, *Caulobacter*, *Methylobacterium*, *Bosea*, and *Sphingomonas* have not been previously identified as hosts for BHR plasmids (3, 9, 17, 18, 32, 35, 36).

Effect of the plasmid donor on diversity of transconjugants. We tested the hypothesis that the plasmid donor has an effect on the diversity of transconjugants in two ways (Fig. 2A; Table 2). First, the relative diversity at the genus level, expressed as the ratio of the number of different genera of transconjugants to the total number of transconjugants obtained with a specific donor, was compared between donors and shown to be clearly different. *S. meliloti* had the highest ratio (0.10), indicating this group of transconjugants was the most diverse, followed by *P. putida* (0.07) and *R. eutropha* (0.05). The most striking donor-dependent observation was the detection of eight unique genera of transconjugants when *Sinorhizobium meliloti* was used as plasmid donor (Fig. 2A). Second, a statistical test was employed to determine if these observations at the genus level were the result of a significant effect of the plasmid donor on the diversity of transconjugants as defined by their 16S rRNA gene sequence divergence. Using the 16S rRNA gene sequences of all transconjugants, grouped according to the donor used to obtain them, the averages of the three subgroup diversity indices (θ_w) and one overall group diversity index were calculated (θ_T), resulting in an F_{ST} value of -0.0082 . This F_{ST} value calculated from our observed data set fell outside of the range of F_{ST} values of 1,000 data sets obtained through permutation between donors, under the null hypothesis that the donor does not affect the transconjugant diversity. Therefore, we rejected this null hypothesis ($P < 0.002$) and concluded that

TABLE 2. Identification of transconjugants from the activated-sludge community that received pB10::rfp

