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Chromosomal Gene Capture Mediated by the *Pseudomonas putida* TOL Catabolic Plasmid

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The *Pseudomonas putida* TOL plasmid pWW0 is able to mediate chromosomal mobilization in the canonical unidirectional way, i.e., from donor to recipient cells, and bidirectionally, i.e., donor→recipient→donor (retrotransfer). Transconjugants are recipient cells that have received DNA from donor cells, whereas retrotransconjugants are donor bacteria that have received DNA from a recipient. The TOL plasmid pWW0 is able to directly mobilize and retromobilize a kanamycin resistance marker integrated into the chromosome of other *P. putida* strains, a process that appears to involve a single conjugational event. The rate of retrotransfer (as well as of direct transfer) of the chromosomal marker is influenced by the location of the kanamycin marker on the chromosome and ranges from 10^{-3} to less than 10^{-8} retrotransconjugants per donor (transconjugants per recipient). The mobilized DNA is incorporated into the chromosome of the retrotransconjugants (transconjugants) in a process that seems to occur through recombination of highly homologous flanking regions. No interspecific mobilization of the chromosomal marker in matings involving *P. putida* and the closely related *Pseudomonas fluorescens*, which belongs to rRNA group I, was observed.

Horizontal gene transfer refers to the transfer and maintenance of DNA from one bacterium to another. This phenomenon is well established in prokaryotes. Conjugative plasmids represent the most widespread vehicle for unidirectional (donor→recipient) gene flux, not only among virtually all eubacteria, but also from eubacteria to yeast and plant cells (see reference 2 for a review). Self-transmissible plasmids (*mob*$^+$, *tra*$^+$) encode the elements required for their own intercellular movement, whereas mobilizable (*mob*$^+$, *tra*) plasmids require helper functions for intercellular movement.

The bidirectional transfer of DNA (donor→recipient→donor) mediated by IncP1 plasmids has been described previously. This phenomenon, called retrotransfer, includes the retromobilization of *mob*$^+$, *tra* plasmids and *mob*, *tra* plasmids, as well as that of chromosomal markers (11, 13). The kinetics of retrotransfer are similar to those of conventional direct mobilization, but there is disagreement as to whether only one or two conjugational events are involved (4, 9, 22).

The approximately 115-kb *Pseudomonas putida* TOL plasmid pWW0 (Table 1) belongs to the IncP9 group and encodes a set of enzymes for the catabolism of toluene/xylenes via benzoate/toluates and catechol/alkylcatechols through a *meta*-cleavage pathway (26). The set of toluene-degrading (*xyl*) genes on the TOL plasmid is located in the 56-kb transposon Tn*4651* (23), and this element is in turn included within a 70-kb transposon designated Tn*4653* (24, 25). Constitutive expression of the *tra*$^+$ genes on the TOL plasmid pWW0 accounts for the large number of pili on the surface of *P. putida* (5). The pWW0 plasmid has been shown to be able to transfer between and to be maintained stably in *Escherichia coli*, *Erwinia chrysanthemi*, and members of the family *Pseudomonadaceae* belonging to rRNA group I (17). We now show that the pWW0 plasmid can intraspecifically mobilize, both uni- and bidirectionally, a kanamycin marker inserted on the *P. putida* chromosome. The rates of direct transfer and retrotransfer were influenced by the location of the marker on the chromosome.

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MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this research are listed in Table 1. Bacteria were grown at 30°C in Luria-Bertani (LB) broth and in modified M9 minimal medium with glucose (0.5% [wt/vol]), benzoate (15 mM), or 3-methylbenzoate (15 mM). When appropriate, antibiotics were added at the following concentrations (micrograms per milliliter): kanamycin, 50; tetracycline, 20 to 50; streptomycin, 50 to 100; chloramphenicol, 30; nalidixic acid, 20; and rifampin, 50.

Filter matings. Standard filter matings between appropriately marked donor and recipient strains involved about 10^8 cells of each, deposited on 2-cm-diameter filters which were placed on the surface of an LB agar plate and were kept at 30°C for 12 to 16 h unless otherwise stated. Donor and recipient controls were used without mixing.

DNA manipulations. Total DNA was prepared basically as described by Robson et al. (18). The methods used for DNA restriction, separation of DNA fragments by electrophoresis on agarose gels, transfer to nitrocellulose membranes, and DNA fixation have been described previously (15, 17). The DNA probes were randomly labelled with digoxigenin according to the manufacturer's instructions (Boehringer Mannheim). Strict hybridization conditions (50% [vol/vol] formamide) were used as described previously (17).

