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Conjugative Transfer of Chromosomal Genes between Fluorescent Pseudomonads in the Rhizosphere of Wheat

JOSEPH TROXLER,[1] PIERRE AZELVANDRE,[2] MARCELLO ZALA,[1]
GENEVIÈVE DÉFAGO,[1] AND DIETER HAAS[2]*

Institute of Plant Sciences/Phytopathology, Eidgenössische Technische Hochschule, CH-8092 Zürich,[1] and Laboratoire de Biologie Microbienne, Université de Lausanne, CH-1015 Lausanne,[2] Switzerland

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Bacteria released in large numbers for biocontrol or bioremediation purposes might exchange genes with other microorganisms. Two model systems were designed to investigate the likelihood of such an exchange and some factors which govern the conjugative exchange of chromosomal genes between root-colonizing pseudomonads in the rhizosphere of wheat. The first model consisted of the biocontrol strain CHA0 of *Pseudomonas fluorescens* and transposon-facilitated recombination (Tfr). A conjugative IncP plasmid loaded with transposon Tn*5*, in a CHA0 derivative carrying a chromosomal Tn*5* insertion, promoted chromosome transfer to auxotrophic CHA0 recipients in vitro. A chromosomal marker (*pro*) was transferred at a frequency of about 10^{-6} per donor on wheat roots under gnotobiotic conditions, provided that the Tfr donor and recipient populations each contained 10^6 to 10^7 CFU per g of root. In contrast, no conjugative gene transfer was detected in soil, illustrating that the root surface stimulates conjugation. The second model system was based on the genetically well-characterized strain PAO of *Pseudomonas aeruginosa* and the chromosome mobilizing IncP plasmid R68.45. Although originally isolated from a human wound, strain PAO1 was found to be an excellent root colonizer, even under natural, nonsterile conditions. Matings between an auxotrophic R68.45 donor and auxotrophic recipients produced prototrophic chromosomal recombinants at 10^{-4} to 10^{-5} per donor on wheat roots in artificial soil under gnotobiotic conditions and at about 10^{-6} per donor on wheat roots in natural, nonsterile soil microcosms after 2 weeks of incubation. The frequencies of chromosomal recombinants were as high as or higher than the frequencies of R68.45 transconjugants, reflecting mainly the selective growth advantage of the prototrophic recombinants over the auxotrophic parental strains in the rhizosphere. Although under field conditions the formation of chromosomal recombinants is expected to be reduced by several factors, we conclude that chromosomal genes, whether present naturally or introduced by genetic modification, may be transmissible between rhizosphere bacteria.

Certain root-colonizing bacteria are able to protect plants from soilborne fungal pathogens and therefore may be useful as biocontrol agents in greenhouses and in the field. A number of disease-suppressing or plant growth-promoting rhizobacteria that have been well characterized with respect to their mode of action belong to the genus *Pseudomonas* (11, 61). The ability of these bacteria to suppress root diseases depends on several traits, including root colonization, antibiotic production, induction of resistance in the plant, and competition for nutrients (especially iron) (9, 11, 14, 32). Several studies have shown that antibiotic-negative mutants of biocontrol pseudomonads display reduced disease suppression abilities (14, 26, 68, 70). Conversely, in some cases it has been possible to improve the biocontrol performance genetically by overexpression of bacterial antibiotic production (54, 55) or by introduction of genes coding for the biosynthesis of an additional antibiotic (9). In general, the genes involved in biocontrol traits appear to be chromosomal in fluorescent pseudomonads (61, 68), and for stability reasons, any genetic modification that might be introduced into biocontrol strains would preferentially be integrated into the chromosome (1).

The population size of beneficial pseudomonads on plant roots is an important parameter determining biocontrol effectiveness. Below a certain threshold population density (usually about 10^5 CFU per g of root), little disease suppression is observed (5, 45, 70). Effective seed treatments typically involve 10^6 to 10^9 CFU per seed (5, 30, 45). For soil inoculation, pseudomonads have been introduced at 10^7 CFU per g of soil in seed beds (71). Thus, in field applications, large numbers of biocontrol bacteria may be released to achieve protective root colonization.

The deliberate release of biocontrol bacteria, whether genetically modified or not, may have an impact on indigenous soil microorganisms. Potential hazardous events include the dissemination of genes from the introduced bacteria to resident microorganisms by conjugation, transduction, or transformation (56). In fluorescent pseudomonads, the exchange of genetic material is promoted by conjugative plasmids and transducing phages (22). Transfer of conjugative plasmids between fluorescent pseudomonads and to other gram-negative bacteria has been demonstrated in soil (17, 31, 52, 57, 64). Plant roots greatly stimulate bacterial growth as well as conjugative plasmid transfer, by providing a nutrient-rich surface (31, 64). In contrast, little is known about the transfer of chromosomal DNA between bacteria in soil. Hfr (high frequency of recombination) donors and F^- recipients of *Escherichia coli*, when mating in soil, are able to produce chromosomal recombinants (29). However, *E. coli* is not considered a typical soil inhabitant, and quantitative experimental data for soil bacteria are lacking (1, 65).