Closest relative to transconjugant in RDP type strain database	No. of clones related to type strain	Avg % sequence divergence (±SD)		No. of clones isolated per donor			No. of clones isolated per mating and selection protocol			
		To type strain	Within group	<i>P. putida</i>	<i>R. eutropha</i>	<i>S. meliloti</i>	Plate		Liquid	
							R2A	DAB	R2A	DAB
α-Proteobacteria										
<i>Agrobacterium sanguineum</i> (T); IAM 12620	2	1.64 ± 0.48	NC ^a	0	0	2	0	0	0	2 ^{*b}
Alpha proteobacterium 34635 (T) (<i>Bosea</i> sp.) ^c	5	1.53 ± 0.32	0.56 ± 0.48	0	0	5	0	0	2*	3*
<i>Aminobacter aminovorans</i> (T); DSM7048T	3	0.69 ± 0.00	0.00 ± 0.00	0	0	3	0	0	2	1*
<i>Caulobacter vibrioides</i> (T); CB2	1	NC	NC	0	0	1	0	0	1*	0
<i>Defluviobacter lusitiae</i> (T); DSM11099T	1	NC	NC	0	0	1	0	0	1	0
<i>Ensifer adhaerens</i> (T); ATCC 33212	21	1.26 ± 0.88	1.19 ± 0.89	8	2	11	3	10	0	8
<i>Mesorhizobium chacoense</i> (T); PR5	1	NC	NC	0	0	1	0	0	1	0
<i>Methylobacterium organophilum</i> (T); JCM2833	1	NC	NC	0	0	1	0	0	0	1*
<i>Ochrobactrum grignonense</i> (T); OgA9a	13	1.00 ± 0.68	0.97 ± 0.78	5	2	6	8	3	2	0
<i>Ochrobactrum tritici</i> (T); SCII24	37	0.18 ± 0.21	0.20 ± 0.03	16	15	6	30	6	0	1
<i>Phenylobacterium immobile</i> (T); E (T)	1	NC	NC	0	0	1	0	0	1*	0
<i>Rhizobium gallicum</i> (T); R602sp.	25	2.3 ± 0.22	0.22 ± 0.32	4	10	11	4	6	1	14
<i>Rhizobium huautilense</i> (T); SO2	2	2.37 ± 0.19	NC	0	0	2	0	0	1	1
<i>Rhizobium radiobacter</i> (T); ATCC19358	14	0.07 ± 0.16	0.12 ± 0.21	2	8	4	0	8	0	6
<i>Rhizobium yanglingense</i> (T); SH22623	34	2.12 ± 0.17	0.20 ± 0.26	1	18	15	1	9	6	18
<i>Sinorhizobium morelense</i> (T); Lc04	1	NC	NC	0	1	0	0	1*	0	0
<i>Sphingomonas</i> sp. (T); BN6	1	NC	NC	0	0	1	0	0	1	0
<i>Sphingomonas yanoikuyae</i> (T); GIFU9882	5	0.26 ± 0.24	0.38 ± 0.25	0	3	2	0	5	0	0
γ-Proteobacteria										
<i>Pseudomonas citronellolis</i> (T); DSM 50332T	5	0.05 ± 0.1	0.11 ± 0.13	0	2	3	0	5	0	0
<i>Pseudomonas fragi</i> (T); ATCC 4973	5	1.02 ± 0.57	1.63 ± 0.51	0	1	4	0	5	0	0
<i>Pseudomonas frederiksbergensis</i> (T); JAJ28	2	0.88 ± 0.02	NC	1	1	0	1	1	0	0
<i>Pseudomonas fulva</i> (T); IAM1529	1	NC	NC	0	1	0	1	0	0	0
<i>Pseudomonas gessardii</i> (T); CIP 105469	7	1.47 ± 0.00	0.00 ± 0.00	5	2	0	7	0	0	0
<i>Pseudomonas graminis</i> (T); DSM 11363	1	NC	NC	0	1	0	0	1	0	0
<i>Pseudomonas kilonensis</i> (T); 520–20	4	1.6 ± 0.27	0.93 ± 0.79	1	1	2	3	1	0	0
<i>Pseudomonas koreensis</i> (T); Ps 9–14	15	0.73 ± 0.31	0.38 ± 0.44	2	9	4	10	5	0	0
<i>Pseudomonas migulae</i> (T); CIP 105470	23	1.18 ± 0.12	0.19 ± 0.47	3	8	12	23	0	0	0
<i>Pseudomonas monteilii</i> (T); CIP 104883	14	0.51 ± 0.29	0.36 ± 0.33	1	8	5	4	10	0	0
<i>Pseudomonas nitroreducens</i> (T); IAM1439	17	1.19 ± 0.16	0.16 ± 0.18	0	8	9	2	15	0	0
<i>Pseudomonas plecoglossicida</i> (T); FPC951	18	1.65 ± 1.39	1.34 ± 1.87	12	5	1	13	5	0	0
<i>Pseudomonas putida</i> (T); DSM 291T	3	0.00 ± 0.00	0.00 ± 0.00	1	0	2	3	0	0	0
<i>Pseudomonas putida</i> (T); IFO 14164	9	0.29 ± 0.10	0.20 ± 0.38	0	2	7	5	4	0	0
<i>Pseudomonas syringae</i> (T); LMG 1247t1T	1	NC	NC	0	0	1	0	1	0	0
<i>Pseudomonas umsongensis</i> (T); Ps 3–10	7	2.11 ± 1.76	2.25 ± 1.72	2	2	3	3	4	0	0
<i>Pseudomonas veronii</i> (T); CIP 104663	3	0.22 ± 0.00	0.00 ± 0.00	0	2	1	2	1	0	0
<i>Pseudomonas viridiflava</i> (T); LMG 2352T	1	NC	NC	1	0	0	1	0	0	0
<i>Stenotrophomonas maltophilia</i> (T); ATCC 13637T	1	NC	NC	1	0	0	1	0	0	0
<i>Stenotrophomonas maltophilia</i> (T); LMG 958-T	1	NC	NC	1	0	0	1	0	0	0
Total	306			67	112	127	126	106	19	55

^a NC, not calculated.^b *, only isolated at second isolation round.^c Type strain of α -proteobacterium 34635 (T) is classified in the RDP database under the genus *Bosea*.

there was a clear effect of the plasmid donor on the diversity of transconjugants.

Since it may be argued that the transconjugant diversity per donor subgroup could be correlated with the size of the transconjugant pool obtained for each donor, we examined this possibility by calculating the slope of a linear regression performed on the diversity indices θ for each donor subgroup as a function of the number of transconjugants in that subgroup (slope = -0.0001479). Next, its position was determined in the

distribution of slopes under the null hypothesis (slope is not different from zero), obtained through permuting the data as described before. We were unable to reject the null hypothesis ($P = 0.498$; 1,000 permutations), indicating that the differences in the transconjugant numbers for each donor did not correlate with the transconjugant diversity measures.