Delivery of minitransposons into the chromosome of *P. putida* EEZ15. About 10^8 cells of *E. coli* CC118λPIR(pUT-Km) as a donor, *P. putida* EEZ15 as a recipient, and *E. coli* HB101(pRK600) as a helper strain were mixed and deposited on a filter laid on the surface of a solid LB plate. After 12 to 16 h, cells were resuspended and serial dilutions were spread on

TABLE 1. Plasmids and strains used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
Plasmids		
pWW0	Archetypical TOL plasmid, 3MB$^+$	26
pWW0::Ω	Sm^r derivative of pWW0, 3MB$^-$	12
pWW0-EB6	pWW0 derivative bearing a point mutation in *xylE*	15
pRK600	Suicide helper plasmid, Cm^r	10
pUT-Km	Ap^r Km^r mini-Tn*5*-Km delivery plasmid	10
pUT-Tc	Ap^r Tc^r mini-Tn*5*-Tc delivery plasmid	10
pJB3Tc19	pRK2 replicon, Tc^r	J. Blatny
Strains		
P. putida 2440	*hsdM*$^+$ *hsdR*	3
P. putida UWC1	Rif^r derivative of 2440	M. Day
P. putida UWC1Sm	Sm^r derivative of UWC1	This study
P. putida EEZ15	PT^r derivative of 2440	16
P. putida EEZ15K-x	Km^r mini-Tn*5* derivatives of EEZ15	This study
P. putida PaW340	Nal^r Sm^r Trp$^-$	S. Harayama
P. fluorescens EEZ23(pWW0)	Nal^r Rif^r 3MB$^+$	16
E. coli CC118λPIR	Rif^r *recA1* λpir	V. de Lorenzo

[a] PT^r, Sm^r, Km^r, Nal^r, Cm^r, and Rif^r, resistant to phosphinothricin, streptomycin, kanamycin, nalidixic acid, chloramphenicol, and rifampin, respectively. 3MB, 3-methylbenzoate.

M9 minimal medium plates with benzoate as a C source, 1 mM phosphinothricin (PT), and kanamycin. These plates are selective for Km^r derivatives of *P. putida* EEZ15. The Km^r transconjugants were further purified on LB plates supplemented with kanamycin. Independent clones were selected for experiments and were called EEZ15K-x, where x is 1,2,3....34.

Second insertion of a mini-Tn*5*-Tc into the chromosome of *P. putida* EEZ15K-x. The mating conditions were as described above except that the donor strain was *E. coli* CC118λPIR (pUT-Tc) and the recipients were either *P. putida* EEZ15K-3, EEZ15K-8, or EEZ15K-11. As a helper, plasmid pRK600 was used. Km^r Tc^r *P. putida* EEZ15K-x transconjugants were selected on M9 minimal medium plates with benzoate and were supplemented with 1 mM PT, kanamycin, and tetracycline; these plates are selective for EEZ15K-x acquiring the Tc marker delivered by the pUT-Tc plasmid. Three independent Km^r Tc^r clones of each recipient were selected for further assays and were called EEZ15K3-Tc1, EEZ15K3-Tc2, etc.

RESULTS

Low exclusion surface determined by the TOL plasmid. The TOL plasmid pWW0::Ω carries the omega interposon at the 5′ end of the *meta*-operon (12), so *Pseudomonas* sp. strains harboring this plasmid are resistant to streptomycin and cannot grow on 3-methylbenzoate. We did filter matings between *P. putida* (pWW0::Ω) as the donor and PT-resistant and kanamycin-resistant *P. putida* EEZ15K-8 as the recipient. The frequency of Sm^r PT^r Km^r transconjugants was about 0.6 to 1 transconjugant per recipient. Matings were also done between the donor *P. putida* (pWW0::Ω) and the recipient PT^r Km^r *P. putida* EEZ15K-8 harboring the TOL plasmid pWW0-EB6. The latter had a mutation in the *xylE* gene, so that the resulting catechol 2,3-dioxygenase was resistant to inactivation by 4-ethylcatechol (1, 15). The frequency of Sm^r PT^r Km^r transconjugants was as high as 1.5×10^{-2} per recipient. This is only about 2 orders of magnitude lower than when the recipient bacterium was TOL plasmid free. These results suggest that TOL plasmid pWW0 derivatives have a low surface exclusion coefficient. Alternatively, plasmid incompatibility may also have caused the observed decrease in the frequency of pWW0:Ω transfer (7).

