Manure Enhances Plasmid Mobilization and Survival of *Pseudomonas putida* Introduced into Field Soil

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We report a field study on plasmid mobilization in an agricultural soil. The influence of pig manure on the mobilization of the IncQ plasmid pIE723 by indigenous plasmids or by the IncP α plasmid pGP527 into the recipient *Pseudomonas putida* UWC1 (Rif^r Nal^r) was studied in field soil. Six plots were prepared in duplicate, three of which were treated with manure prior to inoculation of the donor and recipient strains. As a donor strain, either *Escherichia coli* J53(pIE723) or *E. coli* 600(pIE723, pGP527) was used. Putative transconjugants obtained on a selective medium were confirmed by DNA hybridization and PCR. Plasmid mobilization by indigenous mobilizing plasmids was observed on two occasions in manured soil. Manuring of soil significantly enhanced the frequency of pIE723 mobilization by pGP527, since mobilization frequencies into *P. putida* UWC1 were at least 10-fold higher in manured soil than in nonmanured soil. Enhanced numbers of *P. putida* UWC1 transconjugant and recipient colonies could be observed in manured soil throughout the 79-day field test. Transfer of pIE723 or pGP527 into indigenous soil or rhizosphere bacteria could not be detected when indigenous bacteria isolated by selective cultivation were screened for the presence of these plasmids by DNA hybridization. Furthermore, the presence of IncN-, IncP-, or IncQ-specific sequences was confirmed in total community DNA extracted directly from the manured or nonmanured soil by PCR. IncW plasmids were detectable only in manured soil, indicating entry of these plasmids into soil via manure.

The possibilities of using genetically modified microorganisms (GMOs) for environmental or agricultural purposes raise questions about a possible dissemination of engineered genes into the indigenous microflora. Conjugation and mobilization have been considered major processes of horizontal gene transfer (29, 33). The capacity of the indigenous soil bacteria to mobilize introduced transgenic DNA should be taken into consideration when estimating the probability of gene exchange between GMOs and indigenous bacteria. Studies on plasmid mobilization and retromobilization in sterile and non-sterile soil microcosms with defined soil and/or incubation conditions have been reported by several investigators (7, 8, 20, 22, 31, 38, 40). Henschke and Schmidt (8) first reported the mobilization of an IncQ derivative in soil microcosms. Smit et al. (31) found 100-fold-lower mobilization frequencies of the IncQ derivative pSKTG in soil with triparental matings in which the mobilizing Tra⁺ IncP plasmid and the Tra⁻ Mob⁺ IncQ plasmid were inoculated in two separate strains than with biparental matings. This finding was explained by an impediment of cell-to-cell contact of the three mating partners in soil. However, in situ plasmid mobilization by indigenous mobilizing elements has not yet been described (7, 20, 31). On the other hand, the presence of mobilizing plasmids in terrestrial and aquatic environments has recently been demonstrated by several authors (10–12, 39) by use of an exogenous triparental plasmid isolation technique. A variety of factors have been reported to stimulate plasmid transfer in soil, such as nutrient availability, temperature, selective pressure, root exudates, plasmid-encoded pilus shape, soil texture, clay content, and pH (2, 16, 20, 24, 32, 33, 38, 40, 44, 46, 50). The effect of manure slurry on plasmid transfer in soil has not yet been studied.

Manure represents an environment rich in nutrients and plasmid-containing bacteria (3, 41, 42). In light of the large-scale application of manure slurry onto agricultural soils, the possible influence of manure on plasmid mobilization and on the colonization of inoculated strains should be considered.

Our knowledge of plasmid transfer in soil under field conditions is still rather limited, since only a few studies on plasmid transfer in the open field have been performed to date (17). Although there is evidence that conjugative plasmids occur in polluted and nonpolluted soils, none of the studies performed to date could show mobilization of Tra⁻ Mob⁺ plasmids by indigenous mobilizing elements in soil. Since the mobilization of a Tra⁻ Mob⁺ plasmid by indigenous mobilizing plasmids requires the contact of three different cells or in the case of retromobilization two cells, low cell densities might reduce the probability of successful cell-to-cell contact (31). Furthermore, the physiological state of donor cells has been shown to be crucial for plasmid transfer (18).