Effect of different mating and isolation conditions on the diversity of transconjugants. Different mating conditions (agar plate versus liquid sludge) and isolation strategies (medium

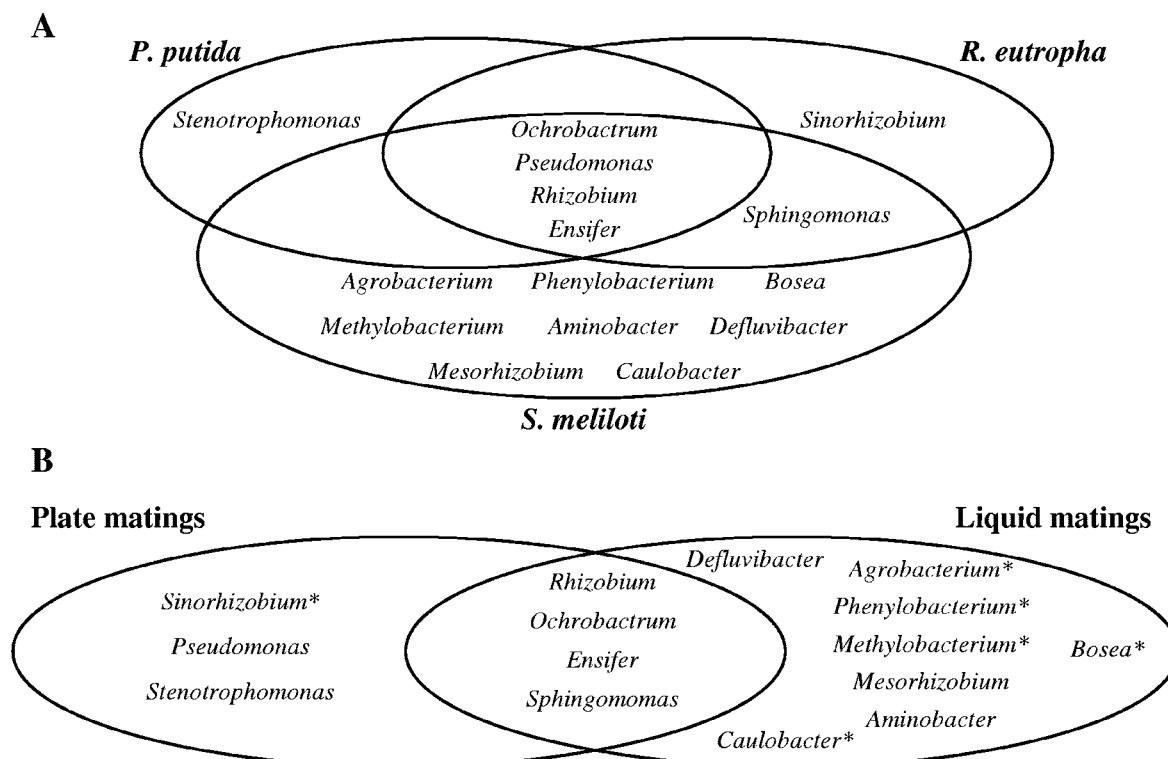


FIG. 2. Diversity of transconjugants obtained depends on the donor used in the matings (A) and the type of matings (on agar medium versus in liquid sludge) (B). *, genus only isolated after prolonged incubation.

and incubation time) (Fig. 1) affected the diversity of transconjugants obtained. Three genera of transconjugants were only recovered after plate matings and eight only after liquid matings (Fig. 2B). *Pseudomonas* sp. and *Ochrobactrum* sp. isolates constituted the majority of transconjugants found in plate matings (183/232), while *Rhizobium* sp. transconjugants represented the main group recovered from liquid matings (47/74) (Table 2). The effect of the mating conditions on the 16S rRNA gene sequence diversity of transconjugants was shown to be statistically significant ($F_{ST} = 0.268$; $P < 0.002$; 1,000 permutations).