In the present study, we have measured the frequencies of conjugative chromosome transfer between root-colonizing, fluorescent pseudomonads with biocontrol potential. The donor

* Corresponding author. Mailing address: Laboratoire de Biologie Microbienne, Université de Lausanne, CH-1015 Lausanne, Switzerland. Phone: 41 21 692 56 31. Fax: 41 21 692 56 35. E-mail: Dieter.Haas@lbm.unil.ch.

TABLE 1. Strains of *P. aeruginosa* and *P. fluorescens* and plasmids

Strain or plasmid	Genotype or phenotype	Reference
P. aeruginosa strains		
PAO1	Wild type	21
PAO25	*argF10 leu-10*	19
PAO512	*argH32 lysA58 nalA7*	20
PAO513	*argB18 lysA60 nalA8*	20
P. fluorescens strains		
CHA0	Wild type	58
CHA116	*trp*::Tn*5* derivative of CHA0	This work
CHA171	*pro ade nal* derivative of CHA0	This work
CHA2613	*ade leu nal* derivative of CHA0	This work
Plasmids[a]		
R68.45	IncP Tra Cb Km Tc Cma	19
pLG221	IncI1 Tra *drd-1 cib*::Tn*5*	4
pME461	IncP Cb Tc *trfA*(Ts) Tn*801*::Tn*5*[b]	50
pME462	IncP Cb Tc *trfA*(Ts) *kfrA*::Tn*5*[c]	49

[a] Plasmid phenotypes and genotypes: IncP, incompatibility group P; Tra, conjugative transfer; Cb, carbenicillin resistance; Km, kanamycin resistance; Tc, tetracycline resistance; Cma, chromosome mobilization ability; IncI1, incompatibility group I1; *drd*, derepressed for conjugative transfer; *cib*, colicin Ib; *trfA*, transacting factor of replication; *kfrA*, gene involved in plasmid replication control.

[b] The parental plasmid of pME461 and pME462 is pME305, a deletion derivative of RP1 (50). The *trfA*(Ts) mutation causes temperature-sensitive plasmid maintenance at 43°C in *E. coli* and *P. aeruginosa* (51).

[c] The site of Tn*5* insertion in pME462 was deduced from restriction mapping data (49) and the RP1/R68 physical map (42).

strains used contain broad-host-range (IncP) plasmids displaying chromosome mobilizing ability (Cma). We have deliberately refrained from using genetically engineered Hfr strains of *Pseudomonas* (47), as similar donor strains have not been found in natural environments (48). Amino acid-auxotrophic mutations were used in the recipients and donors for selection and counterselection, respectively. The rationale is that root exudates contain enough amino acids to support the growth of the introduced bacteria during the duration of the experiment. Starting with gnotobiotic conditions, we show that chromosomal gene transfer can also be detected in the rhizosphere of wheat plants grown in natural, nonsterile soil.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Strains and plasmids are listed in Table 1. Plasmids pME461 and pME462 were transferred from *E. coli* to *Pseudomonas fluorescens* as previously described (66). The Cma of plasmid R68.45 was tested prior to each experiment according to the method of Haas and Holloway (19). For experiments in vitro, *P. fluorescens*, *Pseudomonas aeruginosa*, and *E. coli* were grown in nutrient yeast broth (NYB), on nutrient agar (NA), or on minimal medium S (MMS) (20, 36). Amino acid and adenine supplements were added to a final concentration of 1 mM when required. Antibiotic concentrations for *P. fluorescens* were as follows: kanamycin, 25 μg/ml; nalidixic acid (NAL), 200 to 1000 μg/ml; and tetracycline (TC), 50 μg/ml in minimal medium or 125 μg/ml in complex medium. Antibiotic concentrations for *E. coli* and *P. aeruginosa* have been given previously (50). Liquid cultures were grown with aeration by shaking. *E. coli* and *P. aeruginosa* were grown at 37°C. *P. fluorescens* strains were incubated at 30°C, except when cells were used as recipients in crosses with *E. coli* plasmid donors; in this case, *P. fluorescens* was grown at 35°C prior to mating in order to reduce the activity of its restriction system (67). For gene transfer experiments in soil and in the rhizosphere of wheat, *P. aeruginosa* and *P. fluorescens* strains were cultivated overnight at 27°C on King's medium B (27) supplemented with appropriate antibiotics (50 μg of TC/ml for R68.45 maintenance in *P. aeruginosa*, 125 μg of TC/ml for pME461 and pME462 in *P. fluorescens*, or 200 μg of NAL/ml for recipient strains). The bacteria were scraped from the plates with 10 ml of sterile distilled water and washed twice in sterile distilled water. The desired cell concentrations were adjusted according to the optical density at 600 nm.

