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Plasmid Transfer between Spatially Separated Donor and Recipient Bacteria in Earthworm-Containing Soil Microcosms†

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Most gene transfer studies have been performed with relatively homogeneous soil systems in the absence of soil macrobiota, including invertebrates. In this study we examined the influence of earthworm activity (burrowing, casting, and feeding) on transfer of plasmid pJP4 between spatially separated donor (*Alcaligenes eutrophus*) and recipient (*Pseudomonas fluorescens*) bacteria in nonsterile soil columns. A model system was designed such that the activity of earthworms would act to mediate cell contact and gene transfer. Three different earthworm species (*Aporrectodea trapezoides*, *Lumbricus rubellus*, and *Lumbricus terrestris*), representing each of the major ecological categories (endogeic, epigeic, and anecic), were evaluated. Inoculated soil microcosms, with and without added earthworms, were analyzed for donor, recipient, and transconjugant bacteria at 5-cm-depth intervals by using selective plating techniques. Transconjugants were confirmed by colony hybridization with a *mer* gene probe. The presence of earthworms significantly increased dispersal of the donor and recipient strains. *In situ* gene transfer of plasmid pJP4 from *A. eutrophus* to *P. fluorescens* was detected only in earthworm-containing microcosms, at a frequency of $\sim 10^2$ transconjugants per g of soil. The depth of recovery was dependent on the burrowing behavior of each earthworm species; however, there was no significant difference in the total number of transconjugants among the earthworm species. Donor and recipient bacteria were recovered from earthworm feces (casts) of all three earthworm species, with numbers up to 10^6 and 10^4 bacteria per g of cast, respectively. *A. trapezoides* egg capsules (cocoons) formed in the inoculated soil microcosms contained up to 10^7 donor and 10^6 recipient bacteria per g of cocoon. No transconjugant bacteria, however, were recovered from these microhabitats. To our knowledge, this is the first report of gene transfer between physically isolated bacteria in nonsterile soil, using burrowing earthworms as a biological factor to facilitate cell-to-cell contact.

Advances in biotechnology have led to the production of genetically engineered microorganisms (GEMs) which have the ability to improve agriculture or remediate environmental hazards (50). Environmental risk assessment pertaining to the release of GEMs into the environment must take many factors into account. These include survival, persistence, and replication; vertical movement and dispersal through soil and water; the potential for genetic exchange with indigenous microorganisms; and possible ecosystem effects (17). For this reason there has been a heightened interest in microbial ecology specifically relating to the fate of introduced microorganisms (35).

Several studies have examined conjugal gene transfer between cointroduced donor and recipient bacteria in nonsterile soil (23, 24, 30, 34, 41, 47, 49). More recently, an increasing number of studies have been performed with indigenous soil microflora as potential recipients (4, 7, 9, 10, 16, 20, 34, 40, 41). However, there have been few investigations on the influence of invertebrates on gene transfer between soil microorganisms or GEMs (1, 2, 7, 19, 21). In addition, survival and gene transfer studies are usually performed in more-or-less homogeneous systems in which the inoculated bacteria are mixed throughout the entire soil volume. To our knowledge there have been no studies performed in which the donor and recipient bacteria were spatially separated within a nonsterile soil microcosm. In such a system, gene transfer between bacteria can occur only if abiotic or biotic factors disperse or transport the inoculated bacteria.

Invertebrates such as earthworms are often overlooked in the development of models of microbial risk assessment. Earthworms are virtually ubiquitous terrestrial invertebrates, with population sizes ranging between 0 and 400 m^{-2}. However, densities of up to 2,000 m^{-2} in soils with a high organic matter content have been reported (26). Earthworms directly and indirectly alter the biological and physiochemical properties of the soil, mainly through the processes of feeding, burrowing, and egg capsule (cocoon) and fecal (cast) deposition (26, 37, 51). The influence of earthworms on soil processes varies depending on the ecological strategies of individual species. There are over 3,000 species of earthworms, which can be placed in three broad ecological categories based on physiological, morphological, and behavioral differences (26). Epigeic earthworms are those that live in litter or on the immediate surface and do not burrow into the soil. Endogeic earthworms are considered true soil inhabitants, since they feed, burrow, and cast within the mineral soil horizon. In contrast, anecic earthworms bury surface litter in deep, vertical, semipermanent burrows and cast on the soil surface.

Recently, we demonstrated that surface-inoculated *Pseudomonas fluorescens* C5t was transported down a 40-cm soil column to depths which depended on the ecological category of the earthworm species (7). In the absence of worms, however, the *P. fluorescens* strain exhibited only limited movement. Other studies have shown that the penetration of bacteria into deeper soil layers is limited in the absence of transport mechanisms, such as burrowing earthworms or percolating water (18, 28, 31, 46). In addition, we previously demonstrated that in

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TABLE 1. Bacterial strains used in this study

Species and strain	Relevant characteristic(s)[a]	Source or reference
A. eutrophus		
JMP134(pJP4)	Hg^r Tfd^+	11
JMP222	Str^r; cured derivative of JMP134	11
JMP222N	Nal^r; mutant of JMP222	This study
JMP222N(pJP4)	Str^r Nal^r Hg^r	This study
P. fluorescens		
C5t	[R2f(Tn5::*tox*)] Rif^r Kan^r	48
C5t(pJP4)	Rif^r Kan^r Hg^r	7
E. coli		
HB101	Str^r	36
DH5α(pLD-1)	Amp^r Hg^r	7

