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Intergeneric Transfer of Conjugative and Mobilizable Plasmids Harbored by *Escherichia coli* in the Gut of the Soil Microarthropod *Folsomia candida* (Collembola)

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The gut of the soil microarthropod *Folsomia candida* provides a habitat for a high density of bacterial cells (T. Thimm, A. Hoffmann, H. Borkott, J. C. Munch, and C. C. Tebbe, Appl. Environ. Microbiol. 64:2660–2669, 1998). We investigated whether these gut bacteria act as recipients for plasmids from *Escherichia coli*. Filter mating with *E. coli* donor cells and collected feces of *F. candida* revealed that the broad-host-range conjugative plasmid pRP4-luc (pRP4 with a luciferase marker gene) transferred to fecal bacteria at estimated frequencies of 5.4×10^{-1} transconjugants per donor. The mobilizable plasmid pSUP104-luc was transferred from the IncQ mobilizing strain *E. coli* S17-1 and less efficiently from the IncF1 mobilizing strain NM522 but not from the nonmobilizing strain HB101. When S17-1 donor strains were fed to *F. candida*, transconjugants of pRP4-luc and pSUP104-luc were isolated from feces. Additionally, the narrow-host-range plasmid pSUP202-luc was transferred to indigenous bacteria, which, however, could not maintain this plasmid. Inhibition experiments with nalidixic acid indicated that pRP4-luc plasmid transfer took place in the gut rather than in the feces. A remarkable diversity of transconjugants was isolated in this study: from a total of 264 transconjugants, 15 strains belonging to the alpha, beta, or gamma subclass of the class *Proteobacteria* were identified by DNA sequencing of the PCR-amplified 16S rRNA genes and substrate utilization assays (Biolog). Except for *Alcaligenes faecalis*, which was identified by the Biolog assay, none of the isolates was identical to reference strains from data banks. This study indicates the importance of the microarthropod gut for enhanced conjugative gene transfer in soil microbial communities.

Gene transfer is a process by which bacterial populations substantially increase their rates of evolution and adaptation (12, 59). Particularly, plasmid-located genes, which are transferred by conjugation from donor to recipient cells, can disseminate rapidly between even phylogenetically different bacterial groups (17, 36, 41) and microbial communities in different spatial habitats (34, 71). Such microbial genetic networks should be considered in risk assessments of releases of genetically engineered microorganisms into the environment (22, 37, 43). The probability and rate of plasmid transfer from a donor to indigenous microorganisms in a given habitat are influenced by plasmid-borne genes which determine the type of transfer mechanism (self-transmissible or mobilizable) and the host range of autonomous plasmid replication. Additionally, specific physicochemical conditions, such as temperature, water potential, and the availability of energy (substrates) for donor and recipient cells, are important factors influencing gene transfer rates in terrestrial and aquatic environments (23, 53, 64).

The spread of plasmid-borne genes is still extremely difficult to predict for terrestrial habitats, since a large variety of microhabitat conditions which are not well characterized exists. In bulk soil under laboratory conditions, conjugative gene transfer from recombinant bacterial donor strains to indigenous soil bacteria has been found only under specific selective conditions or on rare occasions (11, 20, 24, 27, 50, 61). Several studies failed to detect such transfer events, and it was concluded that heterogeneity and low densities of recipient cells, as well as a lack of substrates for microbial metabolism, prevented efficient plasmid transfer in bulk soil (19, 49, 54, 75). Plant exudates increased rates of gene transfer in soil (33, 48), and higher rates of gene transfer were found in rhizospheres than in bulk soil (50, 61). It was assumed that other microsites which favor gene transfer in terrestrial habitats are associated with soil invertebrates (74). However, to date little experimental evidence to prove this assumption is available.

Intraspecies transconjugants of added *Enterobacter cloacae* donor and recipient cells could be isolated from microcosm experiments with the variegated cutworm, *Peridroma saucia*, and plant material (2). The investigators in that study concluded that gene transfer events happened, most likely, in the digestive tracts or in the feces of the insects. Another recent report demonstrated that a conjugative plasmid was transferred between fed *Escherichia coli* strains in the guts of *Rhabditis* nematodes (1). Earthworms mediated transport and enhanced plasmid transfer from added donor cells to added recipients and to indigenous bacteria in soil (14, 15). High rates of intraspecies plasmid transfer, comparable to those obtained in pure broth cultures, were detected with *Bacillus thuringiensis* in infected lepidopterous larvae (31).

Microarthropods (collembolans and mites) are the most abundant invertebrate group in the majority of soils (5) but

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TABLE 1. *E. coli* strains and plasmids used in this study