Isolation of *P. fluorescens* mutants. Strain CHA116 is a tryptophan auxotrophic mutant of *P. fluorescens* CHA0 obtained by Tn*5* insertion mutagenesis with pLG221 as described elsewhere (67). In vitro, Trp^+ revertants occurred at 5×10^{-8}; they had all lost the kanamycin resistance marker, indicating excision of Tn*5* from a *trp* biosynthetic gene. The double auxotrophs CHA171 and CHA2613 were derived from strain CHA0 by two rounds of chemical mutagenesis with ethyl methanesulfonate (69). An overnight culture of strain CHA0 was diluted 20-fold into fresh NYB and grown to exponential phase. Ethyl methanesulfonate was added to 1% (vol/vol) with vigorous mixing. After incubation at 30°C for 1 h without shaking, the culture was diluted 20-fold into NYB and incubated with shaking for 6 h to permit expression. Cells were washed twice by centrifugation and resuspended at 10^7 cells/ml in carbon- and nitrogen-free MMS. After incubation overnight without aeration, the starved cells were diluted 10-fold into complete MMS containing 5.6 mg of D-cycloserine/ml and incubated with aeration for 36 h. After serial dilution, 0.1-ml aliquots were plated on NA and incubated for 1 day. Auxotrophic mutants were recognized by replica plating onto MMS plates. Spontaneous NAL-resistant mutants were isolated by plating 10^8 cells on supplemented MMS containing 1,000 μg of NAL/ml. Since strain CHA0 shows signs of genetic instability in vitro resulting in tryptophan side chain oxidase-negative, antibiotic-negative phenotypes (68), the presence of the tryptophan side chain oxidase-positive marker was routinely verified (40).

***P. fluorescens* matings in vitro.** For a demonstration of plasmid or chromosome transfer between *P. fluorescens* strains, about 10^9 donor cells (CHA116/pME461 or CHA116/pME462), which had been grown overnight in NYB containing 125 μg of TC/ml, were mixed with about 10^9 recipient cells (CHA171 or CHA2613), washed together in saline (0.85% [wt/vol] NaCl), and plated on MMS supplemented with adenine and proline (for crosses involving CHA171) or adenine and leucine (for crosses involving CHA2613). The mating mixture was confined to an area of about 4 cm^2 and incubated at 30°C for 1 day. The mating mixture was harvested in 1 ml of saline, and appropriate dilutions were plated on MMS selective for chromosomal recombinants, plasmid transconjugants, donors, or recipients. Colonies were counted after 3 days of incubation at 30°C.

Conditions of horizontal gene transfer in the rhizosphere of wheat. In a gnotobiotic system previously described (25), conjugative gene transfer ability of *P. aeruginosa* and *P. fluorescens* was tested. The artificial soil contained pure vermiculitic clay, quartz powder, and quartz sand of different particle sizes but no organic substrates and was moistened with distilled water (10%, wt/wt). Erlenmeyer flasks (1 liter) were filled with 300 g of artificial soil, closed with cotton wool stoppers, and autoclaved at 121°C for 30 min. Wheat (*Triticum aestivum* L. "Arina") seeds were surface disinfected in 5% (wt/wt) sodium hypochlorite for 10 min, rinsed with distilled water, and germinated on 0.85% water agar (Difco) for 2 days. Each bacterial suspension (10 ml) required for gene transfer experiments was added to the artificial soil. In experiments conducted with *P. aeruginosa*, the soil was inoculated simultaneously with recipient and donor strains. Control treatments consisted of donor or recipient strains in 20 ml of sterile distilled water. Then five seedlings of wheat were planted per flask, and 10 ml of modified Knop nutrient solution (25) was added. Flasks were incubated in a growth chamber at 70% relative humidity and 20°C with light (160 microeinsteins m^{-2} s^{-1}) for 16 h, followed by an 8-h dark period at 17°C. Experiments with *P. fluorescens* followed the same protocol but were modified such that donor strains were injected with a syringe into the rhizosphere, 7 days after inoculation of the recipient into soil and planting of wheat.

Experiments with *P. aeruginosa* were also done in microcosms containing natural soil. The soil was collected from the upper layer (20 cm) of an agricultural field in Eschikon, near Zurich, Switzerland; passed through a 5-mm-mesh screen; and stored at 15°C prior to use. The physical properties of this sandy loam soil (cambisol) have been described elsewhere (38). When required, the soil was autoclaved twice for 60 min with an interval of 1 day. To achieve a uniform bacterial distribution, suspensions of *P. aeruginosa* donors and recipients were spread onto a sterile sand layer (0.8 to 1.2 mm, 5% [vol/vol] of total soil) and mixed with the soil with a sterile spatula. Sterile flowerpots were filled with 300 g of the inoculated soil, five seedlings of wheat were planted, and 10 ml of modified Knop nutrient solution (25) was added. Pots were incubated in a growth chamber under conditions similar to those described for the gnotobiotic system; soil was kept moist at field capacity. Nonsterile soil was inoculated by an analogous procedure.

After growth for 14 to 21 days, roots were carefully harvested and loosely adhering soil was removed. The roots of five plants from one flask or pot were weighed and transferred to a 100-ml flask containing 20 or 40 ml of sterile distilled water, shaken at 300 rpm for 1 h, and then mixed on a Vortex shaker for 15 s. The resulting extracts were serially, decimally diluted and plated on MMS selective for chromosomal recombinants, plasmid transconjugants, donors, or recipients; details are given in the legends of Tables 2 and 3. Colonies were counted after 3 or 4 days of incubation at 27°C. One experiment consisted of five to six replicates per treatment. Formation of revertants was checked in controls, including five pots inoculated with donor or recipient strains alone.

