APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1996, p. 2621–2628 Vol. 62, No. 7
0099-2240/96/$04.00+0
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# Detection and Characterization of Broad-Host-Range Plasmids in Environmental Bacteria by PCR

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Received 21 December 1995/Accepted 11 April 1996

**Primer systems for PCR amplification of different replicon-specific DNA regions were designed on the basis of published sequences for plasmids belonging to the incompatibility (Inc) groups IncP, IncN, IncW, and IncQ. The specificities of these primer systems for the respective Inc groups were tested with a collection of reference plasmids belonging to 21 different Inc groups. Almost all primer systems were found to be highly specific for the reference plasmid for which they were designed. In addition, the primers were tested with plasmids which had previously been grouped by traditional incompatibility testing to the IncN, IncW, IncP, or IncQ group. All IncQ plasmids gave PCR products with the IncQ primer systems tested. However, PCR products were obtained for only some of the IncN, IncP, and IncW group plasmids. Dot blot and Southern blot analyses of the plasmids revealed that PCR-negative plasmids also failed to hybridize with probes derived from the reference plasmids. The results indicated that plasmids assigned to the same Inc group by traditional methods might be partially or completely different from their respective reference plasmids at the DNA level. With a few exceptions, all plasmids related to the reference plasmid at the DNA level also reacted with the primer systems tested. PCR amplification of total DNA extracted directly from different soil and manure slurry samples revealed the prevalence of IncQ- and IncP-specific sequences in several of these samples. In contrast, IncN- and IncW-specific sequences were detected mainly in DNA obtained from manure slurries.**

Plasmids play a major role in bacterial adaptation to environmental or man-made stress. The rapid dissemination of antibiotic resistance genes in bacterial populations as a consequence of the intensive use of antibiotics in medicine and agriculture can be partly attributed to plasmid-mediated horizontal transfer. Plasmids capable of being transferred and stably maintained in a wide range of bacteria, the so-called broad-host-range plasmids, are of special interest with respect to interspecies gene exchange. The molecular biology of the broad-host-range conjugative IncP (8, 14, 15, 17, 19, 24, 25, 31, 41–43), IncN (3, 16), and IncW (20, 21, 23) plasmids and the mobilizable IncQ (6, 9, 11, 27, 28) plasmids has been studied intensively since the beginning of the 1980s. A comprehensive review on broad-host-range conjugative and mobilizable plasmids in gram-negative bacteria has been published (10). The IncPα plasmid RP4 presently is the only conjugative plasmid which has been completely sequenced (24). In addition, the complete sequence of the small mobilizable IncQ plasmid RSF1010 is available (28). For IncN, IncW, and IncPβ plasmids, sequences of several replicon-specific regions have been published.

Surprisingly, very little is known about the prevalence of these plasmids in the environment. Most of the current knowledge comes from plasmids isolated from clinical material or from studies on plasmid-mediated dissemination of antibiotic and/or heavy metal resistance genes (36), in which plasmids were usually isolated after selective cultivation of bacteria with subsequent screening for the presence of plasmids. These studies often focused on members of the family *Enterobacteriaceae*. Plasmid typing has been traditionally performed by incompatibility (Inc) testing, donor-specific phage propagation, and molecular sizing. Recently, a collection of *inc*/*rep* probes has become available (4), allowing replicon typing by DNA hybridization. Another promising approach would seem to be the use of PCR with oligonucleotide primers designed on the basis of replicon-specific DNA sequences. PCR-based detection of broad-host-range plasmids would permit studies on plasmid prevalence to be extended to the nonculturable bacterial fraction present in many environments. This would be analogous to the PCR-based method used by Dahlberg and Hermansson (5) to study the abundance of Tn*3*, Tn*21*, and Tn*501* transposase sequences in bacterial community DNA from marine environments.

The intention of this work was to determine whether PCR could be used to detect and characterize broad-host-range plasmids in pure cultures and environmental samples. PCR detection would require a high specificity of the primers for the Inc group for which they are designed. Additionally, for plasmids belonging to the same Inc group, sequence conservation in the DNA regions chosen for primer annealing would be required for PCR amplification. Little is known about sequence diversity within plasmids assigned to the same Inc group by traditional Inc testing. Therefore, at the beginning of this study it was unclear whether PCR-based detection of broad-host-range plasmids would be feasible. The design of different primer sets for the aforementioned broad-host-range plasmids, the testing of their specificities for the respective Inc groups, and their application to a collection of endogenously isolated plasmids and to total community DNA extracted directly from different soils and manure slurries are described in the paper.

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TABLE 1. Primer systems designed by Oligo on the basis of published sequence data and used for the amplification of broad-host-range IncQ, IncP, IncN, and IncW plasmids