Strain or plasmid	Description	Reference and/or source
E. coli strains		
S17-1	*recA* derivative of *E. coli* 294 (F^- *thi pro hsdR*) carrying a modified derivative of IncPα plasmid pRP4 (Ap^s Tc^s Km^s) integrated in the chromosome, Tp^r	58
S17-1 (λ*pir*)	λ lysogenic S17-1 derivative producing π protein for replication of plasmids carrying *oriR6K*	K. N. Timmis
CC118 (λ*pir*)	Routine strain for maintenance of π-dependent plasmids carrying *ori*R6K	28
NM522	*hsdR* strain with F′ plasmid	Promega Corporation
HB101	F^- *hsdS* (r_B^- m_B^-) *thi pro leu lacY ara xyl supE recA13* Sm^r	10; obtained from Promega Corporation
Plasmids		
pUC18	Cloning vector, Ap^r	77
pUC18-luc	pUC18 carrying a 2.3-kb *npt-2p–luc* cassette inserted into a multicloning site as an *Hin*dIII fragment, confers a bioluminescence phenotype	J. Schiemann
pSUP202	Tra^- Mob^+ derivative of plasmid pBR325, narrow host range of replication, Ap^r Cm^r Tc^r	58
pSUP202-luc	pSUP202 carrying a 2.3-kb *npt-2p–luc* cassette inserted into an *Hin*dIII site, confers a bioluminescence phenotype, Ap^r Cm^r	J. Schiemann
pSUP104	Tra^- Mob^+ derivative of plasmid pACYC184, broad host range of replication, Cm^r Tc^r	46
pSUP104-luc	pSUP104 carrying a 2.3-kb *npt-2p–luc* cassette inserted into an *Hin*dIII site, Cm^r	This study
pUT-*luxAB*	Tra^- Mob^+ derivative of pUT (*oriR6K*) with a miniTn*5* transposon and carrying promoterless *luxAB* and *luxB* genes, Tc^r	18
pRP4	Tra^+, IncPα broad-host-range plasmid, Ap^r Km^r Tc^r	68
pRP4-luc	pRP4 carrying a 2.3-kb *npt-2p–luc* cassette cloned into an *Hin*dIII site, confers a bioluminescence phenotype, Ap^r Tc^r	This study

have not been recognized, so far, for their impact on microbial gene transfer. There are some indications that microarthropods harbor a large variety of microorganisms in their guts and thereby contribute to microbial biodiversity in terrestrial environments (7, 9, 57). In the accompanying paper, we have described the gut of *Folsomia candida* (Collembola) as a habitat and species-specific vector for microorganisms (67). The gut of this soil-dwelling insect, which has a volume of only several nanoliters, was found to be densely colonized, predominantly by rod-shaped bacterial cells. We were interested to know whether such bacterial cells act as recipients for plasmids and thereby promote gene transfer in microbial communities. *F. candida* feeds, under natural conditions, on bacteria (3), fungal mycelia (6, 66), and nematodes (35). Here, we report on the results of experiments in which plasmid-bearing *E. coli* strains were fed to *F. candida* in microcosms. Self-transferable plasmids, as well as mobilizable plasmids with different host ranges, and a nonmobilizable plasmid were included in this study in order to determine the specific capacities of these different classes of plasmids to spread into indigenous bacterial populations. For detection purposes, all plasmids were engineered by the insertion of the luciferase-encoding marker gene *luc* or *lux* (30, 47).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. *E. coli* donor strains and plasmids used in this study are shown in Table 1. Cloning of the luciferase genes did not affect maintenance or transfer functions of the individual plasmids. All strains were cultivated on Luria-Bertani (LB) medium (51). In order to maintain the plasmids, filter-sterilized antibiotic stock solutions were added to the autoclaved media to the following final concentrations for the listed plasmids: 10 μg of tetracycline ml^{-1} for pRP4-luc and pUT*luxAB*, 100 μg of ampicillin ml^{-1} for pUC18-luc, 100 μg of ampicillin and 50 μg of chloramphenicol ml^{-1} for pSUP202-luc, and 50 μg of chloramphenicol ml^{-1} for pSUP104-luc.

All media inoculated with feces from *F. candida* were amended with cycloheximide (100 μg ml^{-1}) in order to inhibit growth of eukaryotic microorganisms. The total number of bacteria (heterotrophic, aerobic, and culturable) from feces and the gut of *F. candida* was determined, in this study, on nonamended LB agar. Agar medium cultures were incubated at 28°C for 2 days. Donor strains were enumerated after growth on LB agar with the appropriate antibiotic additions at 37°C. Recipients were obtained on M9 minimal growth medium (51) with purified agar (Merck, Darmstadt, Germany) and benzoic acid (2.5 mM) as the sole source of organic carbon. Transconjugants were grown on M9 benzoic acid growth agar with plasmid-selective antibiotics. Antibiotic concentrations were identical to those indicated above for LB medium. Transconjugants were cultivated routinely onto M9 agar with benzoic acid and plasmid-selective antibiotics.

Construction of pRP4-luc. A 2.3-kb *nptII* promoter–*luc* cassette (56) was ligated as an *Hin*dIII fragment to *Hin*dIII-digested pRP4, and the ligation mixture was transformed into *E. coli* HB101. Transformants were selected on LB agar with tetracycline. Of 100 tetracycline-resistant clones tested, 20 conferred a bioluminescence phenotype. Restriction enzyme analysis of a selected plasmid designated pRP4-luc confirmed the presence of a single 2.3-kb *Hin*dIII fragment in pRP4-luc (data not shown).