[a] Hg^r, Str^r, Nal^r, Rif^r, Kan^r, and Amp^r, resistance to mercury, streptomycin, nalidixic acid, rifampin, kanamycin, and ampicillin, respectively; Tfd^+, ability to grow on 2,4-D.

situ gene transfer of plasmid pJP4 from surface-inoculated *P. fluorescens* C5t to indigenous soil bacteria was significantly influenced by the presence of earthworms (7). However, whether plasmid transfer occurred at the initial zone of inoculation or at the depth to which the donor was transported could not be resolved. The goal of the present study was to develop a heterogeneous soil microcosm which would unequivocally determine whether earthworm activity was solely responsible for gene transfer. To do this, we investigated the influence of diverse earthworm species on gene transfer between spatially separated donor and recipient bacteria in nonsterile soil. In addition, earthworm casts and cocoons were analyzed to determine their potential to act as microhabitats for gene transfer and dispersal.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Strains were maintained on selective media at 4°C, and long-term storage was in 50% glycerol at −80°C. *Alcaligenes eutrophus* and *P. fluorescens* strains were grown at 28°C on TY (3) and King's B (22) agar media, respectively. *Escherichia coli* strains were grown at 37°C on Luria-Bertani medium (36). Filter-sterilized antibiotic and metal stock solutions were added to media after autoclaving. The final concentrations of selective agents in media were as follows: streptomycin, 500 μg ml^{-1}; nalidixic acid, 30 μg ml^{-1}; rifampin, 30 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; ampicillin, 50 μg ml^{-1}; and $HgCl_2$, 10 μg ml^{-1}. Media used for isolation of bacteria from soil, casts, and cocoons contained cycloheximide and pimaricin at concentrations of 100 and 25 μg ml^{-1}, respectively.

The donor organism used for all soil experiments was *A. eutrophus* JMP222N containing pJP4. pJP4 is an IncP, broad-host-range plasmid containing genes for mercury resistance and 2,4-dichlorophenoxyacetic acid (2,4-D) degradation (11). Plasmid pJP4 was transferred to *A. eutrophus* JMP222N from *P. fluorescens* C5t(pJP4) by patch mating (24). The plasmid recipient strain for all soil experiments was *P. fluorescens* C5t. Transconjugant bacteria isolated from soil, earthworm casts, and cocoons were selected on King's B medium containing rifampin, kanamycin, and $HgCl_2$.

For inoculation of soil, donor and recipient bacteria were grown in selective TY broth medium to the mid- to late log phase of growth and harvested by centrifugation at 5,000 × *g* for 10 min. Cells were washed three times in a 0.1× mineral salts solution (6) (pH 7.0) and resuspended in the same solution to obtain 10^9 cells ml^{-1}.

Soil. A Hubbard loamy sand (Udorthentic Haploboroll) was used for all experiments. The soil texture for the top 30 cm of soil consisted of 85% sand, 9% silt, and 6% clay, and the soil organic matter in the surface horizon was 1.4%. The soil was freshly collected from the surface, under rye grass, at the University of Minnesota Sand Plain Experimental Station at Becker, Minn., and stored at 20 to 25°C in sealed plastic bags at field moisture content (9.1%). Before each experiment, the soil was air dried to approximately 4% moisture, sieved (2-mm pore size), and adjusted to pH 6.8 by the addition of $CaCO_3$.

For all experiments, soil was mixed with bacterial suspensions or a 0.1× mineral salts solution (pH 7.0) to give a final moisture content of 16.5%. Each microcosm was filled with soil to yield a bulk density of 1.2 g cm^{-3}.

Earthworms. Adult earthworms were provided by Edwin Berry, National Soil Tilth Laboratory, USDA Agricultural Research Service, Ames, Iowa. The earthworms were maintained at 20°C in Hubbard loamy sand soil amended with composted cow manure and dried oak leaves, with a final moisture content of 18 to 20%. According to the taxonomy cited by Fender (14), the earthworm species used in this study were the epigeic *Lumbricus rubellus* Hoffmeister 1843, with each worm weighing 0.80 to 1.21 g; the endogeic *Aporrectodea trapezoides* Dugès 1828, with each worm weighing 2.15 to 2.57 g; and the anecic *Lumbricus terrestris* Linnaeus 1758, with each worm weighing 5.08 to 6.23 g. Prior to introduction into soil microcosms, earthworms were randomly chosen from stock cultures, washed with distilled water, and placed in fresh Hubbard loamy sand soil for 2 days.

Nonsterile soil-earthworm microcosms. The microcosm design is shown in Fig. 1 and consisted of a polyvinyl chloride tube (50 by 10 cm [inside diameter]) which was cut into eight 5-cm segments. The remaining 10-cm segment served as the headspace. The segments of each tube were taped together, and the bottom was capped. Columns were disinfected with 2% *N*-alkyl dimethyl benzyl ammonium chloride (Chemical Specialties Laboratory, University of Minnesota, Minneapolis), thoroughly rinsed with distilled water, and air dried. The experimental design consisted of soil microcosms containing spatially segregated, coinoculated donor and recipient strains, inoculated control (donor or recipient strain only) soil microcosms, and uninoculated control soil microcosms. Triplicate microcosms for each of the four treatments (i.e., no worms, *L. rubellus*, *A. trapezoides*, and *L. terrestris*) were used.