Since longer incubation times were required for some transconjugants to form visible colonies on the selective agar media, a second isolation round after 3 or 4 weeks increased the diversity of transconjugants obtained. Overall, six genera of transconjugants were only detected after the second isolation round (Fig. 2B). The extended incubation time particularly increased the diversity of transconjugants isolated from liquid matings, since 5 of the 12 genera were only isolated in the second round. The effect of incubation time on the diversity of transconjugant 16S rRNA gene sequences was found to be statistically significant ($F_{ST} = 0.304$; $P < 0.002$; 1,000 permutations).

Some genera and several species of transconjugants were only detected on one of the two types of agar media (R2A versus DAB-SAC) (Table 2), which corresponded to a significant effect of medium on transconjugant diversity ($F_{ST} = 0.029$; $P < 0.002$; 1,000 permutations). In sum, our findings show that employing multiple experimental methods signifi-

cantly increased the transconjugant diversity compared to any method alone.

DISCUSSION

Previous investigators have suggested that the “flow” of a plasmid within a community could depend on the original plasmid donor (13) and that the genetic background of the host might influence the conjugational transfer frequency and host range of the plasmid (25). However, prior to this study, no experimental data were available to support this hypothesis. Our finding that transconjugants belonging to the genera *Aminobacter*, *Bosea*, *Defluviibacter*, *Mesorhizobium*, *Agrobacterium*, *Phenylobacterium*, *Methylobacterium*, and *Caulobacter* were only isolated from sludge when *Sinorhizobium meliloti* was the plasmid donor was the most striking example of donor-dependent plasmid host range. Moreover, the statistically significant effect of donors on the diversity of obtained transconjugants in the F_{ST} test (29) clearly demonstrates that the transfer range of pB10::rfp was affected by the donor delivering the plasmid to the bacterial community. In general, these results suggest that the range of hosts to which a plasmid can spread in a bacterial community depends on the hosts already carrying the plasmid. In other words, the host range of a plasmid in a community is not a fixed trait that is dependent only on the properties of the plasmid itself.

There are at least five different mechanisms by which a donor-dependent effect on plasmid host range might occur. First, differences in strain survival, plasmid maintenance, and

conjugative plasmid transfer efficiencies will determine the number of successful conjugation events, which might lead to an apparent increase in the diversity of recipients. In the present case this can be rejected, since there was no statistically significant correlation between the number of transconjugants obtained per donor subgroup and the corresponding diversity indices. A second possibility is that host regulation of *tra* genes could affect the efficiency of plasmid transfer, as has been shown for F-like plasmids (25). While this might influence the efficiency or frequency of transfer, it is difficult to envision how this would influence the range of recipients to which the donor transfers its plasmid. Third, restriction-modification (R-M) systems of the donor and recipient strains might limit the acquisition of foreign DNA, as restriction enzymes cleave DNA not modified by the appropriate modification enzymes (14). This constitutes a frequently encountered barrier to the acquisition of foreign DNA lacking the imprinted modification pattern characterizing the recipient DNA as self (57). Since conjugative transfer of RP4(IncP-1 α) and R751(IncP-1 β) is known to be sensitive to restriction (58), even the transfer of these BHR plasmids, which evolved systems to acquire a general promiscuity, can still be negatively affected by R-M systems of certain recipient strains. Conversely, a high similarity of R-M systems between donors and recipients might increase the success of plasmid transfer. Fourth, the proximity of donors and recipients in the environment will determine the likelihood of contact between donors and recipients. At least two factors play a role in determining this proximity. The first is the physiology of donors and potential recipients, since donors with strictly aerobic metabolism are more likely to be adjacent to other aerobic organisms, for example. The nonrandom spatial distribution of microbial populations is commonplace and has been shown to occur in biofilms (28), sludge granules (46), termite gut (45), microbial mats (56), soil (20), and activated sludge (2). The second factor is the physiochemical characteristics of outer cell surfaces of the donor and recipient. For example, cells with a hydrophobic surface are more likely to associate with hydrophobic particulate matter in an environment. It has been shown that the adherence of bacterial isolates from wastewater treatment plants to flocs is increased by higher cell surface hydrophobicity (59). Both of these factors help determine the "nearest neighbors" of donors and thereby influence the chances of plasmid acquisition by a potential recipient. Finally, biophysical interactions existing between bacterial surfaces, such as electrostatic, hydrophobic, and Van Der Waals forces (53), could influence the ability of cells to be in close apposition so that assembly of bacterial mating pairs and the formation of conjugative pores can occur (25, 27). In RP4-bearing *E. coli*, changes in membrane physiology that allow formation of conjugational junctions have been postulated to be essential for conjugative success and might be key to binding to cell walls or membranes from diverse taxa, including gram-positive bacteria and yeast (42). As these cell surface changes may be more efficient in some donors than in others, donor-related influences on mating pair formation might exist. Given our limited knowledge of plasmid transfer processes in the environment, it is not possible to know the extent to which these or possibly other factors influence the host range of a plasmid in a community.