The main purpose of this study was to investigate the influence of pig manure on the mobilization of an IncQ plasmid into an introduced *Pseudomonas putida* recipient strain in soil. In contrast to other investigations of plasmid mobilization in soil, this study was performed under field conditions. An *Escherichia coli* strain harboring the IncQ plasmid pIE723 was introduced into soil. The detection of transconjugants would indicate the presence of indigenous mobilizing elements. Since it was supposed that plasmid mobilization by indigenous mobilizers might be a very rare event in the soil (30, 31), an *E. coli* donor strain harboring pIE723 and the mobilizing IncP α plasmid pGP527 were also used. Both plasmids have been recently isolated from environmental samples (5, 35). Since no natural background of these plasmids could be detected in soil, the transfer of plasmids pIE723 and pGP527 into indigenous soil or rhizosphere bacteria of the manured and nonmanured soil could be traced by selective cultivation in conjunction with DNA hybridization.

To investigate the possibility of an entry of broad-host-range

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TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristics	Drug resistance markers ^a	Reference(s)
pIE723	IncQ, 9.5 kb, Tra ⁻ Mob ⁺	Gm (<i>aadB</i>), Km, Sm, Su	23, 35
pGP527	IncP α , 75 kb, Tra ⁺	Ap, Km, Tc, Sm, St (<i>sat-2</i>)	5

^a Ap, ampicillin; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Su, sulfonamide; St, streptothricin; Tc, tetracycline; *sat*, streptothricin acetyl transferase.

plasmids via manure into soil, the occurrence of IncN-, IncP-, IncW-, and IncQ-specific sequences was determined in manured and nonmanured soil by PCR amplification of total community DNA with primer systems for replicon-specific regions of these Inc groups (5).

MATERIALS AND METHODS

Bacterial strains and plasmids. The plasmids used in this study are listed in Table 1. Plasmid pGP527 was previously isolated from pig manure (5). *E. coli* C600 harboring pIE723 and pGP527, used as donor 1, and *E. coli* J53 harboring only pIE723, used as donor 2, were kindly provided by E. Tietze and H. Tschäpe. A spontaneous mutant of *P. putida* UWC1 resistant to rifampin and nalidixic acid was used as the recipient; *P. putida* UWC1 was obtained from J. Fry, Cardiff, United Kingdom. To study back-transfer of pGP527 from soil into *E. coli*, strain CV601 (Thr⁻ Leu⁻ Thi⁻ Rif^r) (20) was used as a recipient in the filter matings.

Media and growth conditions. (i) **Total aerobic plate counts.** Culturable bacteria from soil and pig manure were enumerated on plate count agar (PCA; Merck, Darmstadt, Germany) after 2 days of incubation at 28°C. Cycloheximide (200 µg/ml) was added to selective media to suppress fungal growth on plates.

(ii) **Generation of inoculants.** Donors and recipients were grown overnight in Luria-Bertani broth (25) supplemented with appropriate antibiotics. Cells were harvested by centrifuging overnight cultures, washing them once, and resuspending the pellet in 0.85% NaCl.

(iii) **Enumeration of recipient and transconjugants.** The recipient *P. putida* UWC1 was enumerated on Caso-Agar (Merck) supplemented with 350 µg of nitrofurantoin, 2 µg of crystal violet, 100 µg of rifampin, 20 µg of nalidixic acid, 30 µg of chloramphenicol, and 200 µg of cycloheximide, each per ml. Transconjugants with pIE723 were obtained on the medium selective for the recipient supplemented with 20 µg of gentamicin per ml and 50 µg of kanamycin per ml, whereas those with pIE723 and pGP527 were selected on this medium supplemented with 100 µg of nourseothricin per ml in addition. Transconjugants and recipients were counted after 48 h of incubation at 28°C.

(iv) **Detection of putative indigenous transconjugants.** To detect transfer of pIE723 or pGP527 into indigenous soil or rhizosphere bacteria, soil or rhizosphere bacteria were grown on PCA supplemented with 20 µg of gentamicin, 30 µg of streptomycin, and 200 µg of cycloheximide or 30 µg of kanamycin, 20 µg of tetracycline, and 200 µg of cycloheximide, each per ml.