The bottom 25 cm of the columns was filled with bulk uninoculated soil. This was followed by 10 cm of soil that was mixed with a suspension of recipient cells to obtain 10^8 cells g^{-1}. The remaining top 5 cm consisted of 2 cm of uninoculated bulk soil which separated the donor and recipient cells and 3 cm of soil that was mixed with a suspension of donor cells to obtain 10^8 cells g^{-1}. The inoculated control microcosms, containing either donor or recipient cells, were treated similarly, except that the corresponding recipient or donor bacterium was replaced with uninoculated bulk soil. Uninoculated control microcosms consisted of columns filled with bulk unamended soil to a depth of 40 cm.

For soil microcosms receiving earthworms, three adult earthworms of each species were randomly selected from 48-h soil cultures (see above), washed in sterile water, and added to the surface of the respective column. Each column received only one earthworm species. The final earthworm density was equivalent to approximately 100 earthworms per m^2 of soil. The tops of all columns were covered with Saran Wrap, containing air holes, and placed randomly in a moist air incubator at 20°C for 2 weeks. After 24 h of incubation, the surface of each column received earthworm food, consisting of 1.5 g of sterile dried, ground (particle size, <2 mm) oat grass and 0.5 g of oat straw moistened to 16.5% with sterile water.

Extraction and enumeration of bacteria from soil. Microcosms were analyzed for inoculated donor, recipient, and transconjugant bacteria at the end of the 2-week incubation period. Columns were sampled every 5 cm, starting at the surface. Each segment was carefully removed so as not to disturb soil and earthworms, and with the exception of the 0- to 5-cm depth, a 100-g sample of

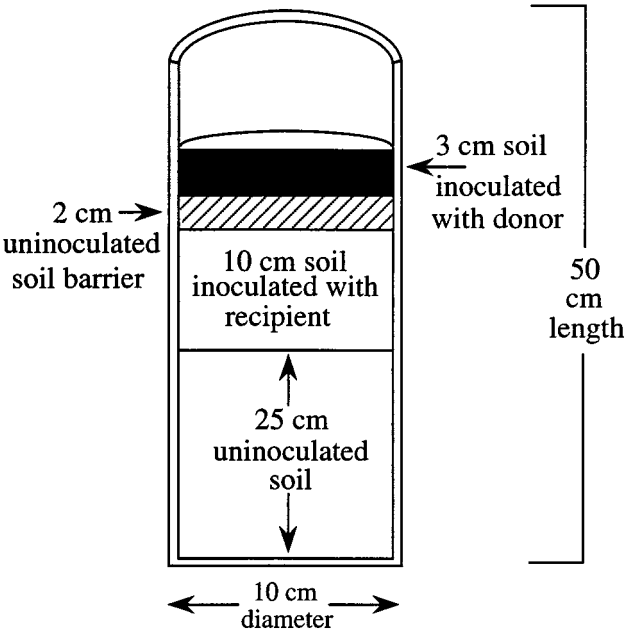

FIG. 1. Microcosm design for the introduction of donor [*A. eutrophus* JMP222N(pJP4)] and recipient (*P. fluorescens* C5t) bacteria.

soil was obtained by coring through the segment with a sterile 2.5-cm diameter cork borer. Three core samples were randomly taken near the center to avoid soil in contact with the polyvinyl chloride surface. For the 0- to 5-cm segment, a 100-g sample of soil was taken from the top 2 cm with a sterile spatula. A 100-g soil sample was also obtained for the 3- to 5-cm depth. Nine samples were taken from each soil microcosm, representing the depths 0 to 2, 3 to 5, 5 to 10, 10 to 15, 15 to 20, 20 to 25, 25 to 30, 30 to 35, and 35 to 40 cm. Observations regarding production of surface casts, depths and extent of burrows, and location of earthworms and cocoons were made.

Bacterial cells were extracted from the soil with 400 ml of sterile extractant solution containing 90% 0.1 M $NH_4H_2PO_4$ and 10% hydrolyzed gelatin (24). Four drops of Tween 80 detergent and 2 drops of silicone antifoam (Sigma Chemical Co., St. Louis, Mo.) were added to the extractant solution. Soil suspensions were shaken for 30 min at 250 rpm on a horizontal shaker and allowed to settle for 10 min. A 10-ml portion of the upper, soil-free phase was serially diluted in 0.01 M phosphate-buffered saline (pH 7.1) containing 0.03% (vol/vol) Tween 80 (PBS-T) and spread plated, in duplicate, onto appropriate selective media. Donor and recipient bacterial counts were enumerated at 3 and 2 days, respectively, while counts of transconjugants were determined after 1 week of incubation at 28°C. Donor, recipient, and transconjugant counts are reported as CFU per gram of dry soil. This method of analysis allowed the detection of approximately log 1.36 donor and recipient CFU and log 1.07 transconjugant CFU per g of dry soil.

To evaluate the possibility that plasmid transfer occurred during bacterial cell extraction or on plates, a sampling control procedure similar to that described by Smit and van Elsas (39) was performed. Sampling control treatments consisted of bacterial cell extractions from the donor-only and recipient-only microcosms. These bacterial extracts were mixed together and plated onto agar medium selective for transconjugants. For microcosms devoid of earthworms, bacterial cell extractions representing the 0- to 2-cm depth from the donor-only microcosms were mixed together and plated with the 5- to 10-cm-depth soil extractions obtained from the recipient-only microcosms. For microcosms containing earthworms, bacterial cell extractions representing the depths 0 to 2, 3 to 5, and 5 to 10 cm for each of the donor-only microcosms were mixed and plated with the same corresponding depth extractions obtained from the recipient-only microcosms.