| Inc group | Region | Primer sequence | Product size (bp) | Annealing temp (°C) | Primer position | Accession no. |
|---|---|---|---|---|---|---|
| IncN | *rep* 1 | AGT TCA CCA CCT ACT CGC TCC G | 164 | 55 | 1017 | X52972 |
| | *rep* 2 | CAA GTT CTT CTG TTG GGA TTC CG | | | 1158 | |
| | *kikA* 1 | ACT TAC CTT TAT CAA CAT TCT GGC G | 329 | 55 | 97 | M88549 |
| | *kikA* 2 | CGA CTG GTT ACT TCC ACC TTC GC | | | 403 | |
| | *oriT* 1 | TTG GGC TTC ATA GTA CCC | 349 | 49 | 36 | M30197 |
| | *oriT* 2 | GTG TGA TAG CGT GAT TTA TGC | | | 364 | |
| IncP | *oriT* 1 | CAG CCT CGC AGA GCA GGA T | 110 | 57 | 4642 | X54459 |
| | *oriT* 2 | CAG CCG GGC AGG ATA GGT GAA GT | | | 4729 | |
| | *trfA1* 1 | ATG ACG ACC AAG AAG CG | 889 | 57 | 721 | X00713 |
| | *trfA1* 2 | AAC CCC CAG CCG GAA CTG | | | 1592 | |
| | *trfA2* 1 | CGA AAT TCR TRT GGG AGA AGT A | 241 | 57 | 1334 | X00713 |
| | *trfA2* 2 | CGY TTG CAA TGC ACC AGG TC | | | 1555 | |
| | *korA* 1 | ATG AAG AAA CGG CTN ACC GA | 294 | 52 | 441 | X00082 |
| | *korA* 2 | TTC CTG TTT YYT CTT GGC GTC | | | 714 | |
| | *traG* 1 | CTG CGT CAC GAT GAA CAG GCT TAC C | 762 | 63 | 795 | X54459 |
| | *traG* 2 | ACT TCC AGC GGC GTC TAT GTG G | | | 1535 | |
| IncQ | *repB* 1 | TCG TGG TCG CGT TCA AGG TAC G | 1,160 | 62 | 2816 | M28829 |
| | *repB* 2 | CTG TAA GTC GAT GAT CTG GGC GTT | | | 3952 | |
| | *oriV* 1 | CTC CCG TAC TAA CTG TCA CG | 436 | 57 | 2328 | M28829 |
| | *oriV* 2 | ATC GAC CGA GAC AGG CCC TGC | | | 2743 | |
| | *oriT* 1 | TTC GCG CTC GTT GTT CTT CGA GC | 191 | 57 | 2988 | M28829 |
| | *oriT* 2 | GCC GTT AGG CCA GTT TCT CG | | | 3159 | |
| IncW | *oriV* 1 | GAC CCG GAA AAC CAA AAA TA | 1,140 | 58 | 91 | M95790 |
| | *oriV* 2 | GTG AGG GTG AGG GTG CTA TC | | | 1211 | |
| | *oriT* 1 | TCT GCA TCA TTG TAG CAC C | 317 | 51 | 8 | X51505 |
| | *oriT* 2 | CCG TAG TGT TAC TGT AGT GG | | | 305 | |
| | *trwAB* 1 | AGC GTA TGA AGC CCG TGA AGG G | 141 | 57 | 355 | X60085 |
| | *trwAB* 2 | AAA GAT AAG CGG CAG GAC AAT AAC G | | | 471 | |

## MATERIALS AND METHODS

**Strains and plasmids.** *Escherichia coli* K53 (*met pro*) was the host for reference plasmids and the endogenously isolated plasmids used in this study (2). The plasmids endogenously isolated from sewage, manure slurries, and clinical specimens originated from the plasmid type collection of the Robert-Koch-Institut in Wernigerode, Germany (see Tables 2 to 4). Plasmids were grouped phenotypically by Inc testing (segregation of respective plasmid doubles) and sensitivity to pilus-specific phages (37, 38). The IncPβ plasmids R751, pSS50, and pJP4 were provided by M. Mergeay (Mol, Belgium). The IncPα plasmid pG527 was isolated by exogenous plasmid isolation from pig manure slurry by using *Pseudomonas putida* UWC3 ($Rif^r$) as a recipient as described by Bale et al. (1). Reference plasmids used to test the specificities of the primer systems belonged to the following Inc groups: IncB (R16), IncC (R40a), IncOF (pIE509), IncFI (R386), IncFII (R1), IncH1 (R27), IncH2 (R478), IncI1 (R64), IncI2 (pIE636), IncJ (R391), IncK (R387), IncM (R446), IncN (RN3), IncPα (RP1), IncPβ (R751), IncQ (RSF1010), IncT (Rts), IncU (RA3), IncW (R388), IncX (R6K), and IncZ (R545) (37, 38).

*E. coli* strains hosting the respective plasmids were grown by shaking inoculated Luria-Bertani broth cultures at 28°C overnight or by plating on minimal medium (M9) supplemented with methionine and proline and at least one antibiotic for which a resistance was encoded by the plasmid under investigation.

**Primer design.** Oligonucleotide primers to amplify different plasmid "backbone" regions related to replication (IncP, *trfA1/2* and *korA*; IncN, *rep*; IncW, *oriV*; and IncQ, *oriV* and *repB*) and to transfer (IncP, *oriT* and *traG*; IncN, *oriT*; and IncW, *oriT* and *trwAB*) or the so-called *Klebsiella oxytoca* killing gene *kikA* of IncN plasmids were designed on the basis of published sequences by using the Oligo program (National Biosciences, Plymouth, United Kingdom). Table 1 summarizes information on primer sequences, annealing temperatures, product sizes, and references for the respective sequences used.

**PCR conditions.** PCR was performed with heat-denatured cells as targets (1 μl of a 1:10-diluted overnight broth). PCR mixtures contained 5 U of *Taq* polymerase (Stoffel fragment), Stoffel buffer, 0.2 mM deoxynucleoside triphosphates, 3.75 mM $MgCl_2$ (4.75 mM for IncQ *oriT*), and 0.2 μmol each of primer I and primer II. Usually, after a 5-min step at 94°C, 35 cycles of amplification consisting of 1 min of denaturation at 94°C, 1 min of primer annealing at the annealing temperature according to the Oligo program (Table 1), and 1 min of primer extension at 72°C, followed by a 10-min final extension step at 72°C, were performed. PCR products were analyzed in 1% agarose gels with Tris-borate-EDTA buffer, Southern blotted, and hybridized with respective digoxigenin-labeled probes. Positive controls were RN3 for IncN, R388 for IncW, pIE723 (which is identical to RSF1010 in the region amplified) for IncQ, RP4 for IncPα, and R751 for IncPβ. Negative controls contained heat-denatured plasmid-free *E. coli* J53 cells or water.

**Preparation of plasmid DNA.** Bacteria were harvested from 5-ml overnight cultures. Plasmid extractions were performed with the Qiagen midi prep (Qiagen, Hilden, Germany) or as described by Ish-Horowicz and Burke (13) with a modification involving phenol extraction of the potassium acetate supernatant (38a).