Mobilization of a chromosomal marker mediated by the TOL plasmid: direct mobilization and retrotransfer of a kanamycin marker. The above results indicate that the TOL plasmid can travel efficiently between cells with or without the catabolic plasmid. To study whether the IncP9 TOL plasmid pWW0 was able to mediate unidirectional and bidirectional (retrotransfer) mobilization of chromosomal DNA, the chromosome of the plasmid-free *P. putida* EEZ15 was labelled at different positions with a kanamycin resistance marker. The Km^r marker was delivered within a *tnpA* mini-Tn*5* provided by the suicide plasmid pUT-Km, whose replication requires the π protein, which is not present in *P. putida* (see reference 10 for details). Independent Km^r *P. putida* EEZ15K-x derivatives (*P. putida* EEZ15K-1 through *P. putida* EEZ15K-34) were selected and purified. To confirm that the insertions of the Km^r marker occurred at physically distinct positions, total DNA of each Km^r strain was prepared and digested with *Hin*dIII to cut once within the DNA encoding the Km marker. The DNA fragments were separated on agarose gels, blotted onto nitrocellulose filters, and hybridized against the whole kanamycin resistance gene, which was labelled with digoxigenin as a probe. Each Km^r *P. putida* EEZ15K-x derivative had a single mini-Tn*5* insertion at different sites, as revealed by the presence of two hybridization bands at different positions (see Fig. 1 for seven mutants).

To determine whether the locations marked were stably maintained, *P. putida* EEZ15K-x was grown for more than 100 generations in the absence of selective antibiotic pressure, and cells were then spread on plates with or without kanamycin. One hundred percent of the colonies were Km^r, suggesting that the insert was stably maintained. Furthermore, the Km^r marker remained at its original location when bacteria were grown in the absence of selective pressure, as shown by the fact that the hybridization pattern in the parent bacteria and in bacteria isolated from cultures grown in the absence of selective pressure was the same. The absence of retransposition of the Km^r marker was also shown by the fact that this marker was not rescued on pJB3Tc19, a Tc-resistant RK2 derivative.

All 34 Km^r PT^r *P. putida* derivative clones were able to receive TOL plasmid pWW0 at frequencies similar to those received by the parental strain, i.e., 0.5 to 1 transconjugant per recipient. Growth of the Km^r PT^r *P. putida* strains bearing pWW0 for more than 100 generations in the absence and in the presence of selective pressure did not have an effect upon the

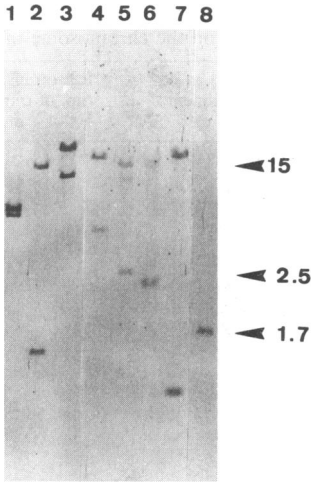

FIG. 1. Differential location of the Km^r marker on the chromosome of *P. putida* EEZ15K-x. Chromosomal DNA from seven independent clones was prepared, and about 1 to 3 μg of DNA was digested with *Hin*dIII for 3 h. Then fragments were separated on 0.8% (wt/vol) agarose gels and were blotted onto nitrocellulose filters. A 1.7-kb *Not*I fragment from pUT-Km bearing the kanamycin marker was labelled with digoxigenin (Boehringer Mannheim kit) and was used as a probe. Hybridization and immunodevelopment of digoxigenin hybrids were as described in reference 17. Lanes 1 through 7, DNA from *P. putida* EEZ15K-1 through EEZ15K-7. Lane 8, positive control: 1.7-kb *Not*I fragment from pUT-Km. Relevant sizes (in kilobases) are indicated by arrowheads.

hybridization patterns with the Km^r determinant as a probe in Southern blots (data not shown), suggesting that the TOL-encoded transposases (23–26) do not allow retransposition of the mini-Tn*5*. To test whether the TOL plasmid was able to mobilize the Km^r marker integrated into the chromosome, we did matings between PT^r Km^r *P. putida* EEZ15K-x(pWW0) cells as donors and the Rif^r Sm^r *P. putida* UWC1Sm as a recipient. The frequency of Rif^r Sm^r 3MB$^+$ *P. putida* UWC1Sm transconjugants was on the order of 0.5 to 1 per recipient, whereas the frequency of Rif^r Km^r 3MB$^+$ *P. putida* UWC1Sm transconjugants was between 4×10^{-4} and $<10^{-8}$ per recipient, depending on the strain used (see Table 2 for eight clones), suggesting that the TOL plasmid was able to directly mobilize chromosomal DNA.

