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Mechanism of Retrotransfer in Conjugation: Prior Transfer of the Conjugative Plasmid Is Required

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Bacterial conjugation normally involves the unidirectional transfer of DNA from donor to recipient. Occasionally, conjugation results in the transfer of DNA from recipient to donor, a phenomenon known as retrotransfer. Two distinct models have been generally considered for the mechanism of retrotransfer. In the two-way conduction model, no transfer of the conjugative plasmid is required. The establishment of a single conjugation bridge between donor and recipient is sufficient for the transfer of DNA in both directions. In the one-way conduction model, transfer of the conjugative plasmid to the recipient is required to allow the synthesis of a new conjugation bridge for the transfer of DNA from recipient to donor. We have tested these models by the construction of a mutant of the self-transmissible, IncP plasmid RK2*lac* that allows the establishment of the conjugation bridge but is incapable of self-transfer. Four nucleotides of the *nic* region of the origin of transfer (*oriT*) were changed directly in the 67-kb plasmid RK2*lac* by a simple adaptation of the vector-mediated excision (VEX) strategy for precision mutagenesis of large plasmids (E. K. Ayres, V. J. Thomson, G. Merino, D. Balderes, and D. H. Figurski, J. Mol. Biol. 230:174–185, 1993). The resulting RK2*lac oriT1* mutant plasmid mobilizes IncQ or IncP *oriT*$^+$ plasmids efficiently but transfers itself at a frequency which is 10^4-fold less than that of the wild type. Whereas the wild-type RK2*lac oriT*$^+$ plasmid promotes the retrotransfer of an IncQ plasmid from *Escherichia coli* or *Pseudomonas aeruginosa* recipients, the RK2*lac oriT1* mutant is severely defective in retrotransfer. Therefore, retrotransfer requires prior transfer of the conjugative plasmid to the recipient. The results prove that retrotransfer occurs by two sequential DNA transfer events.

The exchange of plasmid and chromosomal DNA by conjugation is a significant factor in the genetic variation of bacteria (1, 22, 26). The evolutionary impact of conjugation has been demonstrated most dramatically by the rapid emergence and dissemination of multiple-antibiotic resistance in bacterial strains through the transfer of R plasmids (1, 24). The enormous potential for genetic variation in bacteria by conjugation is perhaps best illustrated by the promiscuous plasmids of incompatibility group P (IncP) (28, 43), which are capable of directing the transfer of DNA to a wide variety of gram-negative and gram-positive bacterial species and even to several yeast species (11, 13).

Conjugation is primarily mediated through self-transmissible plasmids, whose occurrence is common among bacteria (1, 26). These self-transmissible plasmids encode the functions necessary for both mating pair formation and the transfer of DNA (11, 50). Many other plasmids unable to promote mating pair formation nevertheless specify *cis*- and *trans*-acting functions that allow them to be efficiently mobilized by self-transmissible plasmids (20). However, even without specialized functions, virtually any DNA segment, either from the bacterial chromosome or a plasmid, can be conjugally transferred by recombining with a self-transmissible or mobilizable plasmid (34).

Generally conjugal transfer of DNA occurs from a donor strain which contains a self-transmissible plasmid to a recipient which lacks the plasmid (12). However, it has been observed that a donor strain can sometimes acquire DNA from a recipient (27). Both plasmids (6, 14, 17, 30, 44–46) and chromosomal segments (3, 19, 27, 33, 38) can be transferred from recipient to donor. This phenomenon, termed retrotransfer (27), occurs at a low but significant frequency and depends on the presence of self-transmissible plasmids in the original donor cells. Because the frequency of transfer of chromosomal markers from recipient to donor can in some circumstances be comparable to the frequency of marker transfer in the forward direction, Mergeay et al. (27) raised the intriguing possibility that retrotransfer occurs by a mechanism which is distinct from normal conjugal transfer. As a result, two different models have been generally considered to explain the phenomenon of retrotransfer (11, 14). In the "two-way conduction" model, the self-transmissible plasmid in the donor is required for the establishment of the conjugation bridge between donor and recipient cells; DNA can then be mobilized in both directions through this bridge. Thus, the plasmid-containing donor can act as an active recipient that is capable of capturing DNA from otherwise nonconjugative bacteria. In the "one-way conduction" model, the self-transmissible plasmid first uses the conjugation bridge to transfer itself to the recipient. Then it directs the formation of a second conjugation bridge that is used for the unidirectional transfer of DNA from the transconjugant back to the donor. Both models have some experimental support. Top et al. (45) and Ramos-González et al. (33) obtained data on the kinetics of retrotransfer that are consistent with the two-way conduction model but do not rule out the one-way conduction mechanism. In support of the one-way conduction model, Heinemann and Ankenbauer (14) demonstrated that protein synthesis is required in the recipient for retrotransfer to occur.