**Generation of probes.** PCR products obtained with the different primer systems with the respective reference plasmids (IncQ, pIE723; IncPα, RP4; IncPβ, R751; IncN, RN3; and IncW, R388) were sliced out of preparative gels and recovered with Qiagen spin columns or QiaEx according to the manufacturer's instructions. The IncN *oriT* probe was digoxigenin labeled by PCR. In addition, *inc*/*rep* probes for IncN and IncP were obtained from *Eco*RI or *Hae*III restriction digests of pULB2432 or pULB2420 (4), respectively, and digoxigenin labeled. The strains containing the cloned fragments were kindly provided by Martine Couturier (Brussels, Belgium). The DNA fragments were digoxigenin labeled by random-primed digoxigenin labeling according to the manufacturer's protocol (Boehringer, Mannheim, Germany).

**Dot blot and Southern blot hybridizations.** Lysozyme-treated cells (100 μl of an overnight culture incubated with 50 μl of lysozyme [5 mg/ml] for 30 min at 37°C in a microtiter plate) or plasmid DNA was transferred onto a nylon membrane by using a dot blot apparatus. Southern blots were made for analysis of the PCR products obtained, using the different primer systems, and for *Eco*RV-digested plasmid DNA. Dot blotting and Southern blotting were performed by the standard protocols described by Sambrook et al. (26). For the hybridizations, the instructions for the digoxigenin detection kit (Boehringer) were followed.

**Extraction of total community DNA from soils and manure slurries.** Total community DNA was extracted from 2- to 5-g composite samples of different pig manure slurries (two samples from three independent samplings) and five different soils by lysozyme, bead beating, and alkaline sodium dodecyl sulfate treatments followed by cold phenol and phenol-chloroform extraction according to the protocol described by Smalla et al. (29, 30). The DNA (1 μl; approximately 10 to 50 ng of DNA) was purified by cesium chloride, subjected to potassium acetate precipitation and Wizard DNA cleanup (Promega, Madison, Wis.) (39), and used as a template for PCR amplification.

## RESULTS

**Specificities of oligonucleotide primers.** The specificities of primers for each Inc group were tested by using a collection of 21 different reference plasmids belonging to various Inc groups as templates for PCR. The PCR products were analyzed by agarose gel electrophoresis and Southern blot hybridization. The IncN-, IncW-, and IncQ-specific primer systems unequivocally identified the plasmids for which they were designed. All primers tested gave hybridization-positive PCR products of the expected sizes. However, the IncP primers *trfA1* and *traG* (designed on the basis of RP4 sequences) gave PCR products only with the IncPα plasmid RP4, and no PCR product was produced with the IncPβ plasmid R751 or with other plasmids. Thus, these primers seem to be specific for IncPα and suitable to differentiate IncPα from IncPβ plasmids. In contrast, the IncP primers for *oriT*, *trfA2*, and *korA* gave PCR products with both IncPα- and IncPβ-like plasmids. However, differences in the sequences of the respective PCR products became obvious when hybridizing Southern blots at 75 to 100% DNA homology. PCR products generated with the *korA*-, *trfA2*-, or *oriT*-specific primers, which gave strong signals with R751 (IncPβ)-derived probes, gave no hybridization signal with RP4 (IncPα)-derived probes and vice versa. Because of the high specificities of the primers tested, PCR-based detection of broad-host-range plasmids seemed feasible.

**Conservation of DNA sequence among plasmids of the same Inc group.** Since very few plasmids belonging to the same Inc group have been sequenced, the degree of similarity among plasmids of the same Inc group is unknown. Sequence conservation of the region chosen for primer annealing would be particularly important for PCR to serve as an identification tool.

To evaluate the degree of similarity, the various PCR primers were tested with a set of plasmids isolated from clinical and environmental specimens. These plasmids had previously been classified according to Inc testing, donor-specific phage propagation, and molecular size. The PCR products were analyzed by agarose gel electrophoresis and Southern blot hybridizations. To test whether the endogenously isolated plasmids contained the region used for PCR amplification, dot-blotted plasmid DNAs were hybridized with probes obtained by PCR with the reference plasmids as templates.

**(i) IncQ plasmids.** The seven IncQ plasmids (RSF1010 [$Sm^r$ $Su^r$], five pIE639-like plasmids [$Sm^r$ $Su^r$ $Km^r$ $St^r$] and pIE723 [$Gm^r$ $Km^r$ $Sm^r$ $Su^r$]) used yielded hybridizing PCR products of the expected sizes with the IncQ primers *oriV*, *oriT*, and *repB*, suggesting that there was a high degree of conservation of the IncQ replicon. Thus, the primers for amplification of IncQ-specific sequences are well suited for the detection of IncQ sequences in bacterial isolates or in DNA extracted directly from environmental samples.

**(ii) IncN plasmids.** A total of 45 IncN plasmids were tested with the primers specific for the IncN genes (*oriT*, *repA*, and *kikA*) and by dot blot hybridizations of plasmid DNAs (Table 2). When the IncN *oriT* primers were used, PCR products were obtained with 64% of the 45 IncN group plasmids, including three reference plasmids (R15, RN3, and R233), whereas 76% of the plasmids were positive when PCR was done with the *repA* and *kikA* primers. All of the plasmids that gave PCR products with *oriT* primers fell in the group that was positive by PCR with the *repA* and *kikA* primers. Dot blot hybridizations of the 45 IncN group plasmids revealed that only the plasmids positive by PCR with the *repA* and *kikA* primers hybridized with the *rep* (Couturier) and the IncN *oriT* PCR-derived probe (Table 2). The five IncN plasmids that failed to yield a PCR product with *oriT* primers did hybridize in dot blot hybridization of plasmid DNA with the *oriT* probe. Therefore, it is conceivable that primer annealing to these plasmids was inefficient, perhaps because of sequence divergence at the annealing site. Thus, the *kikA* and *repA* primers seem to be a more reliable means to identify IncN plasmids than the *oriT* primers.