Filter mating experiments with *E. coli* donor strains and feces of *F. candida*. Fecal pellets were collected from petri dish microcosms with water-agar (1.5%, pH 7.0), in which a total of 100 specimens of *F. candida* were incubated with one YTP (2.0 g each of yeast extract, tryptone, and peptone $liter^{-1}$) agar cube (1.7-cm^2 surface, 0.5-cm thick) as a sole nutrient source. Microcosms were incubated for 10 days at 18°C in the dark. After the insects and the remaining YTP agar cube were removed, the feces were suspended from the water-agar twice with 1 ml of 0.85% NaCl solution each time. The suspensions were collected in Eppendorf tubes, with one tube per microcosm, and centrifuged for 5 min at 2,700 × *g* in a microcentrifuge. The supernatant was removed, and the pellet was suspended with 200 μl of a donor cell suspension. The donor cell suspension was obtained by the following procedure. Overnight cultures were grown in 75 ml of LB broth with the appropriate antibiotic(s) (dependent upon the plasmid) at 37°C and shaken at 200 rpm on a rotary shaker (TM-3; Infors, Basel, Switzerland). Cells were harvested by centrifugation (5 min at 2,700 × *g* and 4°C), resuspended in 75 ml of 0.85% NaCl solution, centrifuged again, and, finally, resuspended in 2 ml of 0.85% NaCl solution. Suspensions of feces and donor strains (200 μl) were transferred directly onto LB agar (1.2% agar), which had been covered previously with a presterilized nylon filter (8.2 cm in diameter, Hybond-N; Amersham, Braunschweig, Germany). After an incubation at 28°C for 24 h, filters were removed carefully with a sterile forceps and transferred into 50-ml Falcon tubes, and grown cells were suspended in 2 ml of NaCl solution by vortexing at the highest setting. Dilutions were inoculated onto the appropriate media for determination of total numbers of bacteria and donor, recipient, and transconjugant cells. Donor and recipient cells were also inoculated onto separate filters, incubated overnight, diluted, and cultivated on the appropriate media in order to correlate the effect of the transconjugant detection technique with the occurrence of transconjugants (plate mating). This control is required for plasmids, such as pRP4, which have high transfer potentials (63).

Feeding experiments. Microcosms for feeding experiments of *F. candida* with *E. coli* strains consisted of petri dishes with water-agar (1.5%, pH 7.0) and a YTP agar cube (see above) in the center. A total of 200 μl of donor cells (approximately 10^{10} cells), obtained as described above for the filter mating experiments, were loaded carefully onto the YTP agar cube and air dried for 30 min under sterile conditions. Each microcosm was inoculated with a total of 100 specimens of *F. candida* taken from a breeding stock (67) and preincubated (starved) for 24 h in petri dishes with sterile water-agar. Microcosms were kept at 18°C in the dark. All treatments were tested with three replicate microcosms. Before analysis of the microbial populations in the feces, all animals were anesthetized by a CO_2 fumigation and were counted, and specimens were stored at −20°C for further analyses. The YTP agar cube with remaining donor cells was then removed

TABLE 2. Results of filter mating experiments with different plasmid-bearing *E. coli* donor strains and feces collected from *F. candida* after an incubation period of 24 h

E. coli donor strain	Plasmid	log CFU per filter				Estimated plasmid transfer frequency, no. of transconjugant cells per:	
		Total fecal cells	Recipient cells	Donor cells	Transconjugants	Recipient cell	Donor cell
S17-1	pUC18-luc	9.20	8.74	8.80	ND[a]	$<1.0 \times 10^{-9}$	$<1.0 \times 10^{-9}$
	pSUP202-luc	10.27	10.20	8.38	ND	$<6.3 \times 10^{-11}$	$<4.0 \times 10^{-9}$
	pSUP104-luc	10.44	9.70	6.45	6.58	7.7×10^{-4}	1.35×10^{0}
	pRP4-luc	9.43	9.40	8.15	7.60	1.6×10^{-2}	5.4×10^{-1}
HB101	pSUP104-luc	9.37	8.42	8.60	ND	$<3.0 \times 10^{-9}$	$<2.0 \times 10^{-9}$
NM522	pSUP104-luc	9.74	9.14	9.18	6.93	6.2×10^{-3}	5.6×10^{-3}
S17-1 λPir	pUT*luxAB*	8.57	8.43	4.74	ND	$<3.0 \times 10^{-9}$	$<1.8 \times 10^{-5}$
CC118 λPir	pUT*luxAB*	10.39	9.35	9.15	ND	$<4.5 \times 10^{-10}$	$<7.1 \times 10^{-10}$

[a] ND, not detected.

carefully from the water-agar, and the feces which lay on the surface of the water-agar were extracted twice with 1 ml of 0.85% NaCl solution, as described above for the filter mating.

Detection of reporter genes. Two different types of reporter genes were used in this study: (i) a firefly-derived luciferase (*luc*) gene (30, 44) and (ii) a bacterial luciferase-encoding *luxAB* gene (47). Bioluminescence of grown colonies was detected after transfer onto nylon membranes (Hybond-N), as described by Selbitschka et al. (55). The *luxAB*-encoded luciferase was detected by a procedure similar to that used for detection of the *luc* luciferase, with the exception that instead of the substrate luciferin being loaded onto the surfaces of the membranes, 4 drops (20 μl each) of the undiluted substrate *n*-decyl aldehyde (Sigma, St. Louis, Mo.) were placed next to the filter in opposite positions. To detect light emission of *luc* or *luxAB* reporter gene-encoded enzymes, a film (Kodak T-Mat plus DG; Kodak-Pathé, Paris, France) was placed onto the membranes and incubated in a light-tight film cassette at room temperature overnight in a dark room. After development of the film, the number of bioluminescent colonies could be determined.