We cannot rule out the possibility that insertion of the *rfp*

cassette into the *traC* gene of pB10 influenced transfer of the plasmid to certain potential hosts. Plasmid-encoded primase is generally not required for successful transfer and establishment of the plasmid in a transconjugant (26), but exceptions have been reported in at least two donor-recipient combinations (*P. aeruginosa*-*P. stutzeri* and *E. coli*-*Salmonella enterica* serovar Typhimurium) (31, 34). Likewise, some transconjugants may not have been detected because the *rfp* gene was not expressed or the fluorescent protein did not correctly fold (13). These factors might explain why no β -*Proteobacteria* were found among the transconjugants, an observation in contrast with previous studies showing IncP-1 β catabolic plasmids transferring predominantly to β -*Proteobacteria* (17). However, such events would at worst lead to an underestimation of the diversity of transconjugants obtained. It would not account for differences in the range of recipients observed with different donors, and thus the primary observations of our study remain valid.

The experimental conditions used to examine plasmid transfer and the time allowed for growth of transconjugants both affected the diversity of recipients that were recovered. In our study only α -*Proteobacteria* were isolated from matings done in liquid, while both γ - and α -*Proteobacteria* were isolated from plate matings. This could be due to the initial strong proliferation of γ -*Proteobacteria* when the activated-sludge sample was exposed to the agar medium in plate matings (13, 54), whereas the activated sludge in liquid matings was not amended with nutrients. This finding is analogous to those of Goris et al., who found that conjugative transfer of plasmid pC1gfp to an activated-sludge community on LB agar plates yielded mainly *Aeromonas* sp., whereas *Delftia acidovorans* was predominantly isolated from matings done in liquid sludge (17). Since liquid matings are a more accurate representation of plasmid spread in a wastewater treatment plant, the host range found under these conditions probably best represents the natural host range in this ecosystem. We also observed that prolonged incubation of the selective media enhanced the recovery of transconjugants belonging to the slower-growing α -*Proteobacteria* (Fig. 2B). These findings suggest that various mating conditions and longer transconjugant incubation times should be used to expand the range of transconjugants detected and avoid excluding slow and poorly growing community members.

As this is the first demonstration of a donor effect on plasmid transfer in a microbial community, further evaluations including other plasmids, donors, and microbial communities are needed to examine in which instances such a donor effect can occur. Also, the nature of the effect of different donors on the plasmid transfer properties should be investigated. Our results provide a basis for further research in this area, which will offer novel insights into the role of the plasmid donor in natural plasmid spread.