(v) **Exogenous isolation of pGP527 in *E. coli* CV601 from soil.** Yeast extract medium (peptone, 15 g; yeast extract, 10 g; NaCl, 6 g; agar, 13 g; H₂O, 1 liter [pH 7.5]) was used for filter matings. Putative *E. coli* CV601 transconjugants which carried pGP527 were selected on M9 minimal medium (20, 25) supplemented with 30 µg of rifampin per ml, 20 µg of L-threonine per ml, 20 µg of L-leucine per ml, 2 µg of thiamine per ml, trace elements, 30 µg of kanamycin per ml, 20 µg of tetracycline per ml, and 200 µg of cycloheximide per ml.

Soil and manure slurry. The field experiment was performed in the area of the Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA), Braunschweig, Germany. The BBA soil was determined to be a loamy sand (pH 7.0) with an organic matter content of 0.9% and a clay content of 12.0% (data kindly provided by the Institut für Unkrautforschung, BBA). The manure slurry (pH 7.4), which was aged at least 6 months, was obtained from a pig mast in Derenburg, Germany.

Field design and soil inoculation. Six different plots (0.5 m by 0.5 m) were prepared in duplicate and arranged according to a randomized latin square. The plots received the following treatments: plot 1, manure, *P. putida* UWC1, *E. coli* CV600(pIE723, pGP527); plot 2, manure, *P. putida* UWC1, *E. coli* J53(pIE723); plot 3, *P. putida* UWC1, *E. coli* CV600(pIE723, pGP527); plot 4, *P. putida* UWC1, *E. coli* J53(pIE723); plot 5, manure; and plot 6, none. The pig manure slurry was evenly applied to the soil (3 liters/m²) and homogenized into the top 10 cm by raking. Suspensions of the recipient and the donor were introduced into the soil (1 ml/100 g of soil assuming a 10-cm soil depth) approximately 1 h after manure treatment and homogenized by raking. Grass seeds (commercial DSV mixture) were sown into the soil 3 days after manure treatment and strain inoculation. A dense lawn had grown 16 days after sowing.

Sampling and extraction of bacteria from soil, rhizosphere, and manure. Composite soil samples which contained at least four single samples were collected by means of a sampling cane (Pürckhauer Bohrstock; inner diameter, 18 mm) to a depth of 10 cm and homogenized by sieving through a 2-mm-pore-size sieve. Five grams of soil in 0.85% NaCl (45 ml) was treated in a stomacher laboratory blender for 1 min at high speed. Grass rhizosphere bacteria were

extracted from 10-g samples of roots with adhering soil by a protocol modified from that of Herron and Wellington (9) and Jacobsen and Rasmussen (15). The samples were placed in sterile plastic bags and processed twice with 40 ml of phosphate-buffered saline (25) and 10 ml of NDP (0.1 g of sodium deoxycholate and 3 g of polyethylene glycol in 100 ml of double-distilled water) with 1 g of Chelex 100 (9) in a stomacher blender. Separation of bacterial cells from soil and root particles was reached by differential centrifugation.

Pig manure slurry was treated in a stomacher laboratory blender before plating. The resulting suspensions were serially diluted and plated on selective agar in duplicate.

Filter mating. The back-transfer of pGP527 from soil was performed by biparental filter mating as described by Bale et al. (1) with *E. coli* CV601 as the recipient and the manured or nonmanured soil bacterial communities as the donor organisms. The soil samples were preincubated at 28°C for 24 h prior to use.

Verification of transconjugants. The presence of pIE723 or pGP527 in *P. putida* UWC1 was screened by dot blot hybridization with probes specific for pIE723 (*repB* and *aadB* genes) (5, 21) or for pGP527 (IncP α *trfA2* and *sat-2* genes) (5, 34, 36). Transconjugants were analyzed for the acquisition of pIE723 or pGP527 by plasmid extraction by use of a modified protocol described by Holmes and Quigley (13) for IncQ or one described by Ish-Horowitz and Burke (14) that was modified to include a phenol extraction of the potassium acetate supernatant (43). *SphI*-digested IncP α or *NcoI*-digested IncQ plasmid DNA was analyzed by agarose gel electrophoresis, and blots were prepared and hybridized as described below. In addition, plasmid pIE723 or pGP527 was confirmed by PCR with primers specific for IncQ *repB* and the *aadB* gene located on pIE723 (5, 21) or IncP *trfA2* and the *sat-2* gene located on pGP527 (5, 20). PCR products were hybridized with the respective PCR-derived probes as described below. Transconjugants of the filter mating were confirmed by repetitive extragenic palindromic (REP)-PCR fingerprinting (49).