The *luc* luciferase gene was also detected in some experiments by PCR by amplifying an internal 302-bp fragment of the gene with primers P2 and P3 under the conditions described by Dammann-Kalinowski et al. (16).

Characterization of transconjugants. Transconjugants isolated from the gut or feces of *F. candida* were diluted for purification and subcultured on selective growth agar. Grown colonies were transferred, in two replicates for each strain, onto selective agar in order to exclude plasmid segregants from further analyses. Colonies grown on one replicate plate were analyzed for reporter gene expression, as described above, and the corresponding colonies on replicate plates were subcultured further and subjected to amplified ribosomal DNA restriction analysis (ARDRA) (73).

For ARDRA, single colonies with confirmed reporter gene activity were suspended in 50 μl of lysis buffer (0.05 M NaOH, 0.25% sodium dodecyl sulfate) in reaction tubes and incubated at 95°C for 15 min. Subsequently, 450 μl of water was added and the suspension was microcentrifuged, at the highest setting, for 5 min at room temperature. A total of 450 μl was then transferred to a new reaction tube and served as a template for PCR amplifications. PCR was conducted by targeting a 1.2-kb region of the 16S rRNA gene with universal eubacterial primers. Primer sequences were obtained from R. Simon and H.-V. Tichy, Freiburg, Germany. The forward primer (41f) sequence was (5′ to 3′) GCT CAG ATT GAA CGC TGG CG, and the reverse primer (1066r) sequence (5′ to 3′) was ACA TTT CAC AAC ACG AGC TG. PCR was carried out in 50-μl volumes consisting of 5 μl of 10× PCR buffer (100 mM Tris-HCl, 25 mM $MgCl_2$, 500 mM KCl), 1 μl of a deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP [2.5 mM each]) mixture, 1 μl of each primer (10 mM each), of 0.2 μl of *Taq* polymerase (1 U; Pharmacia, Freiburg, Germany), 2 μl of template DNA, and water to reach the final volume. Solutions were covered with 10 μl of Chill-out 14 liquid wax (MJ Research, obtained from Biozym, Hessisch, Oldendorf, Germany). Tubes were incubated in a thermocycler (Omni Gene; Hybaid Limited, Teddington, United Kingdom) for 2 min at 95°C, followed by 30 cycles, each consisting of 1 min at 95°C, 1 min at 50°C, and 1 min at 72°C. Final primer extension was 5 min at 72°C. PCR products were taken directly for restriction enzyme digestions.

A total of 5 μl of PCR product was incubated with 10 U of a restriction endonuclease (*Cfo*I and *Alu*I, separately; both were obtained from Boehringer Mannheim, Mannheim, Germany) and the manufacturer-recommended incubation buffer in a final volume of 20 μl overnight at 37°C. Restriction fragment length polymorphisms were analyzed after agarose gel electrophoreses (2% low-melting-point, ultra-pure agarose; Gibco BRL, Life Technologies Inc., Gaithersburg, Md.) and staining with ethidium bromide (51) on a UV (312 nm)-illuminated table. Representative isolates of each ARDRA group, consisting of isolates with identical fragment length profiles, were then characterized by Gram staining (42).

Phenotypic characterization of the isolates was performed by a microtiter plate-bound substrate utilization assay (Biolog; Biolog Inc., Hayward, Calif.) (8). Substrate utilization patterns were recorded with a microtiter plate spectrophotometer (Vmax; Molecular Devices, Menlo Park, Calif.) and compared to patterns in a database (MicroLog GN 3.50) with Microlog2 software (Biolog Inc.).

Determination and analysis of 16S rRNA gene sequences of transconjugants. Genomic DNAs were prepared from individual colonies picked from agar medium, resuspended in 100 μl of TE buffer (50 mM Tris-HCl, 1 mM EDTA [pH 8.0]), and incubated at 95°C for 5 min. After cooling on ice, the cell debris was pelleted in a microcentrifuge for 30 s. The genomic DNA in the supernatant was concentrated with Microcon-100 spin concentrators (Amicon GmbH, Witten, Germany) and resuspended in 10 μl of TE buffer.

The 16S rRNA genes were targeted for amplification by PCR with aliquots (between 2 and 5 μl) of the DNA suspension, a forward primer hybridizing at the complement of positions 8 to 27, a reverse primer hybridizing at positions 1525 to 1541 (*E. coli* 16S rRNA gene sequence numbering), and reaction conditions described in detail previously (32). The 16S rRNA gene PCR products were purified with Microcon-100 spin concentrators and sequenced directly with a Perkin-Elmer/Applied Biosystems, Inc. (Weiterstadt, Germany) model 373A DNA sequencer according to the protocols of the manufacturer for *Taq* cycle sequencing with fluorescent-dye-labelled dideoxynucleotides.

Sequence data were aligned with reference rRNA and rRNA gene sequences (40, 72) with evolutionarily conserved primary sequence and secondary structure as references (26). Cluster analyses were carried out with programs contained in the Phylogeny Inference Package (PHYLIP), version 3.5c (J. Felsenstein, University of Washington).