For 11 of the 45 IncN plasmids, no DNA fragments were produced with any of these primer sets. To assess whether the PCR- and dot blot-negative plasmids had any DNA sequence similarity to those which did yield PCR products, *Eco*RV digests of three PCR-positive (pIE660, pIE1092, and RN3) and three PCR-negative (pIE330, pIE647, and pIE1037) IncN plasmids were analyzed by Southern blot hybridizations. The PCR-negative plasmids hybridized neither with the IncN *rep* probe (Couturier) nor with the PCR-derived probes for *kikA* and *oriT* (Fig. 1). These data suggested that the regions used for PCR amplification are not present on these plasmids or are less than 75% similar to the probes. However, plasmid pIE1037 DNA did hybridize to PCR- and dot blot-positive plasmids, indicating that there were regions of sequence similarity.

**(iii) IncP plasmids.** PCR amplification with five sets of primers for IncP-specific genes was performed with 15 IncP plasmids, including RP4 and pTH10 (both IncPα) and R751 (IncPβ) as controls (Table 3). Southern blotting showed that 60% of the IncP plasmids gave PCR products with the *korA* and *oriT* primers, and 53% gave products with the *trfA2* primers. These PCR products hybridized either with the RP4-derived probes or with R751-derived probes, indicating that IncPα- and IncPβ-like plasmids could easily be differentiated. Only IncPα-like plasmids gave PCR products with the *trfA1* and *traG* primer systems. Dot blots of PCR-negative plasmid DNA did not hybridize with digoxigenin-labeled *trfA2*, *korA*, and *oriT* probes derived from RP4 or R751 (Table 3), and only two PCR-negative plasmids weakly hybridized to the *repP* probe (pULB2420). The *trfA2*, *oriT*, and *korA* primers seem to be most appropriate for the detection of both IncPα and IncPβ plasmids in bacterial isolates and environmental DNAs. Subsequent hybridization with RP4- or R751-derived probes permits discrimination between IncPα- and IncPβ-like sequences.

**(iv) IncW plasmids.** The hybridization abilities of the primers based on the IncW plasmids R388 and RSa were tested

TABLE 2. Testing of plasmids grouped by traditional Inc testing to IncN by PCR and dot blot hybridizations

| Designation | Origin | Size (kb) | Antibiotic traits[a] | Hybridization[b] | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | IncN *oriT* | | IncN *repA* | | IncN *kikA* (PCR) |
| | | | | PCR | Dot | PCR | Dot | |
| RN3 | Reference data | 54 | Tc, Sm, Su, Hg | + | + | + | + | + |
| R15 | Reference data | 62 | Sm, Su, Hg | − | + | + | + | + |
| pIE233 | Reference plasmid | 48 | Sm | + | + | + | + | + |
| pIE305 | *Shigella sonnei* | 38 | Cryptic | − | + | + | + | + |
| pIE311 | *E. coli* | 106 | Sm, Su, Ap | + | + | + | + | + |
| pIE319 | *Shigella sonnei* | 39 | Tc | + | + | + | + | + |
| pIE322 | *E. coli* | 45 | Tc | + | + | + | + | + |
| pIE330 | *Shigella sonnei* | 48 | Cryptic | − | − | − | − | − |
| pIE331 | *Salmonella dublin* | 52 | Tc | + | + | + | + | + |
| pIE392 | *Erwinia carotovora* | 64 | Cm, Sm, Su | − | + | + | + | + |
| pIE393 | *Shigella sonnei* | 39 | Tc | + | + | + | + | + |
| pIE462 | *E. coli* | 76 | Tc | − | − | − | − | − |
| pIE465 | *Shigella sonnei* | 42 | Tc, Ap, Cm | + | + | + | + | + |
| pIE483 | *E. coli* | 91 | Su, Ap, Gm | + | + | + | + | + |
| pIE500 | *Klebsiella pneumoniae* | 45 | Tc | + | + | + | + | + |
| pIE601 | *Shigella flexneri* | 38 | Tc, Sm, Ap | + | (+) | + | + | + |
| pIE615 | *Shigella sonnei* | 52 | Su, Tp | + | + | + | + | + |
| pIE616 | *Shigella sonnei* | 61 | Cm, Su, Tp | + | + | + | + | + |
| pIE647 | *E. coli* | 61 | Sm, St | − | − | − | − | − |
| pIE660 | *E. coli* | 61 | Sm, St | + | + | + | + | + |
| pIE672 | *Salmonella typhimurium* | 48 | Sm, Su, Tc | − | − | − | − | − |
| pIE764 | *Salmonella isangi* | 41 | Sm, Su, Km, St | − | − | − | − | − |
| pIE765 | *Salmonella typhimurium* | 45 | Tc | + | + | + | + | + |
| pIE767 | *E. coli* | 83 | Sm, Su | − | + | + | + | + |
| pIE768 | *Shigella sonnei* | 48 | Cm, Su, Tp | + | + | + | + | + |
| pIE769 | *Shigella flexneri* | 76 | Tc, Cm, Ap | − | − | − | − | − |
| pIE770 | *Salmonella typhimurium* | 48 | Cm, Sm | + | + | + | + | + |
| pIE771 | *Salmonella typhimurium* | 48 | Sm, Su, Tp | − | − | − | − | − |
| pIE1037 | *Pseudomonas aeruginosa* | 48 | Sm, St | − | − | − | − | − |
| pIE1067 | *Shigella sonnei* | 106 | Cm | + | + | + | + | + |
| pIE1069 | *E. coli* | 58 | Tc | + | + | + | + | + |
| pIE1070 | *E. coli* | 71 | Km | + | + | + | + | + |
| pIE1071 | *E. coli* | 41 | Km | + | + | + | + | + |
| pIE1072 | *E. coli* | 106 | Tc | + | + | + | + | + |
| pIE1073 | *E. coli* | 106 | Sm, St | − | − | − | − | − |
| pIE1074 | *Shigella flexneri* | 70 | Tc, Cm, Sm, Su | − | + | + | + | + |
| pIE1076 | *Shigella sonnei* | 45 | Ap | + | + | + | + | + |
| pIE1091 | *E. coli* | 41 | Km | + | + | + | + | + |
| pIE1092 | *E. coli* | 76 | Sm, Ap, St | + | + | + | + | + |
| pIE1093 | *E. coli* | 39 | Tc | + | + | + | + | + |
| pIE1094 | *Citrobacter freundii* | 42 | Sm, St | − | − | − | − | − |
| pIE1095 | *Salmonella typhimurium* | 65 | Sm, St | − | − | − | − | − |
| pIE1096 | *E. coli* | 36 | Tc | + | + | + | + | + |
| pIE1100 | *E. coli* | 45 | Tc, Hg | + | + | + | + | + |
| pIE1101 | Sewage | 45 | Tc | + | + | + | + | + |