Total community DNA extraction from soils. Manured and nonmanured soils without introduced strains were collected on six different occasions after manure application for total community DNA extractions by a protocol described by Smalla et al. (27) followed by two-step purification and Wizard DNA (Promega, Madison, Wis.) clean-up as described by Van Elsas and Smalla (48). The DNA was used as a template for PCR amplification with different Inc group-specific primers (5).

PCR. The primer systems used in this study and respective PCR conditions are described elsewhere (5, 20, 21, 49).

Dot blot and Southern blot hybridization. Lysozyme-treated cells (100 µl of overnight culture incubated with 50 µl of lysozyme (4 mg/ml) for 2 h at 37°C in a microtiter plate) were transferred onto a Hybond-N nylon membrane with a dot blot apparatus. Dot blot and Southern blot PCR product and plasmid digests were performed as described by Sambrook et al. (25) and hybridized by use of the protocol of the digoxigenin detection kit (Boehringer GmbH, Mannheim, Germany).

Probes. PCR products of respective reference plasmids (for IncQ and the *aadB* gene, pIE723; IncP α , RP4; IncP β , R751; IncN, RN3; IncW, R388) were labeled with digoxigenin by using random-primed digoxigenin labeling as described in the Boehringer protocol (5). The probe specific for the *sat-2* gene has been described elsewhere (36).

Statistics. Plots 1 to 6 were prepared in duplicate. Filter matings and bacterial extraction from composite soil samples were carried out in duplicate; platings were also performed in duplicate. The standard error was calculated for counts of recipients and transconjugants. Student's *t* test was used to determine the significance of differences in CFU values. Differences in transfer frequencies were analyzed by analysis of variance (*P* < 0.5).

RESULTS

Influence of manure slurry on plasmid mobilization in the soil environment. To study the effect of manure on plasmid mobilization, *E. coli* donors harboring either the IncQ plasmid pIE723 or pIE723 and the IncP α plasmid pGP727 and the recipient *P. putida* UWC1 were inoculated into manured and nonmanured soil. The appearance of *P. putida* UWC1 transconjugants was detected by selective cultivation. Plating of the uninoculated soil and the pig manure on the respective selective media resulted in no background growth of rifampin-

FIG. 1. Mobilization of the IncQ plasmid pIE723 by the IncP α plasmid pGP527 (both present in *E. coli* CV600) to the recipient *P. putida* UWC1 in the manured soil environment.

resistant indigenous bacteria. Total aerobic counts of culturable bacteria were approximately 10^7 CFU per g of dry soil and 10^5 CFU per ml of manure slurry. The initial inoculum level of the recipient (plots 1 to 4) was about 10^6 CFU per g of dry soil.

Mobilization of pIE723 into the recipient by the IncP α plasmid pGP527 was detectable in manured and nonmanured soil (Fig. 1 and 2). Moreover, the number of transconjugants harboring pIE723 in manured soil was approximately 3 orders of magnitude higher than that in soil without manure. In manured soil, about 1% of the *P. putida* recipient population contained the IncQ plasmid pIE723. The number of transconjugants harboring both the mobilized plasmid pIE723 and the mobilizing plasmid pGP527 was 10-fold lower than the number of transconjugants harboring only pIE723 (Fig. 1), as determined by selective plating and DNA hybridization. In nonmanured soil, the percentage of *P. putida* containing pIE723 was lower (approximately 0.1%), and thus the transfer frequencies in manured soil were 10-fold higher than that in nonmanured soil. No *P. putida* UWC1 transconjugants containing both pIE723 and pGP527 could be detected in nonmanured soil. In nonmanured soil, transconjugants decreased to the detection limit on day 6 and were then detectable only up to day 36 (Fig. 2).