To test whether the TOL plasmid was able to retromobilize the Km marker, the Nal^r Sm^r *P. putida* PaW340(pWW0) strain was crossed with each of 34 different recipient strains of *P. putida*, EEZ15K-1 through EEZ15K-34. Km^r Nal^r Sm^r *P. putida* PaW340(pWW0) retrotransconjugants were selected on M9 minimal medium supplemented with the three antibiotics plus tryptophan and 3-methylbenzoate as the sole carbon source. The frequency of retrotransconjugants ranged from 3×10^{-4} to 3×10^{-8} per donor (see Table 3 for a few examples). Similar results were obtained in crosses between the Rif^r Sm^r *P. putida* UWC1Sm(pWW0-EB6) and the different Km^r *P. putida* EEZ15K-x derivatives as recipients (data not shown). These results support the hypothesis of the ability of the TOL plasmids to mobilize chromosomal DNA.

TABLE 2. Direct mobilization of a Km^r cassette inserted at different locations on the chromosome of *P. putida*

Donor	Recipient	Transconjugant frequency[a] (no. of transconjugants/no. of recipient cells)
EEZ15K-3(pWW0)	UWC1Sm	$<10^{-8}$
EEZ15K-8(pWW0)	UWC1Sm	5×10^{-5}
EEZ15K-11(pWW0)	UWC1Sm	5×10^{-6}
EEZ15K-12(pWW0)	UWC1Sm	4×10^{-4}
EEZ15K-13(pWW0)	UWC1Sm	4×10^{-4}
EEZ15K-22(pWW0)	UWC1Sm	2×10^{-8}
EEZ15K-27(pWW0)	UWC1Sm	4×10^{-4}
EEZ15K-31(pWW0)	UWC1Sm	2×10^{-7}

[a] Transconjugants are UWC1Sm cells that have received the Km^r marker inserted on the chromosome of EEZ15K-x(pWW0) cells. Transconjugants were Rif^r Sm^r PT^s. The transconjugant frequencies are given as the number of *P. putida* UWC1Sm-Km^r(pWW0) cells versus the total number of UWC1Sm cells.

That direct mobilization and bidirectional retromobilization of the Km^r chromosomal marker was TOL plasmid dependent was confirmed by the finding that matings between Sm^r Rif^r *P. putida* UWC1Sm and a series of *P. putida* EEZ15K-x did not give rise to Km^r clones of UWC1Sm. These results suggest that mobilization of the Km^r marker on the chromosome is TOL plasmid dependent. Furthermore, since the mobilization frequency of the Km^r marker depends on its physical location, it follows that this system makes it possible to locate regions of the chromosome with low or high frequencies of direct mobilization and retromobilization. To test whether the Km^r marker acquired by the retrotransconjugants could now be directly mobilized to a potential recipient, we did matings between the Nal^r Sm^r Km^r *P. putida* PaW340(pWW0) retrotransconjugants, now used as donors, and the Rif^r Sm^r *P. putida* UWC1Sm as a recipient. The frequency of Rif^r Sm^r 3MB$^+$ *P. putida* UWC1Sm transconjugants was on the order of 0.5 to 1 per recipient, whereas the frequency of Rif^r Km^r 3MB$^+$ *P. putida* UWC1Sm transconjugants was between 10^{-4} and 10^{-8} per recipient, depending on the donor used. These results suggested that the Km^r marker that the retrotransconjugants had acquired was not on the TOL plasmid and was most likely incorporated into the chromosome of the retrotransconjugant cell. From this it follows that the Km^r transconjugants that appeared in this assay were due to direct mobilization of the marker integrated on the chromosome of the donor bacterium.

To confirm that the Km^r marker in the retrotransconjugants was indeed incorporated into the host chromosome, chromosomal DNA from two independent retrotransconjugants of eight Km^r retromobilizable positions was prepared, digested with *Hin*dIII, *Cla*I, or *Sma*I, transferred to a nitrocellulose membrane, and hybridized against the Km resistance gene as a probe, as described above. The retrotransconjugants from each mating were mutually isogenic, and the Km^r marker was located at the same chromosomal position as in the recipient strains (see Fig. 2 for results with two of the clones). These results strongly suggested that the retromobilized DNA was incorporated into the chromosome of the donor bacterium through homologous recombination.

Differential mobilization of a tetracycline marker inserted at different positions within the chromosome of a *P. putida* strain exhibiting a defined kanamycin insertion. Three Km^r-labelled strains of *P. putida* (EEZ15K-3, EEZ15K-8, and EEZ15K-11), whose Km^r marker is mobilized at different frequencies (Tables 2 and 3) were randomly labelled with a second marker by using pUT-Tc as described in Materials and Methods. Figure 3 shows that the second insertion occurred at different positions in all three EEZ15K-x clones, as revealed by the hybridization patterns. Three EEZ15K-x-Tc-y clones were kept for further analysis.