[a] Antibiotic resistances (micrograms per milliliter): Ap, ampicillin (30); Cm, chloramphenicol (20); Gm, gentamicin (20); Hg, mercuric chloride (10); Km, kanamycin (50); Sm, streptomycin (30); St, streptothricin (50); Su, sulfonamide (50); Tc, tetracycline (20); Tp, trimethoprim (20).

[b] Hybridization with digoxigenin-labeled, PCR-derived probes (template RN3) and the *rep* Couturier probe. Hybridization signal: +, strong; (+), weak; −, none.

with 24 IncW plasmids. The plasmids originated from different habitats, such as manure, sewage, and surface water. Only 29% of these plasmids gave PCR products with the *oriV* and the *oriT* primers, and only 17% gave products with the *trwAB* primers. Those plasmids that were *trwAB* positive were also *oriV* and *oriT* positive. Dot blots of IncW plasmids hybridized with *oriT*, *oriV*, and *trwAB* probes (Table 4) showed that only plasmids that gave PCR products with the *oriV* and *oriT* primers strongly hybridized with the *oriV* probe. The plasmids that were positive with the *trwAB* primers also hybridized with the *trwAB* probe. Two IncW plasmids, pIE321 and pIE1105, both isolated from *Salmonella dublin* and coding for tetracycline resistance, were shown by means of PCR and dot blot hybridization to contain *oriV* and *oriT* but not *trwAB*. Similarly, only *oriV* was detected for the tetracycline resistance plasmid pIE774 isolated from *Shigella flexneri*. To determine if the IncW plasmids that did not react with IncW primers were related to the PCR-positive plasmids (R388-like), *Eco*RV-digested plasmid DNA was probed with PCR-negative plasmid DNA (pIE1056). None of the PCR-positive IncW plasmids hybridized, indicating a homology of less than 75%. Hybridization of the same Southern blot with the *oriT*, *oriV*, and *trwAB* probes revealed homology with only the PCR-positive plasmids (Fig. 2).

**Detection of replicon-specific regions in total community DNAs.** To investigate the prevalence of broad-host-range plasmids of the IncP, IncQ, IncN, and IncW groups in the environment, total community DNA was extracted from five soils and three pig manure slurries. These DNA preparations were

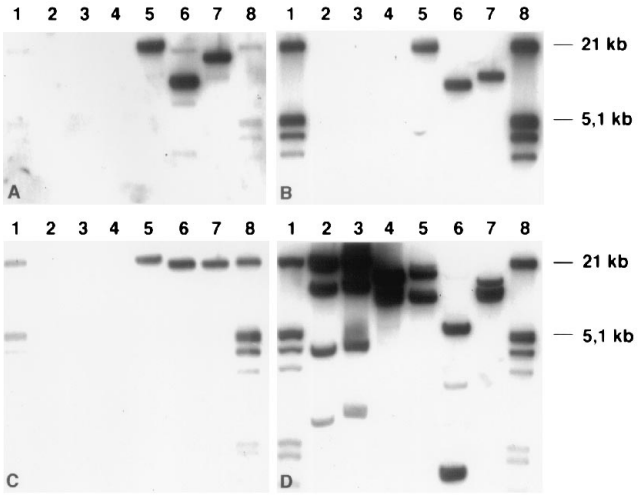


FIG. 1. Southern blot hybridization of *Eco*RV-digested plasmids grouped by traditional Inc testing to IncN. Lanes: 1 and 8, ladder (digoxigenin labeled); 2, pIE1037 (PCR negative); 3, pIE330 (PCR negative); 4, pIE647 (PCR negative); 5, pIE1092 (PCR positive); 6, RN3 (PCR positive); 7, pIE660. The probes (digoxigenin labeled) were *oriT* (A), *rep* (B), *kikA* (C), and pIE1037 (D).

used as targets for PCR amplification with the primers for the IncN (*repA*, *oriT*, and *kikA*), IncW (*oriV* and *oriT*), IncP (*trfA2*, *korA*, and *oriT*), and IncQ (*repB*, *oriV*, and *oriT*) groups (Table 5). IncQ-, IncPα-, IncPβ-, IncN-, and IncW-specific sequences were found in manure samples. IncQ-like sequences seemed very prevalent in manure as judged by the amount of PCR product visible after agarose gel electrophoresis. IncQ (*oriV* and *repB*)- and IncPα and IncPβ (mainly *trfA2*)-specific sequences were detected in some but not all DNAs extracted directly from different soils. More IncP *trfA2* PCR products were obtained for DNAs extracted from soils treated with dichloropropene (40) than for DNAs from untreated soils. IncW *oriV* or *oriT* sequences could not be detected in total DNA extracts from different soils. IncN-specific sequences were detected only in manure slurries, with the exception of one soil from a field planted with transgenic sugar beets.