The mobilization of pIE723 by indigenous plasmids was detected on days 2 and 4 and only in manured soil (Fig. 3). Manure obviously enhanced the mobilizing capacity of the microbial community in soil, resulting in a detectable natural mobilization event. The mobilization frequency of pIE723 by indigenous plasmids on the order of 10^{-7} was 4 orders of magnitude lower than that in manured soil inoculated with *E. coli* C600(pIE723, pGP527).

All transconjugants analyzed (at maximum, 16 colonies per treatment and sampling) were DNA hybridization and PCR positive. *Sph*I and *Nco*I digests of plasmid DNA isolated from

a selection of transconjugants gave no indication of plasmid rearrangements under environmental conditions.

Influence of manure on the fate of the introduced *P. putida* UWC1 in the soil environment. In this study, manure had a stimulating effect on the growth and survival of the introduced recipient *P. putida* UWC1. The numbers of *P. putida* organisms increased from about 10^6 to 10^8 CFU/g of soil after strain inoculation into manured soil (Fig. 1 and 3). A decrease in the numbers of *P. putida* UWC1 was observed only from day 4 on. Ten days after strain inoculation, the CFU of the recipient were comparable to the initial inoculum level. In contrast, the *P. putida* numbers decreased from day 1 in nonmanured soil (Fig. 2). The number of *P. putida* UWC1 was approximately 2 orders of magnitude higher in manured soil than in nonmanured soil, and this was detectable throughout the 79-day field test period. Approximately 5 months after inoculation, the effect of manure on the survival of *P. putida* UWC1 was still detectable. The numbers of UWC1 were also higher in the rhizosphere of manured soil than in the absence of manure (data not shown).

Transfer of plasmids pIE723 and pGP527 into indigenous bacteria. The transfer of pIE723 and pGP527 into indigenous bacteria in manured and nonmanured soil was investigated 90 days after inoculation, and the transfer of pGP527 into bacteria of the grass rhizosphere was investigated 145 days after inoculation. Approximately 1×10^5 CFU/g of soil were grown on PCA supplemented with gentamicin, streptomycin, and cycloheximide, and 5×10^4 CFU/g of soil were grown on PCA with tetracycline, kanamycin, and cycloheximide. Colonies (total, 180; 30 from each treatment) with different morphologies were picked from all dilutions (mainly 10^{-2} and 10^{-3}) and screened by dot blot hybridizations for the presence of pIE723 or pGP527. One hybridization-positive colony was identified as *P. putida* UWC1 by growth on the UWC1-selective medium

FIG. 2. Mobilization of the IncQ plasmid pIE723 by the IncP α plasmid pGP527 (both present in *E. coli* CV600) to the recipient *P. putida* UWC1 in the nonmanured soil environment.

and REP patterns. No indigenous transconjugants from soil or rhizosphere could be detected.

To monitor the fate of pGP527 independently from the culturability of its host, an exogenous plasmid isolation with *E. coli* CV601 as the recipient and the bacterial fraction enriched from manured and nonmanured soil as the donor was performed. No background growth of indigenous bacteria was observed on the CV601-selective agar. Colonies grown after plating the filter mating products of plot 1B (two parallel filter matings, FM1 and FM2) and incubating for 48 h showed two different colony morphologies. REP-PCR fingerprints of the transconjugants were compared with those of *E. coli* CV601 and *P. putida* UWC1. REP fingerprints indicated that both *E. coli* CV601 and *P. putida* UWC1 harboring pGP527 as confirmed by DNA hybridization grew on the plates. However, plasmid pGP527 could be exogenously isolated in *E. coli* CV601 only from manured soil from plot 1B. *E. coli* CV601 transconjugants (9.6×10^2 CFU/ml) were detectable only in one of the parallel filter mating products (FM1) of plot 1B. The transfer frequency of 3.5×10^{-8} indicated a low number of pGP527-carrying host bacteria in the soil environment. *P. putida* UWC1 harboring pGP527 could be isolated on the CV601-selective medium from both parallels (FM1, 1.5×10^3 CFU/ml; FM2, 6×10^1 CFU/ml), indicating that the introduced UWC1 was still present in manured soil approximately 5 months after inoculation. Thus, it is supposed that *P. putida* UWC1(pGP527) served as the donor for the back-transfer of pGP527 to *E. coli* CV601.