Although much is known about the processing of DNA at the origin of transfer (20), remarkably little is known about the molecular details of the transfer of DNA through the conjugal pore. For any model of conjugation, it is clearly important to

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TABLE 1. Plasmids used in this study

Plasmid	Marker(s)	Relevant properties[a]	Description	Reference
pBK1	Ap^r Cm^r	IncQ Rep, Mob^+	Mobilizable by the RK2 transfer system	43a
pEKA28	Ap^r Tc^r	IncQ Rep, Mob^+	Mobilizable by the RK2 transfer system	This study
pEKA33	Cm^r	P15A Rep	pACYC184 vector with the Tc^r marker deleted	This study
pRK2526	Ap^r Km^r Lac^+	IncP Rep, *oriT*$^+$	RK2 with the *E. coli lac* operon inserted into *tetA*	40
pRK21761	Ap^r Km^r Lac^+	IncP Rep, *oriT1*	pRK2526 *oriT1* (Fig. 1 and 2)	This study
pRK21764	Cm^r	P15A Rep, *oriT1*	pACYC184 with the 643-bp *oriT1* region from pRK21761	This study
pRK21765	Cm^r	P15A Rep, *oriT*$^+$	pACYC184 with the 643-bp *oriT*$^+$ region from pRK2526	This study
pVW8703	Cm^r	pMB1 Rep, *traF*$^+$ *G*$^+$	Expresses RK2 *traG*	47

[a] Rep, replicon.

know if DNA can transfer in both directions through a single conjugation bridge established by the donor. To resolve this issue unequivocally, we constructed a mutant of the self-transmissible, IncP plasmid RK2*lac* (40) that is capable of establishing a conjugation bridge and mobilizing DNA to a recipient cell but is incapable of self-transfer. This RK2*lac* mutant is defective in the *nic* site at the origin of transfer (*oriT*). A defined alteration of four nucleotides in *oriT* was constructed directly in RK2*lac* by a modification of our previously described vector-mediated excision (VEX) system for mutating large genomes (2). The resulting RK2*lac oriT1* mutant displayed the expected phenotypes and allowed us to determine if self-transfer is required for retrotransfer.

MATERIALS AND METHODS

Bacteria, plasmids, and bacteriophages. The *Escherichia coli* K-12 strains used were DH5α [*supE44* Δ*lacU169* (ϕ80 *lacZ*Δ*M15*) *hsdR17 recA1 gyrA96 thi-1 relA*] (25); MV10 (*thr-1 leuB6 lacY1 thi-1 tonA21 supE44 rfbD1* Δ*trpE5* λ$^-$) (16); DF4063, a spontaneous nalidixic acid-resistant (Nal^r) mutant of MV10; EKA260 [*leu thi gal-1 gal-2 lac xyl ara hsdR* Δ*trpE5* (λDKC266 P1 *repA*$^+$)] (2); and EKA76, a spontaneous rifampin-resistant (Rif^r) mutant of JA221 (*hsdR lacY leuB6* Δ*trpE5 recA1*) (from C. Yanofsky). The *E. coli* C (r^-m^-) strains used were AS11 [*thr leu ara thi gal lacY tonA malA xyl mtl minA minB* (λ R6K *pir*$^+$)] (39) and C2110 (*polA1 his rha*) (7). *Pseudomonas aeruginosa* PAC452 has previously been described (32).