Data analysis. Root colonization by donor and recipient strains was given as the mean value of five to six replicates. Frequencies of chromosomal recombinants were expressed per donor CFU, as donor cell numbers were usually lower than were recipient cell numbers. The frequencies of chromosomal recombinants varied considerably between replicates, one main source of variation probably being the time when conjugation was initiated. Because prototrophic recombinants of *P. aeruginosa* have a selective advantage over auxotrophic parental strains, early recombination events result in numerous recombinants whereas late recombination produces few if any recombinants. As customary in similar

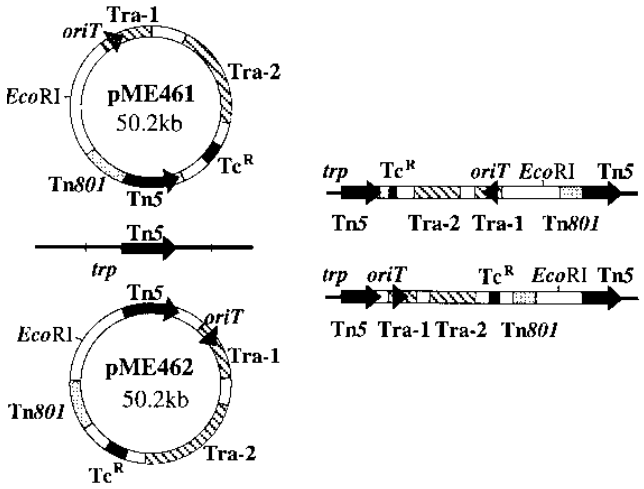

FIG. 1. Tfr donors of *P. fluorescens* CHA0. Transposon Tn*5* was transposed into the RP1 derivative pME305, resulting in pME461 and pME462 (42, 43). Transient integration of pME461 and pME462 into a chromosomal *trp*::Tn*5* locus of strain CHA0 via homologous recombination can produce the Tfr donor strains shown. Tra-1 and Tra-2, transfer gene clusters of RP1; *oriT*, origin of transfer.

situations (13), the median frequencies of recombinants were calculated. Parallel determinations of plasmid transfer frequencies are also given as medians, although plasmid transconjugants did not have a selective advantage; hence, mean values could have been used. However, the medians were similar to the means.

RESULTS

Chromosome and plasmid transfer between auxotrophic mutants of *P. fluorescens* CHA0 in vitro. Strain CHA0 was originally isolated from a plant disease-suppressive soil (58) and has served as a model organism in studies on the mechanisms of biocontrol (11, 68). This strain of *P. fluorescens* is a very poor recipient for common IncP plasmids such as RP1. However, an RP1 derivative (pME305) in which the kanamycin resistance (Km^r) determinant, IS*21*, and the *par* region are deleted is conjugally transmissible in *E. coli* × CHA0 as well as in CHA0 × CHA0 matings (66). The mechanism by which the deletion in pME305 overcomes the conjugation barrier in *P. fluorescens* is unknown; an involvement of restriction or modification is unlikely. Plasmid pME305 was loaded with Tn*5* in either orientation, resulting in pME461 and pME462 (Fig. 1). Both plasmids were transferable with high efficiencies in plate matings of *P. fluorescens* strains with selection being made for antibiotic resistance (Table 2). When pME461 or pME462 was introduced into auxotrophic derivatives of strain CHA0 carrying a Tn*5* insertion in the chromosome, transposon-facilitated (Tfr) donors were obtained (Fig. 1). These transferred chromosomal markers at 10^{-4} to $<10^{-7}$ per donor in plate matings with various auxotrophic recipients. Initially, Tn*5*-induced auxotrophs were tested as recipients. Although they were suitable for crosses in vitro, their reversion (due to Tn*5* excision) was a problem in rhizosphere experiments, as revertants were more frequent than were recombinants in some cases. Therefore, chemically induced, stable, double auxotrophs were used as recipients in subsequent crosses (Table 2). These strains had negligible reversion frequencies. In addition, the recipients were made NAL resistant, whereas the donors were NAL sensitive.

The Tfr donor CHA116 (*trp*::Tn*5*) carrying either pME461 or pME462 produced prototrophic recombinants for the *pro* mutation but not for the *ade* mutation of the recipient strain CHA171 (Table 2). However, another *ade* mutation in strain CHA2613 did yield recombinants with a pME462 donor (Table 2), showing that *ade* mutations need not be refractory to recombination. Matings were carried out on supplemented MMS plates for 20 to 24 h before cells were plated on selective medium containing 1,000 μg of NAL/ml. (Supplemented MMS was used rather than rich NA medium because control experiments had shown that in mating mixtures plated on NA, up to 90% of the recipient cells were killed by the donor within 1 day of incubation.) When mating mixtures were directly plated on selective medium containing NAL, no ($<10^{-8}$) plasmid transconjugants or chromosomal recombinants were recovered, showing that NAL effectively prevents conjugal transfer of *P. fluorescens* in vitro. NAL is known to have the same effect in *P. aeruginosa* (19). In addition, the use of NAL in selective medium helped counterselect the donor. Alone, the Tn*5*-induced auxotrophic marker of the donor CHA116 would have been unsatisfactory for counterselection because of reversion (about 5×10^{-8}).