RESULTS

Plasmid transfer from *E. coli* to fecal bacteria. Filter matings with *E. coli* donor strains and bacteria extracted directly from collected feces of *F. candida* were carried out in order to detect plasmid transfer potentials. Donor counterselection was achieved on minimal medium with benzoic acid as the sole source of carbon, a substrate which could not be used by the *E. coli* strains in this investigation. Table 2 indicates that a significant proportion of fecal bacteria (37.1% ± 33.7% of CFU obtained on nonselective yeast tryptone medium) was capable of using benzoic acid as the sole carbon source.

A total of five different plasmids, two of them in various *E. coli* host strains, were selected. Transconjugants were detected in matings with the self-transferable, broad-host-range IncP1 plasmid pRP4-luc and with the IncQ plasmid pSUP104-luc (Table 2). The latter plasmid was mobilized from *E. coli* S17-1, a strain with chromosomally inserted *tra* (transfer) genes of the IncP plasmid pRP4 (see also Table 1). The IncF′ *E. coli* strain NM522 was also capable of promoting IncQ plasmid transfer into indigenous fecal bacteria. Transfer rates, calculated as transconjugants per donor cell and also as transconjugants per recipient cell, indicated that pSUP104-luc mo-

TABLE 3. Results of plasmid transfer from *E. coli* donor strains to gut bacteria in feeding experiments with *F. candida*

E. coli donor strain	Plasmid	Fecal bacteria (log CFU per specimen) extracted from microcosms							
		After 7 days of feeding				After 14 days of feeding			
		Total cells	Recipient cells	Donor cells	Transconjugant cells	Total cells	Recipient cells	Donor cells	Transconjugant cells
S17-1	pUC18-luc	7.07	6.75	ND[a]	ND	6.46	5.97	1.85	ND
	pSUP202-luc	6.85	7.36	ND	−0.19	6.29	5.45	1.57	−0.98
	pSUP104-luc	6.27	6.00	0.87	0.77	6.74	6.00	2.69	2.82
	pRP4-luc	6.27	5.88	3.75	0.36	6.39	5.48	4.73	1.61
HB101	pSUP104-luc	6.02	5.89	2.08	ND	7.02	4.15	ND	ND
NM522	pSUP104-luc	6.16	6.16	3.21	ND	6.93	6.21	3.76	ND
S17-1 λPir	pUT*luxAB*	6.20	6.08	ND	ND	6.63	4.61	ND	ND
CC118 λPir	pUT*luxAB*	6.00	4.28	1.41	ND	6.22	6.13	2.5	ND

[a] ND, not detectable.

bilization from *E. coli* NM522 was less efficient than that from *E. coli* S17-1. Plasmid pSUP104-luc was not transferred from the nonmobilizing strain HB101. Narrow-host-range plasmid pSUP202-luc was also not transferred to fecal bacteria. Filter matings with the broad-host-range mobilizing strain S17-1 (λPir) and miniTn*5* plasmid pUT*luxAB*, which aimed at detecting transconjugants with chromosomally inserted *luxAB* genes among the fecal bacteria, failed. Additionally, no transconjugants were detected after pUT*luxAB* transfer experiments with CC118λPir as the donor and S17-1 as the mobilizing strain.

Transfer frequencies could only be estimated, because clonal expansion of early occurring transconjugants during the 24-h incubation period could not be excluded. Transfer rates above 1, as determined for *E. coli* S17-1 with pSUP104-luc (Table 2), are indicative of growth of transconjugant populations. Generally, however, the frequencies shown in Table 2 indicate that transfer efficiencies for both pRP4-luc and pSUP104-luc from *E. coli* S17-1 were comparable to those which are observed for intraspecies transfer with added *E. coli* recipients under optimum conditions.

Plasmid transfer to *F. candida*-associated microorganisms in feeding experiments. *F. candida* specimens were fed with the same selection of donor strains as that used in the previously described filter matings. Bacterial populations were analyzed after 7 and 14 days of incubation. After 7 days, 91 specimens (±7.7; $n = 12$) and, after 14 days, 72 specimens (±21.1) of 100 initial specimens were still alive in the microcosms. The specimens which were alive could be distinguished by their behavior: one group was actively feeding and another group, which was occupied with molting, was not feeding. The analysis of feces after 7 and 14 days indicated a high proportion of recipient cells among all culturable (LB) medium cells (Table 3). In contrast to the results of the filter mating experiments, donor cells were recovered only at very low concentrations, indicating that the *E. coli* cells were digested in the gut of *F. candida*. Transconjugants were recovered from microcosms with strain S17-1 harboring pRP4-luc and pSUP104-luc. In contrast to the results obtained from the filter mating experiments, mobilization of pSUP202-luc from *E. coli* S17-1 to gut bacteria was detected. Except for pSUP202-luc, the incidence of transconjugants in the feces was higher after 14 days than after 7 days. Strain NM522 did not mobilize pSUP104-luc in these experiments. No transconjugants of the miniTn*5* delivery plasmid, pUT*luxAB*, were observed.