Detection of broad-host-range replicon-specific sequences in total community DNAs. The occurrence of sequences specific for broad-host-range plasmids in total community DNA from manured and nonmanured soil (plots 5 and 6) was determined by PCR to evaluate the entry of the known broad-

host-range plasmids via manure into soil (Table 2). IncN-, IncP α -, IncP β -, and IncQ-specific sequences could be detected in soil community DNA extracted directly from manured and nonmanured soil. Considering the intensity of hybridization signals, no significant enhancement of plasmids belonging to these Inc groups was found in soil after manure application. However, IncW-specific sequences could be detected only in manured soil with the primer system specific for IncW *oriV*. Thus, it appeared that the prevalence of these plasmids was enhanced in soil by the added pig manure.

DISCUSSION

Plasmid mobilization by the indigenous soil microflora in natural soil was not detected in studies by several investigators (7, 20, 31). It is considered to be a very rare event and thus virtually undetectable in the soil environment (30, 31). Soil is known to be an oligotrophic environment, and the small amount of easily available nutrients limits bacterial growth and activity (47). Several investigators reported a stimulating effect of nutrients on plasmid transfer (2, 16, 18–20, 38, 40, 44, 46). Nutrients were added often by supplementing the soil with Luria-Bertani broth or glucose, which is unlikely to occur in nature. In this study, nutrients were added to the soil via manure. Under field conditions, plasmid mobilization by indigenous bacteria was detected on two occasions but only in manure-treated soil. This is the first time that plasmid mobilization by indigenous mobilizing elements could be observed in soil. Since we could not detect the mobilizing plasmid in the recipient, it remains unclear whether this plasmid originated from manure or soil. In the latter case, it could be supposed that manure-derived nutrients enhanced the population density and activity of indigenous soil bacteria and thus improved

FIG. 3. Mobilization of the IncQ plasmid pIE723 present in *E. coli* J53 into the recipient *P. putida* UWC1 by the indigenous plasmids in the manured and nonmanured soil environments.

the chance for successful conjugation events. Van Elsas et al. (45) reported a threshold cell density of donor and recipient cells in nonsterile soil below which plasmid transfer dropped rapidly. Conjugal transfer of plasmid RP4 was similar, with initial parental inoculum levels of about 10^7 and 10^6 CFU per g of soil. However, the transfer frequency was 100-fold lower in the rhizosphere, with an inoculum level of 10^5 CFU per g of soil, and no transconjugants were detectable in bulk soil. In this study, the initial inoculum level of donor and recipient strains was about 10^6 CFU per g of dry soil, which should be a population density adequate for plasmid transfer to be detected. Manure enhanced the survival and stimulated growth of the introduced recipient in the soil environment. Thus, the chances of cell-to-cell contact as a prerequisite for a successful mating may have been enhanced by the increased population densities of recipient strains in the manured soil. Because the population density of the *E. coli* donor strains harboring pIE723 and pGP527 or pIE723 was not determined (due to the lack of an

appropriate selective medium), it is not known whether manure also affected the survival of the plasmid donor strains. However, it is more probable that entry of mobilizing elements into soil by manure slurry enhanced the gene-mobilizing capacity of the soil since mobilization of IncQ plasmids by soil-indigenous mobilizing plasmids could also not be detected by other investigators in experiments with added nutrients (20). Accordingly, a higher number of transconjugants and higher transfer frequencies were detectable in manured soil. A long-term effect of manuring on the survival of inoculated strains and plasmid mobilization into an introduced recipient strain could be shown. The stimulating effect of manure on the colonization ability of soil inoculants might be of great biotechnological importance since sufficient survival is often critical for microbial inoculants. Different strains and manures should be studied before a general conclusion can be drawn. The *P. putida* UWC1 used as the recipient strain was nalidixic acid resistant. It was used since nalidixic acid is known to inhibit

TABLE 2. Characterization of total community DNA from manured (plot 5) and nonmanured (plot 6) soil extracted on 6 different days after manure application with primer systems specific for broad-host-range plasmids