## DISCUSSION

The application of the primer systems for the detection of plasmids which until now have not been studied at the molecular level is of special interest because little or nothing is known about the diversity at the DNA level of plasmids grouped according to their phenotypic traits (incompatibility, donor-specific phage propagation, and molecular size) to the same Inc group. Sequence conservation, at least for the DNA stretches used for primer annealing, is an absolute prerequisite to detect broad-host-range plasmids by means of PCR. Sequence conservation resulting in positive PCRs was shown for all IncQ group plasmids as well as for almost all IncN, IncW, and IncPα or IncPβ grouped plasmids, for which the sequences chosen for PCR amplification had a DNA similarity of 75 to 100% with the sequenced reference plasmid (dot blot positive). Therefore, the primer systems are appropriate to detect the replicon-specific regions of corresponding plasmids in cultured bacteria or in total community DNA which share sufficient DNA similarity with reference plasmids at the amplified regions. Testing of different primer sets against the collection of plasmids belonging to 21 different Inc groups revealed that each of the primers reacts specifically with the plasmid of the Inc group for which it was designed. However, specificity can be predicted only for the collection of reference plasmids belonging to 21 different Inc groups. The use of *trfA2* and *korA* primer systems for IncP plasmid detection had previously been suggested by Thomas and Thorsted (34). However, the primer systems they proposed have not been tested for specificity against reference plasmids belonging to other Inc. groups.

To clarify the intriguing question of why a certain proportion of IncW, IncN, and IncP group plasmids were not PCR amplifiable by the selected primer systems, plasmid DNAs were

TABLE 3. Characterization of plasmids grouped by traditional Inc testing to IncP by PCR and dot blot hybridizations

| Designation | Origin | Size (kb) | Phenotypic traits[a] | Hybridization[b] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | IncP *trfA1* | | IncP *traG* (PCR [α]) | IncP *oriT* | | | | IncP *trfA2* | | | | IncP *korA* | | | |
| | | | | PCR (α) | Dot (α) | | PCR | | Dot | | PCR | | Dot | | PCR | | Dot | |
| | | | | | | | α | β | α | β | α | β | α | β | α | β | α | β |
| RP4 | *P. aeruginosa* | 60 | Tc, Km, Ap | + | + | + | + | − | + | − | + | − | + | − | + | − | + | − |
| pTH10 | *RP4 rep-ts* | 70 | Tc, Km, Ap | + | + | + | + | − | + | − | + | − | + | − | + | − | + | − |
| R751 | *K. aerogenes* | 53 | Tp | − | − | − | − | + | − | + | − | + | − | + | − | + | − | + |
| pSS50 | *Alcaligenes* sp. | 51 | $Bph^+$, $Cbp^+$ | − | − | − | − | + | − | + | − | + | − | + | − | + | − | + |
| pJP4 | *A. eutrophus* | 80 | Hg | − | − | − | − | + | − | + | − | + | − | + | − | + | − | + |
| pIE521 | *E. coli* | 41 | Tc, Cm, Sm, Su, Ap | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| pIE726 | *E. coli* | 61 | Cm, Su, Ap | − | − | − | − | + | − | + | − | − | − | + | − | + | − | + |
| pIE1057 | *Salmonella enteritidis* | 70 | Sm, Su, Tc, Km, Ap | + | + | + | + | − | + | − | + | − | + | − | + | − | + | − |
| pIE1058 | *Shigella sonnei* | 114 | Tc, Sm, Cm, Su, Ap | − | − | − | − | − | (+) | − | − | − | − | − | − | − | − | − |
| pIE1059 | *P. aeruginosa* | 70 | Tc, Sm, Cm, Su, Ap, Km, Gm, Hg | + | + | + | + | − | + | − | + | − | + | − | + | − | + | − |
| pIE1060 | *Shigella sonnei* | 91 | Tc | − | − | − | − | − | (+) | − | − | − | − | − | − | − | − | − |
| pIE1061 | *Salmonella isangi* | 91 | Tc, Cm, Ap | − | − | − | − | − | (+) | − | − | − | − | − | − | − | − | − |
| pIE1062 | *Salmonella typhimurium* | 52 | Tc, Ap | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| pG527 | Exogenous, pig manure | 45 | Tc, Sm, Ap, Km, St | + | + | + | + | − | + | − | + | − | + | − | + | − | + | − |
| R172 | *E. coli* | 61 | Sm, Tp, Ap | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |

[a] Antibiotic resistances (micrograms per milliliter): Ap, ampicillin (30); Cm, chloramphenicol (20); Gm, gentamicin (20); Hg, mercuric chloride (10); Km, kanamycin (50); Sm, streptomycin (30); Su, sulfonamide (50); Tc, tetracycline (20); Tp, trimethoprim (20). Degradation: $Bph^+$, biphenyl; $Cbp^+$, 4-chlorobiphenyl.

[b] Hybridization with digoxigenin-labeled, PCR-derived probes with either the IncPα (RP4) or IncPβ (R751) template. Hybridization signal: +, strong; (+), weak; −, none.

TABLE 4. Testing of plasmids grouped by traditional Inc testing to IncW by PCR and dot blot hybridizations