Kinetics of pRP4-luc transconjugant occurrence in feeding experiments. A more detailed analysis of the time-dependent occurrence of transconjugants in feces was performed with *E. coli* S17-1 pRP4-luc. *F. candida* specimens were transferred from one microcosm to another every day. Thus, data shown in Fig. 1 indicate the number of fecal bacteria released from the insects over a period of 24 h. Due to the mobility of the insects in the microcosms, it cannot be excluded that some of the donor cells detected on the water-agar were not of fecal origin but were contaminants from the food supply. This may explain the high numbers of donor cells detected after 3 and 8 days. Fluctuations in the numbers of recipients recovered from feces were repetitive every 4 days, which, most likely, was influenced by the molting cycles of the insects. The numbers of total bacteria in the feces, however, did not follow this pattern. Transconjugants occurred for the first time 48 h after the beginning of the experiment and were detected infrequently over the entire period of incubation. The cumulative number of transconjugants detected between days 7 and 14 was higher than between days 1 and 7, which was in accordance with the data shown in Table 3. At the end of the experiment, more transconjugants than donor cells were recovered from the microcosms.

Higher numbers of transconjugant cells than donor cells were also detected when the total gut contents of *F. candida* specimens, fed for 10 days with *E. coli* S17-1 pRP4-luc, were analyzed. An average of 2.32×10^4 CFU animal^{-1} was detected on LB medium. Benzoic acid-degrading bacteria (recipients) occurred at 1.30×10^4 CFU animal^{-1} (56%). Only 1.9

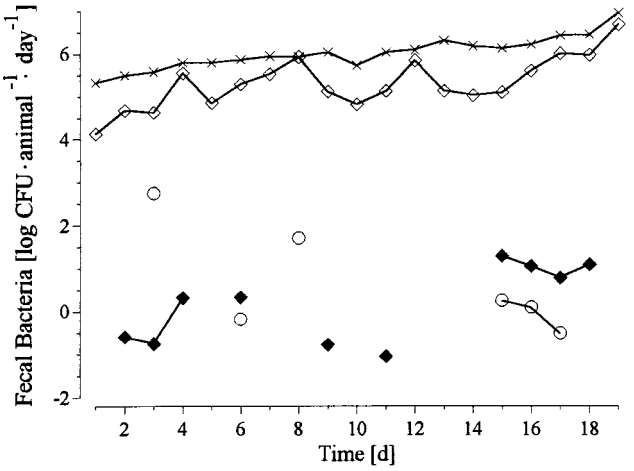

FIG. 1. Feeding of *E. coli* pRP4-luc to *F. candida* in petri dish microcosms. Levels of occurrence, as measured daily, of total bacteria (×), recipients (◇), donor cells (○), and transconjugants (◆) in feces are represented. d, day.

TABLE 4. Effects of nalidixic acid on the recovery of fecal bacteria of *F. candida*[a]

Cell type	No. of cells (log CFU animal^{-1}) extracted from water-agar	
	Without nalidixic acid	With nalidixic acid
Total fecal	6.39	6.06
Recipient	6.03	5.54
Donor[b]	1.17	ND[c]
Transconjugant[b]	0.15	0.25

[a] Nalidixic acid (10 μg ml of water-agar^{-1}) was added or not added to petri dish microcosms of fecal bacteria from *F. candida* in feeding experiments with *E. coli* S17-1 pRP4-luc as a donor strain.
[b] Growth occurred on the appropriate selective medium and was confirmed by luciferase colony detection.
[c] ND, not detected.

CFU of donor cells animal^{-1} was detected. Transconjugants occurred at 4.5 CFU animal^{-1} (not shown).

Localization of plasmid transfer events. The selected method for detecting transconjugants involved, inevitably, the cultivation of a donor-recipient-containing suspension on nutrient agar. Under such conditions, plasmid transfer (plate mating) may occur (60). In order to determine whether transconjugants arose as a result of plate mating or before mating in the gut or feces of *F. candida*, controls in which *E. coli* pRP4-luc and recipient cells were separately preincubated under filter mating conditions were combined and inoculated onto the transconjugant-selective growth agar. No transconjugants could be detected under these conditions. Thus, plate mating did not influence the detection of transconjugants in our investigation (data not shown).

An experiment was conducted in which *F. candida* was fed with *E. coli* pRP4-luc for 7 days in order to determine whether the formation of transconjugants occurred mainly in the gut of *F. candida* or subsequently in the feces. Under these conditions, feces were incubated on water-agar for 1 to 7 days, depending on the experiment. In one set of microcosms, water-agar contained nalidixic acid, an antibiotic which inhibits DNA replication, conjugative gene transfer, and plate mating (39, 60, 65). The selected nalidixic acid concentration (10 μg ml^{-1}) inhibited the growth of *E. coli* S17-1 pRP4-luc. The MICs for eight randomly selected transconjugant strains, determined in separate experiments, were in the range 1 to 50 μg ml^{-1}, with four strains being more resistant than *E. coli* (data not shown). Feces extracted from both types of microcosms, i.e., with and without nalidixic acid, were analyzed for the occurrence of total cells, donor cells, recipients, and transconjugants. The numbers of total cells and recipients were reduced by an order of magnitude of approximately 0.5 (Table 4). This result was in accordance with the previously described tolerance (MIC) of fecal bacteria to this antibiotic. No donor cells were detected on water-agar supplemented with nalidixic acid, indicating efficient diffusion of the antibiotic into the fecal depositions. Transconjugants, however, could be isolated from both types of microcosms, i.e., with and without nalidixic acid. Thus, since donor cells were completely inhibited, it could be concluded that pRP4-luc was transferred from *E. coli* to recipient cells in the gut of *F. candida* and not in its feces.