Day	Hybridization signal ^a											
	IncN <i>oriT</i>		IncN <i>rep</i>		IncPα <i>trfA2</i>		IncPβ <i>trfA2</i>		IncQ <i>rep</i>		IncW <i>oriV</i> ^b	
	Plot 5	Plot 6	Plot 5	Plot 6	Plot 5	Plot 6	Plot 5	Plot 6	Plot 5	Plot 6	Plot 5	Plot 6
1	+	+	+	+	+	+	(+)	(+)	+	+	+, (+)	-, -
2	+	+	+	(+)	+	(+)	+	(+)	+	+	+, +	-, -
6	+	+	+	+	+	+	+	+	+	+	+, +	-, -
13	+	+	(+)	(+)	(+)	(+)	+	+	+	+	+, +	-, -
36	+	+	+	+	(+)	(+)	+	+	+	+	-, +	-, -
79	+	+	(+)	(+)	(+)	(+)	+	+	+	+	+, -	-, -

^a Symbols: +, strong; (+), weak; -, none.

^b Unlike the other PCR columns presented in this table, the results of two parallel PCR runs are shown.

plate matings when supplemented in selective agar via the inhibition of DNA gyrase activity (28). Consequently, plate counts on the selective medium used revealed the numbers of transconjugants that originated from matings in the soil environment. Although natural plasmids were used in this study, unequivocal detection of the introduced plasmids was possible since neither plasmid was found in the soil or manure used. Plasmid pIE723 is the only IncQ plasmid coding for gentamicin resistance (*aadB* gene) isolated to date (35, 37). Similarly, pGP527 is the first IncP plasmid with the Tn7-like transposon Tn1826 carrying the *sat-2* gene described (5, 26, 41, 42).

In this study, transfer of the introduced pIE723 or pGP527 into indigenous bacteria of the manured and nonmanured soil was not detectable although IncQ and IncP plasmids are known to have an extremely broad host range (4, 6). In contrast, several investigators reported plasmid mobilization (8, 31) and conjugal transfer (20, 24, 29) into the indigenous microflora. However, we looked for plasmid transfer into indigenous bacteria only 3 and 5 months after inoculation. Furthermore, the detection of indigenous transconjugants containing pIE723 or pGP527 was impeded by background growth. Although both plasmids were inoculated into soil with rather high numbers of *E. coli* donor strains, a long-term persistence in the fraction of soil bacteria accessible to cultivation techniques was not detected. In light of the PCR-based detection of IncP and IncQ replicon-specific sequences in different soils that was recently reported by Götz et al. (5) and confirmed in this study, the lack of indigenous transconjugants was surprising and one might speculate about their natural hosts. Tracking the fate of the introduced plasmids via PCR-based detection of replicon-specific sequences was impossible due to the natural presence of these sequences in uninoculated soils. This clearly shows the limitations of working with natural plasmids.

Mobilizing plasmids assigned to the groups IncW, IncN, and IncP could be isolated endogenously or exogenously from different manure slurries (41, 42) (unpublished data) and were detectable by PCR in all pig manure samples investigated previously (5). The failure to detect IncW plasmids in nonmanured soils in this study is in agreement with investigations of different soils by Götz et al. (5). In contrast, IncW plasmids were detectable with respective primer systems in all independent pig manure slurries (DNA extraction was done in duplicate) investigated previously (5) and in manured soil. An entry of IncW plasmids via manure into soil could be clearly demonstrated. The detection of IncN-, IncP-, and IncQ-specific sequences by PCR in nonmanured and manured soil indicates the presence of these sequences in soil independent from manure treatment. In conclusion, the data obtained in this study indicate a stimulating effect of pig manure on plasmid mobilization in the soil environment due not only to the presence of a mobilizing plasmid inoculated with high bacterial numbers but also to mobilizing plasmids indigenous to the manured soil. When estimating the risk of GMOs released into the environment, the dissemination of the engineered genes and the fitness of the host possibly enhanced by nutrients and/or pickup of environmental plasmids must be considered. Since manure is known to be rich in nutrients and plasmid-containing bacteria, studies on the influence of manure slurry on horizontal gene transfer in the soil environment can be recommended. Besides, the stimulating effect of manure on bacterial fitness previously introduced into soil should be taken into consideration.

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