Chromosome and plasmid transfer between *P. fluorescens* derivatives in the rhizosphere of wheat under gnotobiotic conditions. The donor-recipient combinations producing the highest yields of recombinants in vitro (CHA116 containing pME461 or pME462 × CHA171) were chosen for gene transfer experiments in the rhizosphere of wheat grown in sterile, artificial soil. NAL was present in all selective media to exclude DNA transfer after the isolation of bacteria from roots. The wild-type strain CHA0 colonizes wheat roots efficiently, reaching 10^8 CFU/g of root within 2 to 3 weeks (26). In preliminary gene transfer experiments, which consisted of inocula of 10^5 CFU/g of soil and lasted for 2 weeks, the population of the tryptophan auxotroph CHA116/pME462 was established at about 10^7 CFU/g of root whereas the proline-adenine double auxotroph CHA171 was a poor root colonizer (about 10^4 CFU/g of root). For this reason and because of instability of pME461 and pME462 in the absence of antibiotic selection, larger bacterial inocula and a 1-week incubation period were used in the following experiments (Table 3). Plasmids pME461 and pME462 were transmissible to strain CHA171 in the rhizosphere. Chromosomal pro^+ recombinants were detected in the CHA116/pME462 × CHA171 cross in three of six replicates, whereas the donor CHA116/pME461 failed to give detectable recombinants (Table 3). Chromosomal recombinants were recovered only from roots that were colonized by $\geq 10^6$ CFU of pME462 donors/g of root. In competition experiments involving the parental strain CHA171 and one of its pro^+

TABLE 2. Plasmid and chromosome transfer in Tfr matings of *P. fluorescens* in vitro

Donor	Recipient	Plasmid transconjugant(s)/donor[a]	Chromosomal recombinant	
			Marker selected	Frequency/donor[b]
CHA116/pME461	CHA171 (*pro ade*)	2×10^{-1}	Pro^+	5×10^{-5}
CHA116/pME462	CHA171	4×10^{-1}	Pro^+	2×10^{-4}
CHA116/pME461	CHA2613 (*ade leu*)	6×10^{-1}	Ade^+	$<10^{-8}$
CHA116/pME462	CHA2613	6.1[c]	Ade^+	6×10^{-8}

[a] Mean values of two replicates. Selection was on MMS plus adenine plus Pro (or Leu) plus TC (50 μg/ml) plus NAL (100 μg/ml).

[b] Mean values from two experiments. Selection was on MMS plus adenine (or Leu) plus NAL (1,000 μg/ml). CHA171 gave $<10^{-8}$ Ade^+ recombinant, and CHA2613 gave $<10^{-8}$ Leu^+ recombinant.

[c] Elevated frequency, apparently due to secondary plasmid transfer and/or instability of the donor.

TABLE 3. Plasmid and chromosome transfer in Tfr matings of *P. fluorescens* in the rhizosphere of wheat under gnotobiotic conditions

Donor	Root colonization (CFU/g of fresh root) by:				Plasmid transconjugant/donor[d]	Pro$^+$ recombinant/donor[e]
	Recipient cells (CHA171)[a]	Donor cells with plasmid[b]	Donor cells having lost plasmid	Heterotrophic bacteria[c]		
CHA116/pME461	2×10^7	3×10^6	5×10^6	1×10^8	4×10^{-4} (2×10^{-3}–1×10^{-6})	$<5 \times 10^{-7}$
CHA116/pME462	3×10^7	6×10^6	2×10^7	2×10^8	9×10^{-4} (1×10^{-3}–2×10^{-4})	1×10^{-6} (8×10^{-6}–$<5 \times 10^{-7}$)

[a] The recipient CHA171 was inoculated at 10^8 CFU per g of soil when wheat seedlings were planted in sterile artificial soil. After 7 days, donor cells were injected with a syringe into the rhizosphere at 10^7 CFU per g soil. Bacteria were enumerated on selective MMS 7 days later.
[b] The presence of pME461 or pME462 was determined on MMS plus Trp plus TC (50 μg/ml).
[c] Wheat seedlings disinfected with sodium hypochlorite (5%, wt/vol) for 20 min and with H_2O_2 (10%, wt/vol) for 10 min still contained bacteria which developed more rapidly than did the *P. fluorescens* mutants.
[d] Median values of six replicates; extreme values are given in parentheses.
[e] Median values of six replicates; extreme values are given in parentheses. In the CHA116/pME462 × CHA171 cross, three replicates yielded $<5 \times 10^{-7}$ recombinant (see the text).

recombinants, both strains grew to similar cell densities on wheat roots (data not shown), suggesting that in root exudates adenine (rather than proline) was a limiting growth factor for the *P. fluorescens* mutants. Spontaneous loss of pME461 and pME462 from the donor CHA116 and strong colonization by heterotrophic bacteria, which had escaped surface disinfection of the wheat seeds and competed with the introduced *P. fluorescens* mutants (Table 3), appear to be the principal reasons for the limited recovery of the *pro*$^+$ recombinants. When the recombinants did arise at 10^{-6}, the frequency of concomitant pME462 transfer was 10^{-3}. Thus, the ratio of *pro*$^+$ recombinants per plasmid transconjugant was 10^{-3}, similar to the ratio found in experiments done in vitro (Table 2).