| Designation | Origin | Size (kb) | Antibiotic traits[a] | Hybridization[b] | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | IncW *oriT* | | IncW *oriV* | | IncW *trwAB* | |
| | | | | PCR | Dot | PCR | Dot | PCR | Dot |
| Rsa | Reference plasmid | 39 | Cm, Sm, Su | + | + | + | + | + | + |
| R388 | Reference plasmid | 33 | Su, Tp | + | + | + | + | + | + |
| pIE306 | *Salmonella typhimurium* | 30 | Ap | − | − | − | − | − | − |
| pIE321 | *Salmonella dublin* | 38 | Tc | + | (+) | + | + | − | − |
| pIE384 | *Shigella sonnei* | 29 | Cryptic | − | − | − | − | − | − |
| pIE385 | *Shigella sonnei* | 33 | Ap | − | − | − | − | − | − |
| pIE522 | *K. pneumoniae* | 38 | Su, Gm | + | + | + | + | + | + |
| pIE638 | *E. coli* | 30 | Sm, St | − | − | − | − | − | − |
| pIE773 | *E. coli* | 30 | Su, Tp | + | + | + | + | + | + |
| pIE774 | *Shigella flexneri* | 38 | Tc | − | (+) | + | + | − | − |
| pIE775 | *E. coli* | 38 | Tc, Cm, Sm, Su | − | − | − | − | − | − |
| pIE1056 | *Salmonella typhimurium* | 30 | Sm, St | − | − | − | − | − | − |
| pIE1075 | *E. coli* | 91 | Tc, Sm, Su, Tp | − | − | − | − | − | − |
| pIE1077 | *C. freundii* | 38 | Tc | − | − | − | − | − | − |
| pIE1078 | *Shigella sonnei* | 35 | Cm, Sm | − | − | − | − | − | − |
| pIE1079 | *E. coli* | 44 | Sm, St | − | − | − | − | − | − |
| pIE1080 | *Enterobacter cloacae* | 38 | Sm, Tp | − | − | − | − | − | − |
| pIE1081 | *E. cloacae* | 38 | Sm, Su, Ap, Gm | − | − | − | − | − | − |
| pIE1095 | *Salmonella typhimurium* | 38 | Sm, St | − | − | − | − | − | − |
| pIE1097 | *Salmonella dublin* | 38 | Tc, Cm, Ap | − | − | − | − | − | − |
| pIE1098 | *Salmonella typhimurium* | 38 | Ap | − | − | − | − | − | − |
| pIE1099 | *E. coli* | 38 | Sm, St | − | − | − | − | − | − |
| pIE1102 | *E. coli* | 39 | Sm, St | − | − | − | − | − | − |
| pIE1105 | *Salmonella dublin* | 30 | Tc | + | + | + | + | − | − |

[a] Antibiotic resistances (micrograms per milliliter): Ap, ampicillin (30); Cm, chloramphenicol (20); Gm, gentamicin (20); Sm, streptomycin (30); St, streptothricin (50); Su, sulfonamide (50); Tc, tetracycline (20); Tp, trimethoprim (20).
[b] Hybridization with the digoxigenin-labeled, PCR-derived probes with R388 as a template. Hybridization signal: +, strong; (+), weak; −, none.

analyzed in dot blots and Southern blots, using different replicon-related probes. Analysis of PCR-negative IncP, IncN, or IncW group plasmids on dot blots and Southern blots showed that PCR amplification failed, not only because of sequence divergence at the sites for primer annealing but because of the fact that the plasmids lacked the required sequence of sufficient homology. Therefore, PCR-negative plasmids must be different, at least for the DNA regions analyzed (homology of less than 75%). It is unclear whether the PCR- and dot blot-negative plasmids assigned to IncN, IncW, or IncP by traditional testing are "outliers" or whether they belong to new groups of broad-host-range plasmids. Interestingly, the PCR-negative IncW plasmids were grouped as an IncW subgroup (IncW-3) only on the basis of phage testing and molecular size and not on the basis of incompatibility with R388. On the basis of our PCR and hybridization results, these broad-host-range plasmids most likely belong to a new Inc group. A more detailed characterization of these plasmids is in progress.

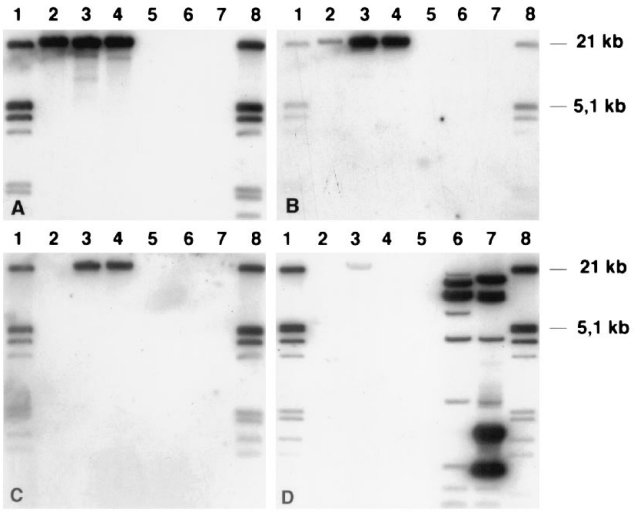


FIG. 2. Southern blot hybridization of *Eco*RV-digested plasmids grouped by traditional Inc testing to IncW. Lanes: 1 and 8, ladder (digoxigenin labeled); 2, pIE774 (PCR positive); 3, Rsa (PCR positive); 4, R388 (PCR positive); 5, empty; 6, pIE1102 (PCR negative); 7, pIE1056 (PCR negative). The probes (digoxigenin labeled) were *oriV* (A), *oriT* (B), *trwAB* (C), and pIE1056 (D).

Our findings support the conclusion that classification of plasmids based on incompatibility, donor-specific phage propagation, and molecular size does not necessarily correspond with plasmid classification based on replicon typing. Plasmid grouping ideally should be based on phenotypic and genotypic characteristics. The consistent application of genotypic and phenotypic tests for plasmid grouping may lead to a reclassification of plasmids comparable with the changes that have occurred in bacterial systematics because of the use of 16S or 23S rRNA gene sequences for determining phylogenetic relationships.

The primer systems described here are appropriate for PCR-based detection of broad-host-range plasmids belonging to the IncQ, IncN, IncW, and IncP groups in culturable and nonculturable bacteria. However, the detection of plasmids will be limited to those replicons which have close sequence similarities with the reference plasmids. The PCR amplification of IncQ *oriV* and *repB* sequences in total community DNA extracted from manure and soil samples showed that IncQ plasmids seem to occur much more frequently than expected. This finding is based on long-term epidemiological studies on the prevalence of antibiotic resistance plasmids in different envi-