Retromobilization assays were then set up with *P. putida* PaW340(pWW0-EB6) as the donor and the nine Km^r Tc^r *P.*

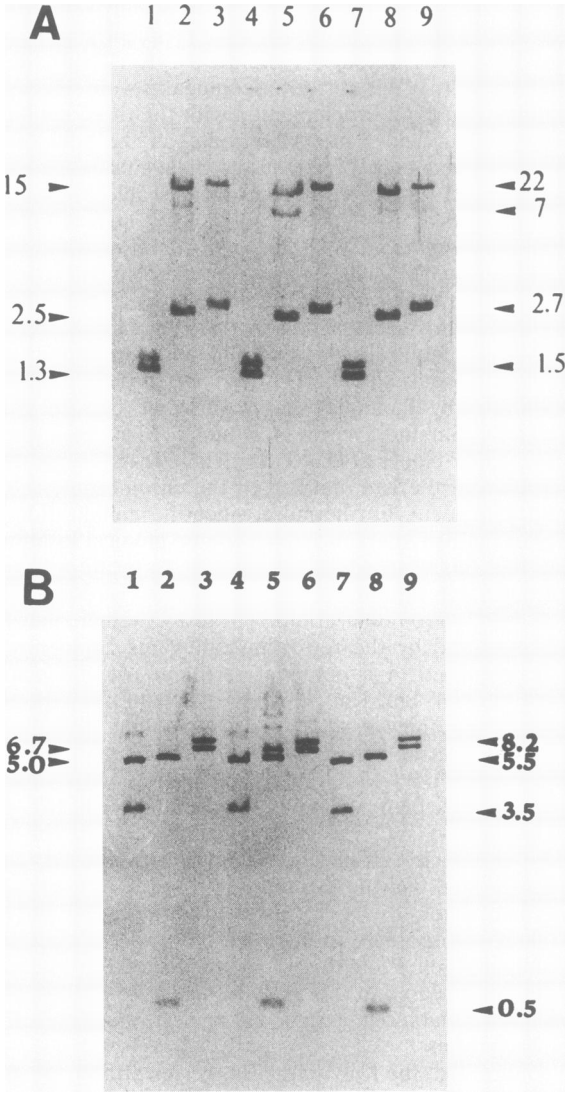

FIG. 2. Chromosomal location of retromobilized Km resistance markers. (A) Southern blot of total DNA of the recipient *P. putida* EEZ15K-5 and two Km^r Nal^r Sm^r PaW340(pWW0) retrotransconjugants that had acquired the Km^r marker from the above recipient. (B) As in panel A, except that the recipients were *P. putida* EEZ15K-8 and two PaW340(pWW0) retrotransconjugants that had acquired the Km^r marker from EEZ15K-8. Lanes 1 to 3, DNA from the recipient bacterium. Lanes 4 to 6 and 7 to 8, DNA prepared from each retrotransconjugant. Lanes 1, 4, and 7, DNA digested with *Cla*I. Lanes 2, 5, and 8, DNA digested with *Hin*dIII. Lanes 3, 6, and 9, DNA digested with *Sma*I. Other conditions were as in the legend to Fig. 1.

putida EEZ15-x-Tc-y clones as recipients. Appropriate controls with unmixed strains were included. The following retrotransconjugants were sought: Nal^r Sm^r Km^r *P. putida* PaW340 (pWW0-EB6), Nal^r Sm^r Tc^r *P. putida* PaW340(pWW0-EB6), and Km^r Tc^r Nal^r Sm^r *P. putida* PaW340(pWW0-EB6). The results obtained are presented in Table 4.

Similar numbers of Km^r retrotransconjugants were found for each clone exhibiting the Km marker in the same positions (Table 4). These numbers were similar to those obtained with the parent strain, which was not labelled with the Tc marker (compare Table 3 with Table 4). However, the frequency of Tc^r clones ranged from 6×10^{-6} to 5×10^{-7} (Table 4), as

TABLE 3. Bidirectional mobilization of a Km^r cassette inserted at different locations on the chromosome of *P. putida*