Chromosome and plasmid transfer between auxotrophic mutants of *P. aeruginosa* PAO in the rhizosphere of wheat. *P. aeruginosa* PAO1 was isolated from a human wound in a Melbourne, Australia, hospital (21) and is the *Pseudomonas* strain that has been most extensively studied by genetic techniques (23). Strain PAO1 was found to be an excellent root colonizer, even in nonsterile soil, and to protect wheat from *Gaeumannomyces graminis* and cucumber from *Pythium ultimum* (15). Although *P. aeruginosa* is not considered a prevalent soil microorganism (7), certain isolates of this species are known to proliferate in the rhizosphere (37) and to have biocontrol activities against phytopathogenic fungi (6, 16). The Cma$^+$ IncP plasmid R68.45 (19) was chosen to assess horizontal gene transfer between doubly auxotrophic derivatives of strain PAO colonizing wheat roots. NAL was used to prevent DNA transfer subsequent to the isolation of the bacteria from the roots. Under gnotobiotic conditions, the donor PAO25/R68.45 (*argF leu*) as well as the recipients PAO512 (*argH lysA nalA*) and PAO513 (*argB lysA nalA*) colonized roots with 10^7 to 10^8 CFU/g of root, indicating that exudates contained sufficient arginine, leucine, and lysine to support these populations.

Two-day-old wheat seedlings were planted into sterilized artificial soil inoculated with the donor PAO25/R68.45 and a recipient strain (PAO512 or PAO513), each at 10^7 CFU/g of soil. After 2 to 3 weeks of incubation under gnotobiotic conditions, R68.45 transconjugants were recovered at about 10^{-4} per donor from the rhizosphere (Table 4). In plate matings in vitro, with selection being made for an antibiotic resistance marker of R68.45, plasmid transfer frequencies are $\geq 10^{-1}$ (19). Strains PAO512 and PAO513 each contain two closely linked auxotrophic markers, such that double prototrophic recombinants are recovered in R68.45-mediated conjugation on plates at about 10^{-4} after 2 days (19). When colonizing wheat roots, strains PAO512 and PAO513 gave similar numbers of prototrophic recombinants (10^{-4} to 10^{-5}) with the R68.45 donor after 3 weeks of incubation (Table 4). Shorter incubation resulted in fewer recombinants; in the case of PAO512 they were below the level of detection (Table 4). In general, donor and recipient populations needed to contain at least about 10^6 CFU/g of root in order to produce detectable numbers of chromosomal recombinants. As shown by competition experiments performed in the rhizosphere of wheat, the prototrophic recombinants grew better (i.e., to higher cell densities) than did the parental auxotrophs, whereas the plasmid R68.45 in an auxotrophic background did not confer any selective advantage on the bacteria (Table 5). As a consequence, chromosomal recombinants were almost as frequent as were plasmid transconjugants, in contrast to the situation in vitro, where plasmid transfer is 1,000 times more frequent.

In unplanted, artificial, sterile soil, strains PAO25/R68.45 and PAO513 (inoculated each at 10^7 CFU/g) failed to produce

TABLE 4. Transfer of plasmid R68.45 and chromosomal markers between *P. aeruginosa* strains colonizing the rhizosphere of wheat under gnotobiotic conditions

Incubation (days)	Recipient[a]	Root colonization (CFU/g of fresh root) by:		Plasmid transconjugant/donor[b]	Recombinant/per donor[b]
		Recipient	Donor		
14	PAO512	7×10^5	7×10^5	2×10^{-4} (5×10^{-4}–1×10^{-4})	$<1 \times 10^{-7}$
	PAO513	6×10^6	5×10^5	9×10^{-5} (1×10^{-3}–3×10^{-5})	2×10^{-4} (4×10^{-3}–4×10^{-5})
21	PAO512	8×10^7	3×10^6	1×10^{-4} (2×10^{-4}–4×10^{-6})	2×10^{-5} (3×10^{-4}–1×10^{-5})
	PAO513	8×10^7	7×10^6	1×10^{-4} (2×10^{-4}–3×10^{-6})	5×10^{-4} (2×10^{-3}–5×10^{-6})

[a] The donor strain was PAO25/R68.45. Parental strains were inoculated at 10^7 CFU per g soil when wheat seedlings were planted in sterile artificial soil. Bacteria were enumerated on selective MMS 14 or 21 days later.
[b] Median values of five replicates; extreme values are given in parentheses.