TABLE 5. Screening of direct DNA extracts originating from pig manure slurries and different soils by means of PCR with subsequent Southern blot hybridizations for the presence of IncQ, IncPα, IncPβ, IncN, and IncW plasmids

| Designation | Origin and description[a] | Hybridization[b] | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | IncQ | | | IncP | | | | | | IncN | | | IncW | |
| | | | | | *trfA2* | | *oriT* | | *korA* | | | | | | |
| | | *repB* | *oriV* | *oriT* | α | β | α | β | α | β | *repA* | *oriT* | *kikA* | *oriV* | *oriT* |
| Manure 3.1 | Derenburg, Germany | + | + | + | + | + | (+) | − | + | + | (+) | (+) | − | − | − |
| Manure 3.2 | Derenburg, Germany | + | + | + | + | + | − | + | + | + | (+) | (+) | − | + | − |
| Manure 4.1 | Derenburg, Germany | + | + | + | + | + | − | + | + | + | + | + | + | (+) | + |
| Manure 4.2 | Derenburg, Germany | + | + | + | + | + | (+) | + | − | + | + | + | + | (+) | + |
| Manure 5.1 | Derenburg, Germany | + | + | + | + | + | − | + | + | + | + | + | + | + | − |
| Manure 5.2 | Derenburg, Germany | + | + | + | + | + | (+) | + | + | + | + | (+) | (+) | + | − |
| Soil 1.1 | Ahlum, Germany | − | − | − | + | − | − | − | (+) | − | − | − | − | − | − |
| Soil 1.2 | Ahlum, Germany | − | − | − | − | − | − | (+) | (+) | − | − | − | − | − | − |
| Soil 2.1 | BBA1, Germany | − | − | − | − | + | − | − | − | − | − | − | − | − | − |
| Soil 2.2 | BBA2, Germany | − | − | − | + | − | − | − | − | − | − | − | − | − | − |
| Soil 3.1 | Oberviehhausen, VR1, Germany | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| Soil 3.2 | Oberviehhausen, VR2, Germany | − | − | − | + | − | − | − | − | − | − | − | − | − | − |
| Soil 3.3 | Oberviehhausen, VR3, Germany | − | − | − | (+) | + | − | (+) | (+) | + | − | − | − | − | − |
| Soil 3.4 | Oberviehhausen, VR4, Germany | − | (+) | − | (+) | + | − | − | (+) | + | − | − | − | − | − |
| Soil 4.1 | Oberviehhausen, VS1, Germany | + | − | − | + | (+) | − | − | − | − | + | + | + | − | − |
| Soil 4.2 | Oberviehhausen, VS7, Germany | − | (+) | − | (+) | (+) | − | (+) | − | (+) | − | − | − | − | − |
| Soil 5.1 | Holland, treated | − | − | − | + | + | − | (+) | − | + | − | − | − | − | − |
| Soil 5.2 | Holland, untreated | + | + | (+) | + | (+) | − | − | − | − | − | − | − | − | − |
| Soil 6.1 | Holland, treated | − | − | − | + | + | − | − | − | − | − | − | − | − | − |
| Soil 6.2 | Holland, untreated | − | − | − | (+) | (+) | − | − | − | − | − | − | − | − | − |
| Soil 7.1 | Holland, treated | + | + | − | + | + | − | − | − | (+) | − | − | − | − | − |
| Soil 7.2 | Holland, untreated | + | + | − | + | (+) | − | − | − | − | − | − | − | − | − |

[a] Ahlum, silt loam; BBA, loamy sand; Oberviehhausen, silt loam (VS, field site planted with sugar beets; VR, plot where sugar beets were incorporated into soil after shredding); Holland, silt loam treated with dichloropropene or untreated.

[b] Hybridization signal: +, strong; (+), weak; −, none.

ronments, which were done by applying traditional endogenous plasmid isolations. The detection of IncQ plasmids in manure and soils is very interesting with respect to antibiotic resistance gene circulation in the environment. Thus, Sundin and Bender (32) and Sundin et al. (33) reported the appearance of streptomycin resistance plasmids in *Pseudomonas syringae* in peach orchards sprayed with streptomycin and copper. Sequence analysis of the plasmid-borne resistance genes showed that the sequence associated with the transposon Tn*5393* was identical to that of the RSF1010 genes *strA and strB* ($Sm^r$). The detection of IncP *trfA2* and *korA* sequences after PCR amplification and hybridization with the respective IncPα- and IncPβ-derived probes in total DNAs from some soil samples or manure slurries is a clear indication that IncP plasmids are prevalent in these habitats. The isolation of IncP plasmids from polluted environments has also been described by others (7, 12, 22, 35). The incidence of IncP plasmids in normal agricultural soils detected by means of PCR is of great importance with respect to the phylogenetic relationship of the P-type conjugative transfer system to the T-DNA transfer system of the self-transmissible Ti plasmid of *Agrobacterium tumefaciens* (18). The presence of plasmids, such as IncP plasmids, in soils indicates that these habitats are likely to have a gene-mobilizing capacity with implications for the potential dissemination of introduced recombinant DNA. In contrast to IncQ and IncP replicon-specific sequences, IncN and IncW plasmid-specific sequences could, with one exception, be detected only in manure slurries. Obviously, these plasmids and their preferred hosts do not appear to be prevalent in soils or are at least below the detection limit (estimated to be $5 \times 10^2$ to $1 \times 10^3$ targets per g of soil).

We have shown clearly that the detection of broad-host-range plasmids belonging to the IncP, IncQ, IncN, and IncW groups by means of PCR is feasible. The systematic application of the selected replicon-specific primers to total DNA extracts from different terrestrial and aquatic samples will enlarge our knowledge of the prevalence and incidence of these plasmids in bacterial communities in the environment. Furthermore, the usefulness of PCR and hybridization with PCR-derived probes for plasmid classification and studies on plasmid diversity could be demonstrated. Further investigations on the genetic relatedness of PCR-negative plasmids and the sequenced reference plasmid are in progress.

## ACKNOWLEDGMENTS

We are grateful to E. Lanka for critical reading of the manuscript and for stimulating discussion. We also thank A. Wolters, G. Carrette, and E. Krögerrecklenfort for their excellent technical assistance. F. Gebhard and B. Engelen are acknowledged for providing soil DNA.

This research was supported by a grant from the EU-BIOTECH programme BIO-CT and by the BMBF project 0310016A.

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