Collection and analysis of earthworm casts and cocoons. Earthworms were surface disinfected and casts were collected, as described previously (7). The fresh casts were weighed, and bacterial cells were extracted from casts by vortexing for 2 min with 9 or 4 ml of sterile PBS-T per g (wet weight) of cast material for *L. rubellus* and *A. trapezoides* or for *L. terrestris*, respectively. The cast extracts were evaluated for donor, recipient, and transconjugant bacteria as described for soil analysis. Bacterial counts are expressed as CFU per gram (fresh weight) of cast material.

Cocoons were analyzed for viability and surface sterilized as described previously (7). Cocoons were checked for sterility by being rolled on the surface of nonselective TY and King's B agar plates. Each cocoon was homogenized, using a sterile tissue grinder, in 2 ml of sterile PBS-T and analyzed for donor, recipient, and transconjugant bacteria as described above for soil analysis. Results are reported as CFU per gram (fresh weight) of cocoon.

Analysis of transconjugant bacteria. All putative transconjugants were transferred to fresh selective media for colony hybridization. Presumptive pJP4 transconjugants were confirmed by Southern hybridization with a ^{32}P-labeled *mer* gene probe as described previously (7). Appropriate positive and negative controls were included. Several of the confirmed transconjugants were backcrossed to *E. coli* HB101, by patch mating (24), to determine their ability to act as plasmid donors.

Statistics. For each experimental treatment, the number of colonies obtained from each microcosm segment, on a per-gram (dry weight) of sample basis, from triplicate soil microcosms was averaged and log transformed. Similarly, the number of colonies obtained from cast analyses, on a per-gram (fresh weight) of sample basis, from each earthworm species was averaged and log transformed. The log-transformed data were analyzed for statistical significance ($\alpha = 0.05$) by using the analysis-of-variance procedures and Duncan's multiple-range analysis using the Statistical Analysis System (SAS, Cary, N.C.). For statistical purposes, values below the limits of detection were considered to be equal to the level of detection.

RESULTS

Distributions of earthworms and cocoons, burrow construction, and cast deposition. The distribution of earthworms recovered from soil microcosms is shown in Fig. 2. A total of 36 earthworms of each species were used, and all were found to be free of injury and disease at the end of the 2-week incubation period. The largest number of *L. rubellus* earthworms, representing 86% of the total population of the epigeic earthworm, were found in the upper 10 cm of soil. The majority of *A. trapezoides* worms were found between the depths of 5 and 20 cm, with the greatest number (47%) recovered at the 10- to 15-cm depth. In contrast, microcosms containing the anecic earthworm, *L. terrestris*, had earthworms at all depths. The distribution, however, revealed three peaks of concentration at the depths of 0 to 10, 15 to 20, and 35 to 40 cm.

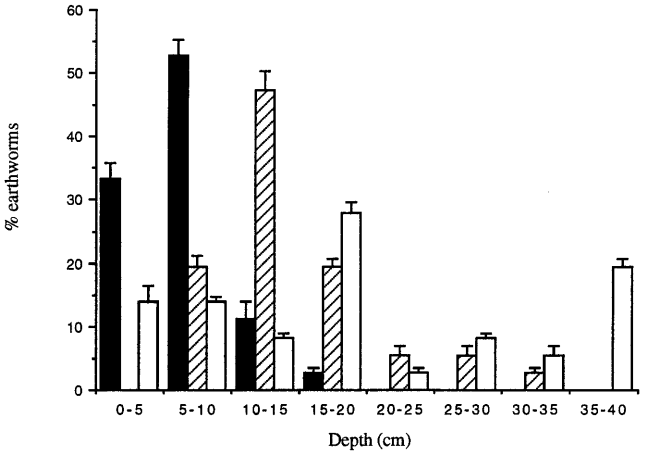

FIG. 2. Recovery of earthworms from inoculated and uninoculated earthworm-containing microcosms. Values are percentages of the total number of earthworms of each species recovered at each depth. Standard errors were calculated based on the mean percentage of earthworms per depth. ■, *L. rubellus*; ▨, *A. trapezoides*; □, *L. terrestris*.

Cast production, degree of branching, and depth of burrow construction were determined for each earthworm species. In a single column, one *L. rubellus* earthworm was recovered at a depth of 20 cm, which represented the maximum burrow depth detected for this species. In all columns inoculated with *L. rubellus*, the soil was extensively worm-worked near the soil surface where casts were being deposited. In contrast, all microcosms containing the endogeic earthworm, *A. trapezoides*, had extensive, branched burrows that reached to a depth of 40 cm. For *A. trapezoides*-inoculated columns, the soil surface contained only a small amount of cast material, which indicated that most of the casts were deposited within the soil column. In microcosms containing *L. terrestris* earthworms, burrows could be detected to 40 cm in most columns and were primarily vertical. Earthworm casts for this anecic species were deposited on the surface as large mounds around each of the burrow entrances.