Phylogenetic diversity of transconjugants. Segregation was observed frequently with all plasmid-bearing transconjugant strains isolated in this study under nonselective conditions. Nevertheless, subcultures which stably maintained the expression of their respective marker genes could be obtained with all transconjugants except those with the narrow-host-range plasmid pSUP202-luc. In the case of transconjugants with pSUP202-luc, bioluminescence faded away during the first three subcultures and detection of the *luc* gene by PCR was positive only during the first five subcultures (data not shown).

The diversity of transconjugants was assessed with a total of 264 pure-culture isolates from both feeding and filter mating experiments. Purified colonies, grown on agar plates, were differentiated first by restriction fragment length polymorphism analysis of their 16S rRNA genes (ARDRA). Fifteen pattern types could be differentiated with two DNA restriction enzyme endonucleases, *Cfo*I and *Alu*I. These ARDRA types were analyzed at a physiological level by microtiter plate-bound substrate utilization assays (Biolog) and at the phylogenetic level by sequencing and comparison of the nearly complete, PCR-amplified 16S rRNA gene. All isolates were gram negative and belonged to the alpha, beta, or gamma subclass of the class *Proteobacteria* (Table 5). All three plasmids transferred from

TABLE 5. Comparison of transconjugants isolated from *F. candida* with data bank strains

Subclass of *Proteobacteria*	Strain	Acquired plasmid	Biolog identification		16S rRNA gene sequencing		
			Closest relative	Similarity coefficient[a]	Closest relative	% Similarity	EMBL accession no.
Alpha	HS1	pSUP202-luc	*Ochrobactrum anthropi*	0.93	*Ochrobactrum anthropi*	99.8	AJ002812
Beta	H151	pSUP104-luc	*Alcaligenes xylosoxidans*	0.86	*Alcaligenes xylosoxidans*	98.6	AJ002802
	H158		*Comamonas acidovorans*	0.90	*Comamonas acidovorans*	99.4	AJ002803
	H159		*Alcaligenes xylosoxidans*	0.98	*Alcaligenes xylosoxidans*	97.9	AJ002804
	HR4	pRP4-luc	*Alcaligenes faecalis*	1.00	*Alcaligenes faecalis*	98.7	AJ002815
	HR5		*Alcaligenes xylosoxidans*	0.74	*Alcaligenes xylosoxidans*	98.5	AJ002808
	HR6		*Alcaligenes xylosoxidans*	0.97	*Alcaligenes xylosoxidans*	97.8	AJ002809
	HR7		*Comamonas testosteroni*	0.82	*Comamonas acidovorans*	96.1	AJ002810
Gamma	HS2	pSUP202-luc	*Pseudomonas cichorii*	0.51	*Pseudomonas agarici*	98.0	AJ002813
	HS3		*Stenotrophomonas maltophilia*	0.88	*Stenotrophomonas maltophilia*	99.5	AJ002814
	H150	pSUP104-luc	*Pseudomonas fluorescens*	0.91	*Pseudomonas fluorescens*	99.1	AJ002801
	HR1	pRP4-luc	*"Pseudomonas maculicola"*	0.80	*Pseudomonas putida*	99.1	AJ002805
	HR2		*Brevundimonas diminuta*	0.65	*Stenotrophomonas maltophilia*	97.6	AJ002806
	HR3		*Aquaspirillum dispar*	0.71	*Steonotrophomonas maltophilia*	97.6	AJ002807
	HR8		*Enterobacter cloacae A*	0.82	*Pantoea agglomerans*	99.4	AJ002811

[a] Readings were taken after 24, 48, and 72 h. A coefficient of 1.0 indicates complete homology to a data bank species.

E. coli to a variety of species. There was accordance between the results obtained from data bank comparisons with Biolog and 16S rRNA gene sequencing. However, at the DNA level no isolate was identical and by Biolog only one isolate (HR4; *Alcaligenes faecalis*) was completely identical to described strains in the data banks.

DISCUSSION

The sensitive detection of gene transfer from donor strains to indigenous microorganisms in soil requires both efficient marker genes and donor counterselection (13, 63). Antibiotic resistance genes, as carried on the plasmids used in our study, may not be sufficient for the detection of gene transfer because natural resistances of indigenous recipients can mask the detection of transconjugants (64). The insertion of constitutively expressing luciferase genes into the plasmids used in our study provided the crucial technique to distinguish transconjugants from a background of antibiotic-resistant or -tolerant indigenous bacteria. By this means we were able to detect even transconjugants which could not stably maintain their acquired plasmid, such as pSUP202 in strain HS2 (*Pseudomonas agarici*). In environmental settings, unstable transconjugants might be able to act as transient hosts and propagate plasmids to other recipients (52). *Pseudomonas* spp. were found to be more persistent in the gut of *F. candida* than *E. coli* (67), and therefore the survival of a plasmid in this particular habitat might be extended, even in transient hosts, compared to its survival in the fed donor strain.