TABLE 5. Root colonization abilities of *P. aeruginosa* parental and recombinant strains in competition experiments under gnotobiotic conditions[a]

Inoculant 1		Inoculant 2	
Strain	Population (CFU/g of root)	Strain	Population (CFU/g of root)
PAO512	2×10^6	PAO512 Arg^+ Lys^+	5×10^8
PAO513	5×10^6	PAO513 Arg^+ Lys^+	4×10^8
PAO25/R68.45	6×10^6	PAO512 Arg^+ Lys^+	4×10^8
PAO25/R68.45	2×10^6	PAO513 Arg^+ Lys^+	7×10^8

[a] Strains were inoculated pairwise at 10^5 CFU per g of soil when wheat seedlings were planted in sterile artificial soil. Bacteria were enumerated on selective MMS 14 days later. Results are mean values of five replicates.

detectable numbers ($\leq 10^{-7}$ per donor) of plasmid transconjugants or chromosomal recombinants within 3 weeks, illustrating the importance of the rhizosphere for conjugative gene transfer as previously shown for plasmid transfer by other authors (31, 64). In sterilized natural bulk soil, which provides abundant substrates to the introduced bacteria and lacks competing microorganisms, plasmid and chromosome transfers were detectable in *P. aeruginosa* at high frequencies, and the wheat rhizosphere did not have a stimulatory effect (Table 6).

In natural, nonsterile soil without plants, neither plasmid nor chromosome transfer was detected within 14 days after inoculation with 10^7 or 10^8 CFU/g of soil. In contrast, in natural, nonsterile soil with wheat plants, strain PAO513 was found to accept R68.45 and to produce about 10^{-6} prototrophic recombinants, whereas crosses with PAO512 produced detectable levels of plasmid transconjugants but no recombinants (Table 6). Chromosome transfer was detectable under these conditions when the donor population was $\geq 10^6$ CFU per g of root. In conclusion, these experiments demonstrate that introduced bacteria can exchange plasmids as well as chromosomal genes in the rhizosphere of wheat grown in natural soil. However, under these conditions, the frequencies of plasmid and chromosome transfer were 10- to 100-fold lower than were the corresponding frequencies in a gnotobiotic system.

DISCUSSION

Chromosomal gene transfer in the rhizosphere is demonstrated here for the first time between introduced *P. fluorescens* or *P. aeruginosa* strains. The donor strains harbor conjugative plasmids whose Cma does not depend on genetic engineering. In *P. fluorescens* CHA0, we used a Tfr donor based on a conjugation-proficient RP1 derivative having undergone a 12-kb deletion in a nonessential plasmid region (50, 66). Deletions of this type are known to be formed spontaneously in IncP plasmids and to facilitate conjugative transfer to *Xanthomonas* spp. (63). Transposon Tn*5*, which was loaded onto the RP1 derivative, is widespread in natural, conjugative plasmids (2). In *P. aeruginosa* PAO, the Cma^+ plasmid used was R68.45, a derivative of the IncP plasmid R68 carrying a tandem duplication of insertion sequence IS*21*. This duplication is crucial for transient integration of R68.45 into the bacterial chromosome and hence for Cma (48). Although R68.45 originated in the laboratory (19), an analogous IncP plasmid carrying an IS*21* tandem and promoting chromosome transfer has been found to occur naturally (53). R68.45 is transmissible in aquatic (24, 41) and terrestrial environments; transfer to indigenous and introduced soil bacteria has been observed (17, 28). Thus, sex plasmids such as those introduced in this study exist and can spread in natural environments. In the laboratory, Hfr strains of *P. aeruginosa* having a chromosomally integrated IncP plasmid are the best chromosome donors (47). However, no naturally occurring Hfr strains of *Pseudomonas* spp. have been reported (48), and for this reason we have not considered using such donors here.

Conjugation requires close cell-cell contacts. These are facilitated by the rhizosphere, a nutrient-rich habitat, as previously noted by other authors (31, 33, 44, 52, 57, 64). Somewhat surprisingly, double auxotrophs of *P. aeruginosa* were able to colonize wheat roots and hence could be used in matings in the rhizosphere. Our experiments provide further evidence that plasmid transfer occurs in the rhizosphere but not in bulk soil, under gnotobiotic conditions as well as in natural nonsterile soil. Stimulation of conjugative transfer by the rhizosphere was also apparent in *P. fluorescens* CHA0 matings, although in this case the auxotrophic derivatives used were poor root colonizers.