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REFERENCES

- Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389–3402.
- Amann, R., J. Snaird, M. Wagner, W. Ludwig, and K.-H. Scheifer. 1996. In situ visualization of high genetic diversity in a natural microbial community. *J. Bacteriol.* **178**:3496–3500.
- Bathe, S., M. Leubner, J. W. Ellwart, S. Würtz, and M. Hausner. 2004. High phylogenetic diversity of transconjugants carrying plasmid pJP4 in an activated sludge-derived microbial community. *FEMS Microbiol. Lett.* **235**:215–219.
- Boyer, H. W., and D. Roulland-Dussoix. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J. Mol. Biol.* **41**:459.
- Christensen, B. B., C. Sternberg, J. B. Andersen, L. Eberl, S. Møller, M. Givskov, and S. Molin. 1998. Establishment of new genetic traits in a microbial biofilm community. *Appl. Environ. Microbiol.* **64**:2247–2255.
- Cole, J. R., B. Chai, T. L. Marsh, R. J. Farris, Q. Wang, S. A. Kulam, S. Chandra, D. M. McGarrell, T. M. Schmidt, G. M. Garrity, and J. M. Tiedje. 2003. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res.* **31**:442–443.
- Davies, J. 1994. Inactivation of antibiotics and the dissemination of resistance genes. *Science* **264**:375–382.
- De Gelder, L., L. J. Forney, and E. M. Top. 2005. Exploring the host range of the broad-host-range plasmid pB10. *Plasmid* **53**:57–58.
- Diaz, E., M. Munthali, V. de Lorenzo, and K. Timmis. 1994. Universal barrier to lateral spread of specific genes among microorganisms. *Mol. Microbiol.* **13**:855–861.
- DiGiovanni, G. D., J. W. Neilson, I. L. Pepper, and N. A. Sinclair. 1996. Gene transfer of *Alcaligenes eutrophus* JMP134 plasmid pJP4 to indigenous soil recipients. *Appl. Environ. Microbiol.* **62**:2521–2526.
- Dröge, M., A. Pühler, and W. Selbitschka. 2000. Phenotypic and molecular characterization of conjugative antibiotic resistance plasmids isolated from bacterial communities of activated sludge. *Mol. Gen. Genet.* **263**:471–482.
- Dunbar, J., D. C. L. Wong, M. J. Yarus, and L. J. Forney. 1996. Autoradiographic method for isolation of diverse microbial species with unique catabolic traits. *Appl. Environ. Microbiol.* **62**:4180–4187.
- Fulthorpe, R., and R. Wyndham. 1991. Transfer and expression of the catabolic plasmid pBRC60 in wild bacterial recipients in a freshwater ecosystem. *Appl. Environ. Microbiol.* **57**:1546–1553.
- Geisenberger, O., A. Ammendola, B. B. Christensen, S. Molin, K.-H. Schleifer, and L. Eberl. 1999. Monitoring the conjugal transfer of plasmid RP4 in activated sludge and in situ identification of the transconjugants. *FEMS Microbiol. Ecol.* **174**:9–17.
- Gerdas, K., S. Ayora, I. Canosa, R. Ceglowski, R. Diaz-Orejas, T. Franch, A. P. Gulyaev, R. B. Jensen, I. Kobayashi, C. Macpherson, D. Summers, C. M. Thomas, and U. Zielonkiewicz. 2000. Plasmid maintenance systems, p. 49–85. In C. M. Thomas (ed.), *The horizontal gene pool*. Hardwood Academic Publishers, Amsterdam, The Netherlands.
- Gibbons, A. 1992. Exploring new strategies to fight drug-resistant microbes. *Science* **257**:1036–1038.
- Good, P. I. 2000. Permutation tests: a practical guide to resampling methods for testing hypotheses, 2nd ed. Springer-Verlag New York, Inc., New York, N.Y.
- Goris, J., N. Boon, L. Lebbe, W. Verstraete, and P. De Vos. 2003. Diversity of activated sludge bacteria receiving the 3-chloroaniline-degradative plasmid pC1gfp. *FEMS Microbiol. Ecol.* **46**:221–230.
- Goris, J., W. Dejonghe, E. Falsen, E. De Clerck, B. Geeraerts, A. Willems, E. M. Top, P. Vandamme, and P. De Vos. 2002. Diversity of transconjugants that acquired plasmid pJP4 or pEMT1 after inoculation of a donor strain in the A- and B-horizon of an agricultural soil and description of *Burkholderia hospita* sp. nov. and *Burkholderia terricola* sp. nov. *Syst. Appl. Microbiol.* **25**:340–352.
- Graw, W. O. K., and O. W. Prozesky. 1973. Drug resistance of coliform bacteria in hospital and city sewage. *Antimicrob. Agents Chemother.* **3**:175–180.
- Grundmann, G. L. 2004. Spatial scales of soil bacterial diversity—the size of a clone. *FEMS Microbiol. Ecol.* **48**:119–127.
- Haagen, J. A. J., S. K. Hansen, T. Johansen, and S. Molin. 2002. In situ detection of horizontal transfer of mobile genetic elements. *FEMS Microbiol. Ecol.* **42**:261–268.
- Herrero, M., V. de Lorenzo, and K. N. Timmis. 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J. Bacteriol.* **172**:6557–6567.
- Jukes, T. H., and C. R. Cantor. 1969. Evolution of protein molecules, p. 21–132. In N. H. Munro (ed.), *Mammalian protein metabolism*, vol. III. Academic Press, New York, N.Y.
- Kessler, B., V. de Lorenzo, and K. N. Timmis. 1992. A general system to integrate lacZ fusions into the chromosomes of gram-negative eubacteria: regulation of the Pm-promotor of the Tol-plasmid studied with all controlling elements in monocopy. *Mol. Gen. Genet.* **233**:293–301.