Donor	Recipient	Retrotransconjugant frequency[a] (no. of retrotransconjugants/no. of donor cells)
PaW340(pWW0)	EEZ15K-3	$<10^{-8}$
PaW340(pWW0)	EEZ15K-8	10^{-4} to 10^{-5}
PaW340(pWW0)	EEZ15K-11	10^{-5} to 10^{-6}
PaW340(pWW0)	EEZ15K-12	3×10^{-4}
PaW340(pWW0)	EEZ15K-13	4×10^{-4}
PaW340(pWW0)	EEZ15K-27	1×10^{-5}
PaW340(pWW0)	EEZ15K-31	2×10^{-7}
PaW340(pWW0)	EEZ15K-34	3×10^{-4}

[a] Retrotransconjugants were PaW340(pWW0) cells that have acquired the Km^r marker inserted on the chromosome of *P. putida* EEZ15K-x cells. The retrotransconjugant frequencies are given as the number of PaW340-Km^r-(pWW0) cells versus the total number of PaW340(pWW0) cells.

expected from the differential location of the Tc^r marker. No Km^r Tc^r clones were found, which suggested that the two antibiotic markers in the nine independent clones were not closely linked (data not shown).

Retrotransfer seems to be a single-step process. The fact that the frequencies of retrotransfer and direct transfer of the Km^r marker in a given position were similar suggested that the retrotransfer of DNA is a single-step process. To test this hypothesis, a triparental mating was done five independent times involving *P. putida* PaW340(pWW0-EB6) as a donor, *P. putida* EEZ15K-8 as a recipient (selected because the Km^r marker was retromobilized [mobilized] at a relatively high frequency), and *P. putida* UWC1Sm, which was used as a plasmid-free Km^r-catcher. We reasoned that because the presence of a TOL plasmid in the cell involves an exclusion coefficient of about 10^{-2} (see text above) and the rate of TOL transfer is about 10^{-1}, then if the retrotransfer process in-

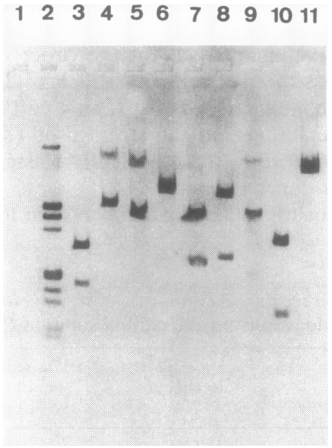

FIG. 3. Chromosomal locations of the Tc^r marker on the chromosome of *P. putida* EEZ15K-x-Tc-y. Chromosomal DNA from independent clones was prepared, and about 2 μg of DNA was digested with *Eco*RV for 3 h. Separation of fragments, blotting, hybridization conditions, and immunodevelopment of digoxigenin hybrids were as in the legend to Fig. 1, except that the probe was a 2-kb *Sfi*I fragment from pUT-Tc, bearing the tetracycline marker. Lanes: 1, *P. putida* EEZ15 used as a negative control; 2, lambda *Hin*dIII markers; 3 to 5, DNA from *P. putida* EEZ15-K3Tc1 through EEZ15K3-Tc3; 6 to 8, DNA from *P. putida* EE15-K8-Tc1 through EEZ15K8-Tc3; and 9 to 11, DNA from *P. putida* K11-Tc1 through EEZ15K11-Tc3.

TABLE 4. Bidirectional mobilization of a Km^r cassette and/or a Tc^r cassette inserted at different locations on the chromosome of *P. putida*

Donor	Recipient	Retrotransconjugant[a]	
		Km^r PaW340	Tc^r PaW340
PaW340(pWW0-EB6)	EEZ15K3-Tc1	$<5 \times 10^{-7}$	2×10^{-6}
PaW340(pWW0-EB6)	EEZ15K3-Tc2	$<5 \times 10^{-7}$	$<5 \times 10^{-7}$
PaW340(pWW0-EB6)	EEZ15K3-Tc3	$<5 \times 10^{-7}$	$<5 \times 10^{-7}$
PaW340(pWW0-EB6)	EEZ15K8-Tc1	4×10^{-4}	1×10^{-5}
PaW340(pWW0-EB6)	EEZ15K8-Tc2	4×10^{-4}	2×10^{-6}
PaW340(pWW0-EB6)	EEZ15K8-Tc3	4×10^{-4}	$<1 \times 10^{-7}$
PaW340(pWW0-EB6)	EEZ15K11-Tc1	1×10^{-6}	3×10^{-6}
PaW340(pWW0-EB6)	EEZ15K11-Tc2	5×10^{-5}	$<1 \times 10^{-7}$
PaW340(pWW0-EB6)	EEZ15K11-Tc3	2×10^{-6}	8×10^{-6}

[a] Retrotransconjugants were PaW340(pWW0-EB6) cells that had acquired either the Km^r or the Tc^r marker inserted on the chromosome of *P. putida* EEZ15K-x-Tc-y. The retrotransconjugant frequencies are given as the number of PaW340-Km^r(pWW0-EB6) or PaW340-Tc^r(pWW0-EB6) versus the total number of PaW340(pWW0-EB6) cells.

volved a single step, the Km^r retrotransconjugants should appear before the Km^r-catcher did. Equal numbers of each strain were mixed by centrifugation, washed in LB, and deposited on a series of filters which were then laid on an LB plate. One filter was removed immediately and the others after 1, 2, 3, 4, and 6 h. Figure 4 shows the results obtained, which indicated that Km^r retrotransconjugants appeared earlier than did the transconjugants of the Km^r-catcher, corroborating the single-step hypothesis.