A total of 14 *A. trapezoides* cocoons were found deposited between the depths of 5 and 20 cm. The coinoculated columns contained three cocoons at the 5- to 10-cm depth, while columns inoculated with only the donor strain had a total of six cocoons (two at the 5- to 10-cm depth and four at the 10- to 15-cm depth). The uninoculated columns contained five cocoons (two at the 5- to 10-cm depth, one at the 10- to 15-cm depth, and two at the 15- to 20-cm depth). No cocoons were recovered from *A. trapezoides*-containing columns inoculated with only recipient bacteria; however, in an identical experiment performed in duplicate at a different time, a total of two cocoons were found deposited at the 5- to 10-cm and 20- to 25-cm depths. No cocoons were found in the *L. rubellus*- or *L. terrestris*-containing microcosms.

Survival and transport of donor and recipient bacteria in soil. Survival and transport of donor [*A. eutrophus* JMP222N (pJP4)] and recipient (*P. fluorescens* C5t) bacteria inoculated into separate areas of a 40-cm soil column with and without earthworms were investigated. In the absence of earthworms, donor bacteria were detected to a depth of 10 cm, and recip-

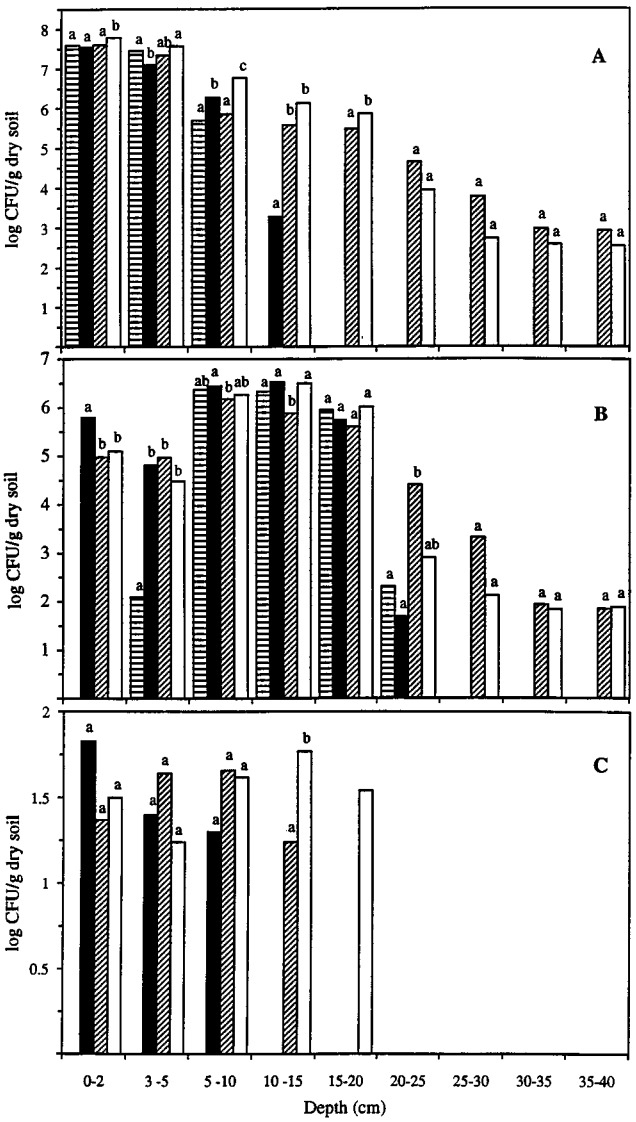

FIG. 3. Numbers of donor (A), recipient (B), and transconjugant (C) bacteria recovered from spatially separated, earthworm-containing soil microcosms. Bacteria were recovered from microcosms at 5-cm intervals, except for the top 5 cm, which was analyzed at 0 to 2 and 3 to 5 cm. Values are means of triplicate microcosms. The absence of a bar indicates that the value was below the level of detection (log 1.36 CFU/g of soil for donor and recipient bacteria; log 1.07 CFU/g of soil for transconjugants). Statistical analyses were done separately for donors, recipients, and transconjugants. Values at each depth with the same letter are not statistically different at $\alpha = 0.05$. ▤, no earthworms; ■, *L. rubellus*; ▨, *A. trapezoides*; □, *L. terrestris*.

ient bacteria were recovered between the 3- and 25-cm soil depths (Fig. 3A and B). The numbers of donor and recipient bacteria at other depths were below the level of detection (log 1.36 CFU per g of dry soil). These results indicate that there was limited movement of the inoculated bacteria in the absence of earthworms.

In columns containing *L. rubellus* earthworms, the donor strain was transported to a maximum depth of 15 cm, while recipient bacteria were recovered between the 0- and 25-cm soil depths (Fig. 3A and B). In contrast, the donor and recipient strains were detected at all soil depths in columns inoculated with *A. trapezoides* or *L. terrestris*. All inoculated soil microcosms had the greatest number of donor organisms in the 0- to 2-cm column depth, with decreasing cell numbers found distal to the inoculation zone (Fig. 3A). There were significantly greater numbers of donor bacteria found at the 0- to 2-, 5- to 10-, and 15- to 20-cm soil depths in *L. terrestris*-inoculated microcosms than in the no-earthworm, *L. rubellus*-inoculated, and *A. trapezoides*-inoculated microcosms. Microcosms containing earthworms had significantly greater numbers of donor organisms transported below the 5- and 10-cm soil depths than microcosms without earthworms. Also, the depth that the donor strain was transported down the soil column was dependent on the earthworm species present. *A. trapezoides* and *L. terrestris* earthworms significantly transported the donor strain below 10 cm, compared to *L. rubellus.*

All microcosms containing earthworms had significantly greater numbers of recipient bacteria found at the 0- to 5-cm soil depth than did microcosms devoid of earthworms (Fig. 3B). In addition, *L. rubellus*-containing microcosms had significantly larger numbers of recipient bacteria found at the 0- to 2-cm soil depth than did microcosms containing the other earthworms. Moreover, *A. trapezoides* and *L. terrestris* significantly distributed the recipient strain below the 25-cm soil depth, compared to columns without earthworms or those containing *L. rubellus.*

For the control microcosms, which contained either donor or recipient bacteria alone, only the inoculated bacteria were recovered. The survival and distribution results for the control microcosms were similar to those reported for coinoculated microcosms, and no donor or recipient bacteria were isolated from uninoculated soil microcosms (data not shown).