- Koraimann, G. 2004. Bacterial conjugation: cell-cell contact-dependent horizontal gene spread, p. 111–124. In R. V. Miller and M. J. Day (ed.), *Microbial evolution: gene establishment, survival, and exchange*. ASM Press, Washington, D.C.
- Krishnapillai, V. 1988. Molecular genetic analysis of bacterial plasmid promiscuity. *FEMS Microbiol. Rev.* **54**:223–238.
- Lawley, T., B. M. Wilkins, and L. S. Frost. 2004. Bacterial conjugation in gram-negative bacteria, p. 203–226. In B. E. Funnell and G. J. Phillips (ed.), *Plasmid biology*. ASM Press, Washington, D.C.
- Manz, W., K. Wendt-Potthoff, T. R. Neu, U. Szewzyk, and J. R. Lawrence. 1999. Phylogenetic composition, spatial structure, and dynamics of lotic bacterial biofilms investigated by fluorescent in situ hybridization and confocal laser scanning microscopy. *Microb. Ecol.* **37**:225–237.
- Martin, A. P. 2002. Phylogenetic approaches for describing and comparing the diversity of microbial communities. *Appl. Environ. Microbiol.* **68**:3673–3682.
- McClure, N., A. Weightman, and J. Fry. 1989. Survival of *Pseudomonas putida* UWC1 containing cloned catabolic genes in a model activated-sludge unit. *Appl. Environ. Microbiol.* **55**:2627–2634.
- Merryweather, A., P. T. Barth, and B. M. Wilkins. 1986. Role and specificity of plasmid RP4-encoded DNA primase in bacterial conjugation. *J. Bacteriol.* **167**:12–17.
- Mølbak, L., T. R. Licht, T. Kvist, N. Kroer, and S. R. Andersen. 2003. Plasmid transfer from *Pseudomonas putida* to the indigenous bacteria on alfalfa sprouts: characterization, direct quantification, and in situ location of transconjugant cells. *Appl. Environ. Microbiol.* **69**:5536–5542.
- Muyzer, G., S. Hottenträger, A. Teske, and C. Wawer. 1996. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA. A new molecular approach to analyze the genetic diversity of mixed microbial communities, p. 3.4.4.1–3.4.4.22. In A. D. L. Akkermans, J. D. van Elsas, and F. J. de Bruijn (ed.), *Molecular microbial ecology manual*. Kluwer Academic Publishing, Dordrecht, The Netherlands.
- Nash, J., and V. Krishnapillai. 1988. Role of IncP-1 plasmid primase in conjugation between *Pseudomonas* species. *FEMS Microbiol. Lett.* **49**:257–260.
- Newby, D. T., T. J. Gentry, and I. L. Pepper. 2000. Comparison of 2,4-dichlorophenoxyacetic acid degradation and plasmid transfer in soil resulting from bioaugmentation with two different pJP4 donors. *Appl. Environ. Microbiol.* **66**:3399–3407.
- Pukall, R., H. Tschäpe, and K. Smalla. 1996. Monitoring the spread of broad host and narrow host range plasmids in soil microcosms. *FEMS Microbiol. Ecol.* **20**:53–66.
- Qiagen. 2001. Sample preparation and lysis protocol for bacteria, p. 40–43. *QIAGEN genomic DNA handbook*, vol. August 2001. Qiagen, Valencia, Calif.
- Ramos-Gonzalez, M.-I., E. Duque, and J. L. Ramos. 1991. Conjugational transfer of recombinant DNA in cultures and in soils: host range of *Pseudomonas putida* TOL plasmids. *Appl. Environ. Microbiol.* **57**:3020–3027.
- Reinthal, F., J. Posch, G. Feiler, G. Wust, D. Haas, G. Ruckebauer, F. Mascher, and E. Marth. 2003. Antibiotic resistance of *E. coli* in sewage and sludge. *Water Res.* **37**:1685–1690.
- Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**:406–425.
- Sambrook, J., and D. W. Russell. 2001. *Molecular cloning: a laboratory manual*, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Samuels, A. L., E. Lanka, and J. Davies. 2000. Conjugative junctions in RP4-mediated mating of *Escherichia coli*. *J. Bacteriol.* **182**:2709–2715.
- Sandaa, R.-A., and Ø. Enger. 1994. Transfer in marine sediments of the naturally occurring plasmid pRAS1 encoding multiple antibiotic resistance. *Appl. Environ. Microbiol.* **60**:4234–4238.
- Schlüter, A., H. Heuer, R. Szczepanowski, L. J. Forney, C. M. Thomas, A. Pühler, and E. M. Top. 2003. The 64,508 bp IncP-1 β antibiotic multiresistance plasmid pB10 isolated from a wastewater treatment plant provides evidence for recombination between members of different branches of the IncP-1 β group. *Microbiology* **149**:3139–3153.
- Schmitt-Wagner, D., M. W. Friedrich, B. Wagner, and A. Brune. 2003. Axial dynamics, stability, and interspecies similarity of bacterial community structure in the highly compartmentalized gut of soil-feeding termites (*Cubitermes* spp.). *Appl. Environ. Microbiol.* **69**:6018–6024.
- Sekiguchi, Y., Y. Kamagata, K. Nakamura, A. Ohashi, and H. Harada. 1999. Fluorescence in situ hybridization using 16S rRNA-targeted oligonucleotides reveals localization of methanogens and selected uncultured bacteria in mesophilic and thermophilic sludge granules. *Appl. Environ. Microbiol.* **65**:1280–1288.
- Séveno, N. A., D. Kallifidas, K. Smalla, J. D. van Elsas, J.-M. Collard, A. D.