By introducing both donor and recipient bacteria into microcosms, we created idealized mating conditions, allowing us to observe the formation of chromosomal recombinants. The gnotobiotic conditions used for the *P. fluorescens* matings reported here represent a worst-case scenario, in which natural competition between rhizosphere microorganisms is strongly reduced (Table 3) or absent. Instability of the chromosome-mobilizing plasmids pME461 and pME462 and poor root colonization abilities of the *P. fluorescens* auxotrophs precluded experiments to be done in natural soil. In contrast, the *P. aeruginosa* matings could be performed in the rhizosphere both under gnotobiotic conditions and in natural soil, as R68.45 is a relatively stable plasmid and double auxotrophs of strain PAO

TABLE 6. Transfer of plasmid R68.45 and chromosomal markers between *P. aeruginosa* strains in natural bulk soil and in the rhizosphere of wheat

Site of transfer	Recipient[a]	Colonization[b] (CFU/g of fresh root or soil) by:		Plasmid transconjugant/donor[c]	Recombinant/donor[c]
		Recipient	Donor		
Sterilized bulk soil	PAO513	8×10^7	5×10^7	5×10^{-5} (9×10^{-5}–3×10^{-5})	7×10^{-3} (1×10^{-2}–4×10^{-3})
Rhizosphere in sterilized soil	PAO512	2×10^7	3×10^7	4×10^{-6} (1×10^{-4}–7×10^{-8})	6×10^{-6} (1×10^{-3}–3×10^{-8})
	PAO513	1×10^7	5×10^7	5×10^{-5} (9×10^{-5}–4×10^{-5})	5×10^{-3} (9×10^{-3}–4×10^{-3})
Rhizosphere in nonsterile soil	PAO512	5×10^7	1×10^6	7×10^{-6} (2×10^{-5}–4×10^{-6})	$<1 \times 10^{-7}$
	PAO513	4×10^7	1×10^6	1×10^{-5} (5×10^{-5}–2×10^{-6})	8×10^{-7} (4×10^{-6}–1×10^{-7})

[a] The donor strain was PAO25/R68.45.

[b] Parental strains were inoculated at 10^7 CFU per g of natural sterilized soil or at 10^8 CFU per g of natural nonsterile soil. Wheat seedlings were planted at the same time. Bacteria were enumerated on selective MMS 14 days later.

[c] Median values of five replicates; extreme values are given in parentheses.

retain significant root colonization abilities. A natural soil environment reduced the gene transfer frequencies 10- to 100-fold relative to those obtained in the gnotobiotic system (Table 6), underscoring the influence of microbial competition. Under field conditions, a further reduction of horizontal chromosome transfer frequencies may occur (1), mainly because the number of Cma^+ bacteria in the field might well constitute a limiting factor. Campbell et al. (7) found that only 3% of fluorescent *Pseudomonas* strains isolated from unpolluted agricultural soils contained plasmids and these were unrelated to the IncP (= IncP-1 in *Pseudomonas* spp.) group. However, there is evidence for the presence of IncP plasmids in some soils (18); manure and chemical pollution may enrich for plasmid carrier bacteria. Some mobilizing plasmids isolated from these habitats have been assigned to the IncP group (18, 62). The catabolic IncP plasmid pJP4, which codes for mercury resistance and 2,4-dichlorophenoxyacetate degradation, is conjugatively transferable in 2,4-dichlorophenoxyacetate-amended nonsterile soil (12, 39) as well as in unamended soil (10, 28). Some rhizosphere pseudomonads contain plasmids belonging to the IncP-7 group (3). A prominent member of this incompatibility group is the TOL plasmid, which can mobilize the *P. putida* chromosome (46). In phyllosphere bacteria, natural transfer-proficient plasmids have been found repeatedly (43). In summary, the potential for conjugative gene transfer clearly exists in natural habitats and is well documented by the global spread of antibiotic and heavy metal resistance genes (34, 60). Heavy metal or pesticide pollution and the agricultural use of sludge and manure may favor the development of bacterial populations carrying conjugative plasmids.

Our experiments conducted with *P. aeruginosa* illustrate the importance of selective advantage in the recovery of chromosomal recombinants. Prototrophic recombinants were found in relatively high numbers (Tables 4 and 6) because they had a growth advantage over the parental auxotrophs in the rhizosphere of wheat (Table 5). The question of whether soil bacteria can gain a selective advantage from their biocontrol properties arises. It has been shown that *Pseudomonas* strains producing phenazine antibiotics (which are important for biocontrol) have a competitive advantage in raw soil over phenazine-deficient mutants (35). In the event that the phenazine biosynthetic genes are transmissible by conjugation or by another mechanism, the spread of these genes might be favored by enhanced ecological fitness of the antibiotic-proficient recombinants. However, it is uncertain whether antibiotic biosynthesis does enhance the competitiveness of soil bacteria in general. For instance, there is no evidence that the production of 2,4-diacetylphloroglucinol can improve the fitness of *P. fluorescens* (8). Hence, it is difficult to predict whether genetically engineered bacteria, e.g., those overproducing antibiotics, might have a selective advantage over natural bacteria.

In conclusion, our experiments provide evidence that chromosomal genes of *Pseudomonas* spp. can be transmitted by conjugation in the rhizosphere, even under nonsterile conditions. Although various factors, such as low population densities of Cma^+ bacteria, restriction, and nonhomology of recipient DNA undoubtedly reduce the likelihood of chromosomal genes being transferred and established in soil bacteria under field conditions, the formation of recombinant microorganisms in the rhizosphere is a distinct possibility. In the same vein, chromosomal transfer of nodulation genes has been demonstrated between strains of *Rhizobium* spp. in the environment (59).

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