Gene transfer in soil microcosms. The numbers of transconjugant bacteria isolated at different depths from coinoculated soil-earthworm microcosms are shown in Fig. 3C. Transfer of plasmid pJP4 from *A. eutrophus* JMP222N to *P. fluorescens* C5t was detected only in microcosms containing earthworms. The number of transconjugants isolated at various soil depths ranged from log 1.24 to log 1.83 CFU per g of dry soil. The depth at which transconjugants could be recovered from soil depended on the species of earthworm. Soil microcosms containing *L. terrestris* had transconjugants at the greatest depth, reaching 20 cm, while columns inoculated with *L. rubellus* or *A. trapezoides* earthworms had transconjugants isolated at maximum depths of 5 to 10 and 10 to 15 cm, respectively. The largest number of transconjugants, log 1.83 CFU per g of dry soil, was detected at the surface in microcosms containing *L. rubellus*; however, this number was not statistically different from the number obtained for microcosms with the other earthworm species. There were significantly higher numbers of transconjugants recovered in *L. terrestris*-containing microcosms between the 10- and 20-cm soil depths than for the other earthworm treatments. There was a trend found for earthworm species: columns containing *L. rubellus* showed decreasing transconjugant numbers recovered with depth, and those with *L. terrestris* revealed greater numbers of transconjugants at lower depths within the soil column. In contrast, *A. trapezoides*-containing microcosms had the greatest numbers of transconjugants found between the 3- and 10-cm soil depths.

No transconjugants or background mercury-resistant bacteria were recovered in either uninoculated or inoculated control microcosms (data not shown). In addition, sampling controls performed to determine if gene transfer was occurring during bacterial soil extraction or on plates showed no transconjugants. This indicates that transfer of plasmid pJP4 was not occurring during sample processing.

Influence of earthworm activity on survival of inoculated bacteria and transconjugants. The influence of earthworm behavior on the survival of coinoculated donor and recipient

TABLE 2. Total numbers of donor, recipient, and transconjugant bacteria from coinoculated soil microcosms

Treatment	log CFU/g of dry soil[a]		
	A. eutrophus (donor)	*P. fluorescens* (recipient)	Transconjugants
No worms	7.83 A	6.73 A	[0][b]
L. rubellus	7.71 B	6.89 A	2.02 A
A. trapezoides	7.81 AB	6.48 B	2.17 A
L. terrestris	8.03 C	6.80 A	2.30 A

[a] Values are means from triplicate microcosms. Within columns, values followed by the same letter are not significantly different ($\alpha = 0.05$).
[b] Below the level of detection (log 1.07 CFU/g of dry soil).

bacteria and the number of transconjugants is shown in Table 2. These results are the sums of mean plate counts from all of the soil layers. Both donor and recipient bacteria survived relatively well after 2 weeks of incubation at 20°C. *A. eutrophus* JMP222N(pJP4) survived better than *P. fluorescens* C5t, decreasing less than 1 log unit compared to the recipient's almost 2-log-unit decline from the initial inoculation level of log 8.0 cells per g of dry soil. Microcosms containing *L. terrestris* earthworms had significantly more inoculated donor bacteria (log 8.03 CFU per g of dry soil^{-1}) than microcosms in the remaining treatments (log 7.71 to log 7.83 CFU per g of dry soil). Microcosms containing the endogeic earthworm, *A. trapezoides*, showed a slightly lower number of total recipient bacteria (log 6.48 CFU per g of dry soil) than those with the remaining earthworm species and columns without added earthworms (log 6.73 CFU per g of dry soil).

The recovery of transconjugants from spatially separated donor and recipient bacteria was found only in earthworm-containing microcosms. However, there was no statistical difference found between the total numbers of transconjugants isolated from microcosms containing the three different earthworm species. The ratios of transconjugants to recipients were calculated by using the total numbers of recipient and transconjugant bacteria recovered from coinoculated earthworm-containing microcosms. The calculated ratios, expressed on a per-recipient basis, were 1.4×10^{-5}, 3.2×10^{-5}, and 4.9×10^{-5} for *L. rubellus*, *L. terrestris*, and *A. trapezoides*, respectively. In preliminary in vitro experiments to determine maximum conjugation frequencies under optimal (patch-mating) conditions, transconjugants were detected at about 10^{-3} per recipient cell (data not shown).

Enumeration of inoculated bacteria and transconjugants from earthworm casts. The numbers of donor and recipient bacteria detected in casts of earthworms from coinoculated soil microcosms are shown in Table 3. Donor bacteria were found in high numbers for all three earthworm species, with approximately log 6 CFU per g (fresh weight) of cast material. In contrast, recipient bacteria were found at approximately log 4 CFU per g (fresh weight) of cast material for *L. rubellus* and *A. trapezoides* and at around log 2 CFU per g (fresh weight) of cast material for *L. terrestris*. However, no transconjugant bacteria were recovered from casts of these earthworms.