Because we had found that TOL surface exclusion was rather low, we tested whether retrotransfer between two differentially labelled *P. putida* strains bearing distinct TOL plasmids occurred. We mated the Sm^r Rif^r Km^s *P. putida* UWC1Sm(pWW0) as a donor to the Sm^s Rif^s Km^r PT^r *P. putida* EEZ15K-8(pWW0-EB6). pWW0-EB6 has a single point mutation at the *xylE6* allele, so that the mutant catechol

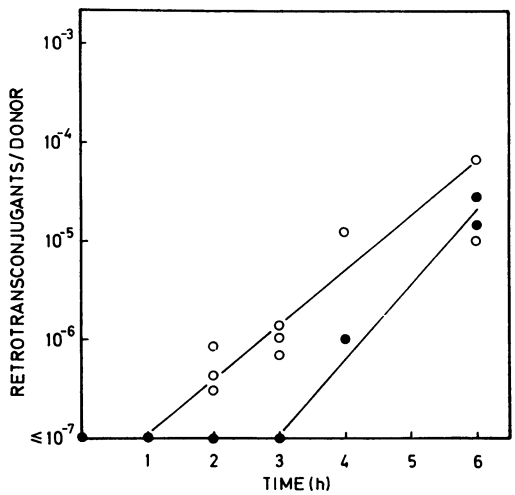

FIG. 4. Time course of the appearance of Km^r clones in a triparental mating. About 10^8 cells of *P. putida* PaW340(pWW0-EB6), *P. putida* EEZ15K-8, and *P. putida* UWC1Sm were mixed, and at the indicated times Km^r Nal^r Sm^r PaW340(pWW0-EB6) retrotransconjugants (○) were counted on M9 minimal medium with 3-methylbenzoate and the corresponding antibiotics, while Km^r Rif^r Sm^r UWC1Sm transconjugants (●) were selected on M9 minimal medium with 3-methylbenzoate and the corresponding antibiotics.

2,3-dioxygenase which it encodes oxidizes 4-ethylcatechol to the corresponding muconic acid semialdehyde, whereas the wild-type enzyme does not (15). Sm^r Rif^r Km^r *P. putida* UWC1Sm retrotransconjugants were found at a rate of 10^{-4} per donor. Ten independent retrotransconjugant colonies were purified and used to determine which of the TOL plasmids were present in the UWC1Sm-Km^r clones: duplicate colonies grown on LB plus 3-methylbenzoate were sprayed with catechol or 4-ethylcatechol. All ten clones showed the wild-type plasmid, as revealed by the positive reaction with catechol and by the lack of reaction with 4-ethylcatechol. These results suggested that the Km^r derivatives of UWC1Sm were true retrotransconjugants, and they also supported the single-step hypothesis for the bidirectional mobilization of chromosomal markers: if the process had involved two steps, at least 50% of the clones would have had the pWW0-EB6 plasmid.

Intraspecific retromobilization of DNA. If homologous recombination is required for stabilization of the retromobilized DNA, the frequency of retromobilization for a given marker should decrease in the course of heterologous matings. To test this hypothesis, we chose *Pseudomonas fluorescens* EEZ23 because it belongs to the *Pseudomonadaceae* rRNA I group, and because direct transfer of pWW0 from *P. putida* to *P. fluorescens* and vice versa occurs at a frequency of about 10^{-1} transconjugants per recipient (16). We set up independent matings between Nal^r Rif^r *P. fluorescens* EEZ23(pWW0) as a donor and three of the Km^r PT^r *P. putida* EEZ15K-x derivatives as recipients (K-3, K-8, and K-11), in which the Km marker was shown to be retromobilized. Km^r PT^r *P. putida* transconjugants able to grow on 3-methylbenzoate were found at a frequency of 10^{-1} per recipient; however, Km^r Nal^r Rif^r *P. fluorescens* retrotransconjugants were not found ($<10^{-8}$ retrotransconjugants per donor). This supported the hypothesis that retromobilization requires highly homologous flanking chromosomal regions, although it did not exclude the possibility of intrageneric retromobilization.