Most of the *E. coli* cells fed to *F. candida* were digested, but counterselection was still necessary to exclude contamination by *E. coli* donor cells. Counterselection was achieved by selecting growth media with benzoic acid as a sole source of carbon. In contrast to results with *E. coli*, a rather high proportion of the culturable gut bacteria were able to use benzoic acid, but this method of donor counterselection inevitably excluded the proportion of indigenous bacteria incapable of growing with benzoic acid. Types of counterselection used in other studies, however, suffered the same disadvantages (61, 69). While recipient exclusion might lead to an underestimation of gene transfer frequencies, as described above, growth and clonal expansion can tend to produce overestimations of transconjugants (19). Since, in some experiments of our study, feces were incubated in microcosms several days before analyses, we could not calculate exact transfer frequencies of the feeding experiments. However, the diversity of indigenous transconjugants, as well as the number of transconjugants detected in experiments by daily analyses, clearly indicated that plasmid transfers in the gut of *F. candida* were not rare events.

Feeding experiments with *E. coli* donor strains were incubated for a period of 7 and 14 days in order to allow for intensive contact between the animals and the donor strains. Also, we anticipated that plasmid transfer events might be restricted to certain periods, correlating with the molting cycles of *F. candida*. Due to the incubation period of 7 days, some of the feces were relatively old before analysis, and it might be speculated that conjugation events may have occurred mostly on the agar surface and not during passage through the gut. One might speculate that the insects contributed to the generation of transconjugants only by providing the ecosystem to mix recipient with donor cells. However, in our study we were able to inhibit plasmid transfer (pRP4-luc) in feces by supplementing water-agar with nalidixic acid but transconjugants could still be isolated. Additionally, data from the feeding experiments indicated that only a few donor cells survived the gut passage. Smit and van Elsas (60) found that plasmid transfer on agar plates (plate mating) did not occur when donor cell numbers were low. Thimm et al. (67) observed with *F. candida* that the gut passage reduced ingested *E. coli* cells over 60,000-fold. Thus, the donor/recipient ratio in the feces was relatively low. Donor/recipient ratios were probably much higher immediately after donor cells were ingested, and thus conditions for conjugative gene transfer were much more favorable than in the feces. In the accompanying paper we have determined that the gut of *F. candida* had an average volume of 10 nl (67). The numbers of recipients in this investigation were approximately 10^6 to 10^7 CFU animal^{-1}. Thus, recipient cell concentrations (10^{11} to 10^{12} CFU ml^{-1}) were comparable to those of stationary-phase batch broth cultures.

Digestion of the ingested *E. coli* cells should result in the release of DNA into the gut, which would thus be a potential substrate for transformation. However, in our investigation, transformation as a process of gene transfer was unlikely because nonmobilizing non-self-transferable pUC18-luc, a cloning vector which can be transferred efficiently by artificial transformation in the laboratory (51), was not transferred from *E. coli* to indigenous gut bacteria of *F. candida*. Our data do not show whether these results were a consequence of the lack of DNA uptake, DNA protection, or expression by the gut bacteria. The narrow host range of replication of pUC (*ori* of ColE1) (4) might also have prevented the detection of transformation.

IncQ plasmids are able to be mobilized by plasmids of different incompatibility groups, e.g., IncP (21). In our investigation, the IncQ mobilizable plasmid pSUP104-luc was mobilized to indigenous bacteria in filter mating and feeding experiments from *E. coli* S17-1, a strain with chromosomally integrated IncP transfer genes of pRP4 (58). In filter matings, the *E. coli* strain NM522, with an F′ plasmid (IncF1), was also capable of mobilizing pSUP104-luc. The efficiency of IncQ mobilization by IncF1 was orders of magnitude below that of IncP mobilization. This result is in accordance with results of a study by Willetts and Crowther (76). However, other pure-culture studies did not detect IncQ mobilization by F plasmids (29). In feeding experiments, NM522 could not mobilize pSUP104-luc, probably because survival rates of the donor strain and therefore the period of contact between donor and recipient cells was, due to digestion, drastically reduced compared to that under filter mating conditions. Mobilization of plasmids in our investigation occurred only when the mobilizing functions were provided by the donor cells themselves. In other studies, IncQ plasmid mobilization in soil was observed from a nonmobilizing donor strain when *E. coli* cells harboring pRP4 were added (48, 62). Recently, IncQ mobilization by indigenous bacteria could be detected in a field experiment with manure-amended soil (25). In this investigation, IncQ mobilization could not be detected in the gut or with fecal bacteria of *F. candida*. However, other studies have shown that self-transferable plasmids and plasmids with mobilization potentials are present in the environment and able to interact with released bacterial cells (38, 45, 70). We assume, therefore, that further analysis will also detect such mobilizing functions in habitats associated with soil microarthropods.

This work demonstrates that the gut of the selected soil microarthropod provided appropriate-to-ideal conditions for conjugation and, thus, should be regarded as a hot spot for gene transfer in soil. If the host range of a plasmid is broad and the biodiversity in a hot spot habitat is uncharacterized, as for most soil microarthropods, the spread of recombinant genes may occur at much higher rates than anticipated by previous risk assessment studies performed with bulk soil microcosms.

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