- Karagouni, and E. M. H. Wellington. 2002. Occurrence and reservoirs of antibiotic resistance genes in the environment. *Rev. Med. Microbiol.* **13**:15–27.
48. Thomas, C. M., and C. A. Smith. 1987. Incompatibility group P plasmids: genetics, evolution, and use in genetic manipulation. *Annu. Rev. Microbiol.* **41**:77–101.
49. Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**:4876–4882.
50. Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**:4673–4680.
51. Top, E., I. De Smet, W. Verstraete, R. Dijkmans, and M. Mergeay. 1994. Exogenous isolation of mobilizing plasmids from polluted soils and sludges. *Appl. Environ. Microbiol.* **60**:831–839.
52. Top, E. M., W. E. Holben, and L. J. Forney. 1995. Characterization of diverse 2,4-dichlorophenoxyacetic acid-degradative plasmids isolated from soil by complementation. *Appl. Environ. Microbiol.* **61**:1691–1698.
53. Ubbink, J., and P. Schär-Zammaretti. 2005. Probing bacterial interactions: integrated approaches combining atomic force microscopy, electron microscopy and biophysical techniques. *Micron* **36**:293–320.
54. Wagner, M., R. Amann, H. Lemmer, and K.-H. Schleifer. 1993. Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. *Appl. Environ. Microbiol.* **59**:1520–1525.
55. Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173**:697–703.
56. Wieland, A., M. Kühn, L. McGowan, A. Fourçans, R. Duran, P. Caumette, T. García de Oteyza, J. O. Grimalt, A. Solé, E. Diestra, I. Esteve, and R. A. Herbert. 2003. Microbial mats on the Orkney Islands revisited: microenvironment and microbial community composition. *Microb. Ecol.* **46**:371–390.
57. Wilkins, B. M. 2002. Plasmid promiscuity: meeting the challenge of DNA immigration control. *Environ. Microbiol.* **4**:495–500.
58. Wilkins, B. M., P. M. Chilley, A. T. Thomas, and M. J. Pocklington. 1996. Distribution of restriction enzyme recognition sequences on broad host range plasmid RP4: molecular and evolutionary implications. *J. Mol. Biol.* **258**:447–456.
59. Zita, A., and M. Hermansson. 1997. Effects of bacterial cell surface structures and hydrophobicity on attachment to activated sludge flocs. *Appl. Environ. Microbiol.* **63**:1168–1170.