No inoculated bacteria or transconjugants were detected in casts of earthworms obtained from uninoculated soil microcosms. In addition, only the inoculated bacteria were isolated from casts of earthworms recovered from control microcosms, and these were at levels similar to those given in Table 3 (data not shown).

Enumeration of bacteria in earthworm cocoons. The length of incubation and environmental conditions were conducive for the production of cocoons only in microcosms containing *A. trapezoides*. All of the *A. trapezoides* cocoons recovered were found to be viable and were analyzed for numbers of donor, recipient, and transconjugant bacteria. The cocoons had a mean fresh weight of 0.037 ± 0.009 g, and nonselective agar plates used for the evaluation of surface sterility showed no growth.

Cocoons collected from the donor-only-inoculated earthworm microcosms contained detectable numbers of donor bacteria, which ranged from log 3.73 to log 7.41 CFU per g (fresh weight) of cocoon. In the present study, no cocoons were recovered from recipient-only microcosms. However, in a previous experiment two cocoons were collected with recipient bacteria detected at log 5.78 and log 6.20 CFU per g (fresh weight) of cocoon^{-1}. A total of three cocoons were found in coinoculated *A. trapezoides* microcosms at the 5- to 10-cm soil depth. All contained detectable numbers of donor bacteria, ranging from log 4.69 to log 6.56 CFU per g (fresh weight) of cocoon. Recipient bacteria were isolated from two of the three cocoons, with numbers ranging from log 6.22 to log 6.41 CFU per g (fresh weight) of cocoon. However, no transconjugant bacteria were found. In a previous experiment performed in duplicate at a different time, donor, recipient, and transconjugant bacteria were detected in 100, 80, and 30% of analyzed cocoons, respectively. In these cocoons, donor bacteria ranged from log 6.30 to log 7.88, recipient bacteria ranged from log 4.70 to log 6.93, and transconjugant bacteria ranged from log 2.11 to log 2.85 CFU per g (fresh weight) of cocoon. In cocoons containing transconjugants, both donor and recipient bacteria were also detected. Cocoons recovered from uninoculated soil-earthworm microcosms revealed no detectable donor, recipient, or transconjugant bacteria. In addition, cocoons collected from control microcosms contained only the inoculated bacteria.

Confirmation of transconjugants. All of the putative transconjugants hybridized with the *mer* gene probe and were considered confirmed transconjugants. Ten randomly chosen, confirmed transconjugants were patch mated with *E. coli* HB101 and found to act as donors with high transfer frequencies (data not shown).

DISCUSSION

Previous studies on horizontal gene transfer have been conducted in relatively small (2 to 350 g of soil), homogeneous soil systems in which the inoculated bacteria were thoroughly mixed, at high population densities, throughout the entire microcosm or washed into deeper layers (49). To date, there have been no reports of studies which have investigated gene transfer between spatially separated bacteria within a nonsterile soil microcosm. While a single study in which separate soil portions were inoculated with either donor or recipient *P. fluorescens*

TABLE 3. Numbers of donor and recipient bacteria recovered from earthworm casts in spatially separated, coinoculated soil microcosms

Treatment	log CFU/g of cast[a]	
	A. eutrophus (donor)	*P. fluorescens* (recipient)
L. rubellus	6.25 AB	4.65 A
A. trapezoides	6.84 A	4.85 A
L. terrestris	6.16 B	2.74 B

[a] Values are means for nine earthworms collected from triplicate soil microcosms. Within columns, values followed by the same letter are not significantly different ($\alpha = 0.05$).

strains was performed, the portions were subsequently mixed together (47). GEMs that are applied to the soil for agronomic purposes may be transported by aerosol (27), water flow (46), or soil animals (7), affecting both bacterial survival and genetic interactions with the heterogeneous soil bacterial community. In the present study, we investigated the abilities of three distinctly different earthworm species to transport and distribute spatially separated donor and recipient bacteria in nonsterile soil microcosms. The model ecosystem was designed to determine whether earthworm activity was responsible for plasmid transfer between physically isolated bacteria.

In soil microcosms devoid of earthworms, the donor bacterium, *A. eutrophus* JMP222N(pJP4), was detected to a depth of 10 cm, and the recipient bacterium, *P. fluorescens* C5t, was found at a depth of 3 to 25 cm. This result shows that some gravimetric water movement was present, but the absence of the recipient strain at the 0- to 2-cm depth revealed that capillary force due to surface evaporation was absent. Several studies have also found only limited movement of inoculated bacteria in the absence of a transport agent (7, 28, 31, 46).

Despite laboratory rearing and incubation in soil columns, each earthworm species revealed typical burrowing and casting behavior as defined by its ecological classification (26). The majority of the epigeic (*L. rubellus*), endogeic (*A. trapezoides*), and anecic (*L. terrestris*) earthworms were found at the depths of 5 to 10, 10 to 15, and 15 to 20 cm, respectively. The earthworms were found to significantly effect the transport and distribution of the inoculated strains in a 40-cm soil column, as compared to microcosms devoid of earthworms. The maximum depth of recovery of both donor and recipient bacteria depended on the burrowing behavior of the individual earthworm species. In a previous study, Daane et al. (7) reported a similar population distribution pattern for the same earthworm species studied, and they were also able to correlate the maximum recovery depth of a surface-inoculated *P. fluorescens* strain with burrowing activity. This result indicated that earthworm behavior was consistent with the model system developed.