DISCUSSION

Plasmids of several incompatibility groups (IncP, IncQ, IncW, IncC, and IncN) can be transferred and stably maintained in a wide range of bacterial species. Some of these self-transmissible plasmids are able to mobilize nonconjugative (Tra$^-$) plasmids and sometimes even chromosomal markers (21). Some IncP1 plasmids are able to mobilize plasmids and chromosomal genes not only in the canonical forward direction, i.e., from donor to recipient, but also in the reverse direction, i.e., from recipient to donor—a phenomenon called retrotransfer.

The *P. putida* TOL plasmid pWW0 belongs to the IncP9 incompatibility group. Its spectrum of hosts includes *Pseudomonas* spp. belonging to the rRNA I group and some members of the family *Enterobacteriaceae* (14, 17). In this study we have shown that the TOL plasmid is able to intraspecifically mobilize antibiotic markers located at physically distinct positions within the chromosome of *P. putida*, both in the canonical way and bidirectionally. Genes coding for antibiotic resistance and delivered via mini-Tn*5* were used as markers for three reasons: (i) the antibiotic marker kanamycin (tetracycline) permits clean phenotypic selection in mobilization studies; (ii) the same piece of DNA can be inserted at different positions within the same replicative unit, whose physical location on the chromosome can be tracked by using the gene as a probe; and (iii) the antibiotic marker is provided within a *tpnA* mini-Tn*5*, which increases the stability of the delivered marker. To allow transposition of the mini-Tn*5*, the suicide plasmid bears a

transposase gene outside the transposon. In fact, the marker remained in its original position for more than 100 generations during growth of the bacteria in the absence of selective pressure.

Our results show that the rates of direct and bidirectional mobilization of the delivered kanamycin resistance marker are similar for each position, although the rates vary over more than 5 orders of magnitude depending on the marker's location. From this it follows that mobilization of chromosomal regions mediated by the TOL plasmid occurred at different frequencies, making it possible to locate regions of high and low mobility. This finding is relevant within the context of dissemination and assessment of the fate of genetically engineered DNA sequences.

Top et al. (22) showed that when relatively high cell densities ($>10^6$ cells cm^{-2}) were used at a donor-to-recipient ratio of 1:1, and when the conjugation time was limited to a maximum period of 2 to 3 h, it was possible to adjust the results in retrotransfer assays to a model that predicted that the retrotransference process involves a single step. However, long-term matings produced steady-state numbers of retrotransconjugants and thus did not distinguish whether retrotransference involved one or two steps. The experiment illustrated in Fig. 4 with triparental matings supports the hypothesis that retrotransference is a single-step process. This can be deduced from the fact that the Km^r retrotransconjugants appeared before the Km^r transconjugant-catcher did.

The idea of a single-step process was further supported by the fact that when retrotransfer assays were done with bacteria having distinct TOL plasmids, all retrotransconjugants had the plasmid originally borne by the donor bacterium.

The molecular events involved in the retromobilization of DNA are unknown. It is nonetheless worth noting that all self-transmissible plasmids described so far which are able to retromobilize chromosomal genes bear transposons (4, 9, 13, 21). In agreement with this observation is the fact that the TOL plasmid pWW0 bears two transposons, Tn*4651* and Tn*4653*, which are members of the Tn*3* family (23). Members of the Tn*3* family of transposons act through the formation of cointegrates (8). Intermediate products may provide the connection between the pWW0 plasmid and the chromosome required for transfer of the chromosomal marker. Because insertion of the Tn*3* family of transposons is thought to be random (8), insertions must be scattered randomly along the chromosome. Why, then, does TOL mobilize the Km^r marker in some chromosomal positions but not in others? A possible explanation is that the supercoiled structure of different regions in the recipient chromosome may favor the transposition event and mobilization of the marker via cointegration with the plasmid. Alternatively, the TOL plasmid may integrate into the recipient chromosome by site-specific recombination, as previously described for the insertion of certain phages on the bacterial chromosome (6). Another possible answer to the above question is that after mobilization of the recipient chromosome to the donor bacterium, the ubiquitous eubacterial Chi sites (19) and RecBC pathway (20), if operative in *Pseudomonas* spp., increase the frequency of homologous recombination in the donor chromosome, thus creating retrotransconjugants. Whatever the mechanism, our results show that the TOL plasmid can function as an active gene capture element that can horizontally spread genes to closely related strains.

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The first two coauthors contributed equally to this work, and both should be considered first authors.

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