Other studies have shown earthworm-mediated vertical transport of bacteria in nonsterile soil; however, transport was rather limited (28, 42). In addition, Doube et al. (12) showed increased root nodulation of subterranean clover by *Rhizobium leguminosarium* biovar trifolii in the presence of *A. trapezoides*. Studies have shown that established populations of introduced earthworms may travel distances of up to 10 m $year^{-1}$ (43) and may migrate on the surface up to 19 m in a single night (29). In addition, burrowing creates large macropores in the soil, producing channels of preferential flow, which contributes to enhanced infiltration rates (26). Therefore, earthworms should not be overlooked as possible vectors for microorganisms which would allow for the colonization of more distant environments and metabolizable substrates.

Earthworms may alter the physical, chemical, and biological properties of the soil by selective grazing, burrowing, and cast deposition (26). In the present study, earthworm activity did not substantially influence the total number of donor or recipient bacteria recovered from soil as compared to that in microcosms devoid of earthworms. Several studies have shown similar declines of introduced bacteria in soil (7, 16, 23, 24, 46–48).

We previously reported that earthworms had a significant influence on bacterial transport and gene transfer (7). However, we were unable to determine if transfer occurred in the homogeneously mixed donor layer or at the depth to which the donor was transported. In the present study, the addition of earthworms was required for the production of transconjugants between spatially separated donor and recipient bacteria. Although there was no significant difference in the numbers of transconjugants recovered for different earthworm species, the depth to which transconjugants could be detected depended on the ecological classification of the earthworm present. Stotzky et al. (44) have reported that a minimum of 10^4 donor and recipient cells must be present for transconjugants to be detected in soil. Therefore, in our system the absence of transconjugants at lower depths in earthworm-containing microcosms may be attributed to the lower numbers of either donor or recipient bacteria. However, this may not be the only cause, since over 10^5 donor and recipient bacteria per g were found at the 15- to 20-cm soil depth in *A. trapezoides*-containing microcosms, and no transconjugants were recovered. A factor of soil mixing due to earthworm activity may also be required for gene transfer. In addition, it should be noted that transconjugants formed in the upper soil depths may have been transported to the lower depths. Nevertheless, this is the first report of intergeneric plasmid transfer between spatially segregated donor and recipient bacteria relying on the activity of earthworms to bring the cells together for necessary cell-to-cell contact.

Transconjugant bacteria were detected only in earthworm-containing microcosms, with total transconjugant counts around log 2 CFU per g of soil for all three earthworm species. Similar numbers of transconjugants have been reported by Kinkle et al. (23) for transfer between two *Bradyrhizobium* species. While Neilson et al. (30) were able to detect in situ pJP4 transfer between *A. eutrophus* and *Variovorax paradoxus* in nonsterile soil, transconjugants were found only after the soil was amended with 2,4-D. In our study, the recovery of transconjugants was not due to any selective pressure but rather relied solely on the activity of burrowing earthworms to mix the physically isolated bacteria.

Most studies involving earthworms have focused on bacterial survival following gut transit, and results have been contradictory (37). Many studies have shown that microbial activity is generally stimulated by gut passage. This is evidenced by higher total microbial plate counts (8, 32, 33) and increased rates of microbial respiration (32, 38), nitrification (32), and denitrification (13) in casts than in bulk soil. More recently, Fischer et al. (15), using 16S rRNA-targeted oligonucleotide probes, analyzed the bacterial community in the gut of *L. terrestris* and found higher numbers of bacteria toward the end of the gut, with the greatest number found in casts. In contrast, studies investigating the survival of specifically inoculated microorganisms have shown earthworm gut passage to be detrimental for some organisms (5, 19, 33, 45) while increasing the survival of or having no influence on others (33). In the present study, both donor and recipient bacteria were recovered from casts of all three earthworm species at relatively high levels; however, their numbers were considerably less than numbers recovered from the bulk soil. The casts from all three earthworm species had lower numbers of recipient bacteria than of donor bacteria. *L. terrestris* earthworms exerted a more detrimental influence of gut passage on the survival of recipient bacteria than the other two earthworm species. Kristufek et al. (25), using plate counts and epifluorescence microscopy, found that different earthworm species may affect the same bacteria in different ways. In their study, total numbers of bacteria increased from the fore- to hindgut in *L. rubellus* earthworms but decreased in *Aporrectodea caliginosa.*

Earthworms reproduce by forming egg capsules (cocoons) that are deposited in the soil. Earthworm cocoons are not sterile but have been found to contain up to 10^8 cells/g of cocoon and may acquire bacteria from the soil environment in which they are formed (7, 51). In the present study, *A. trape-*

zoides cocoons were found to contain both donor and recipient bacteria. While no transconjugants were found in our main study, a previous experiment detected approximately 10^2 transconjugants per g of cocoon. Moreover, we previously reported that an *A. trapezoides* cocoon contained 10^5 transconjugants per g of cocoon (7). We conclude from these results that the recovery of transconjugants from cocoons is a rare but nevertheless significant event. At this time, however, we are unable to determine whether plasmid transfer occurred within the cocoon or preformed transconjugants entered the egg capsule during cocoon formation.

Our model system unequivocally determined that earthworm activity was required for plasmid transfer between spatially separated bacteria. In addition, our results indicate that earthworms from diverse ecological categories may differentially influence the survival and distribution of inoculated bacteria.

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