Relevant plasmids are described in Table 1. The RK2*lac oriT1* mutant (pRK21761) was constructed by a modification of the VEX strategy (Fig. 1) (2). The only requirement for this new strategy is the identification of a restriction endonuclease recognition sequence that is absent in the plasmid to be mutated. Primers J1 (5′-GTCTGGAAGGCAGTACACCTTG-3′) and J2 (5′-TCTAG AATAGGTGAAGTAGGCCACCCG-3′) (Fig. 2) were used to synthesize a 206-bp fragment containing the *oriT* region proximal to the *traJ* coding region and including 67 bp at the 5′ end of the *traJ* gene. Primers K1 (5′-TCTAGACG GCTGACGCCGTTGGATAC-3′) and K2 (5′-CGTGCGAGCGGAACGTCT CGTAGGAGAA-3′) were used to generate a 443-bp fragment containing the *oriT* region proximal to *traK* and including 210 bp of the *traK* coding region. These fragments overlap by 6 bp at the *nic* site of *oriT* (Fig. 2) (48). The internal primers, K1 and J2, were designed to introduce four point mutations at the *nic* site that are predicted to eliminate nicking at *oriT* (48) and create an *Xba*I recognition site. The *Xba*I recognition sequence was used because RK2*lac* contains no *Xba*I sites. The two fragments were cloned into the TA-cloning vector pCRII (InVitrogen), and their nucleotide sequences were confirmed. To construct the pVEX plasmids for homologous recombination, pVEX1212 (2) was inserted into the *Xba*I site of plasmid pCRII-J and then digested with *Eco*RI to produce pVEX1212 carrying the J fragment (pRK21685). The K fragment was cloned similarly into pVEX2211 (2) to produce pRK21686. A single cointegrate formed by homologous recombination between an RK2 derivative with the wild-type *oriT* region and pRK21686 was selected by conjugal transfer, as described previously (2). The double cointegrate formed by homologous recombination between the single cointegrate and pRK21685 was also selected by conjugal transfer. For convenience, the double cointegrate structure was first produced with pRK2013, a kanamycin-resistant (Km^r) ColE1 derivative of RK2 (7), and then easily crossed to pRK2526, a *tetA*::*lacZYA* derivative of RK2 (40), by homologous recombination (2). Because RK2*lac* contains no sites for *Xba*I, digestion of the double cointegrate with *Xba*I and religation resulted in the *oriT1* mutant of RK2*lac* (pRK21761) in which the *nic* site of *oriT* has been replaced by an *Xba*I site.

The ampicillin-resistant (Ap^r), tetracycline-resistant (Tc^r) IncQ plasmid, pEKA28, was constructed by inserting the *Hin*dIII Tc^r-encoding fragment from pHP45ΩTc into pMHL3. pMHL3 was constructed by digesting pMMB67EH (10) with *Pvu*II, ligating with an *Eco*RI linker (5′-GGAATTCC-3′), digesting with *Eco*RI, and religating (21). The chloramphenicol-resistant (Cm^r), tetracycline-sensitive (Tc^s) P15A plasmid, pEKA33, was constructed by digesting pACYC184 (4, 35) with *Hin*dIII and *Hin*cII, treating with *E. coli* DNA polymerase Klenow fragment to blunt the *Hin*dIII cohesive end, and ligating. pRK21762 is the TA-cloning vector pCRII (InVitrogen) with a 643-bp *oriT*$^+$ PCR-generated fragment from pRK2526. pRK21763 is pCRII with the PCR-generated *oriT1* fragment from pRK21761. The 643-bp fragments were synthesized with *Taq* polymerase by using primers J1 and K2 (Fig. 2) and ligated directly to linear T-tailed pCRII (InVitrogen). pRK21764 and pRK21765 were constructed by cloning the *oriT* fragments from pRK21762 and pRK21763, respectively, into *Hin*cII- and *Hin*dIII-cleaved pACYC184. These *oriT* fragments were generated by cleaving pRK21762 and pRK21763 with *Not*I, treating with DNA polymerase Klenow fragment to blunt the ends, and subsequently cleaving with *Hin*dIII.

Media. Luria-Bertani (LB) or M9-CAA medium (25) was used. For *E. coli*, antibiotics were used at the following concentrations (in micrograms per milliliter): chloramphenicol, 50; kanamycin, 50; nalidixic acid, 20; penicillin, 150; rifampin, 100; spectinomycin, 50; and tetracycline, 15. To select *P. aeruginosa* transconjugants, kanamycin was used at 500 μg/ml and tetracycline was used at 100 μg/ml. *P. aeruginosa* PAC452 is intrinsically resistant to 20 μg of nalidixic acid per ml.

DNA procedures. Restriction endonucleases, T4 DNA ligase, *Taq* DNA polymerase, and *E. coli* DNA polymerase I Klenow fragment were purchased from commercial suppliers and used as recommended. Purification of plasmid DNA (18), agarose and polyacrylamide gel electrophoresis (25), transformation of *E. coli* (5), PCR amplification of DNA (36), and DNA sequencing (37) have been described elsewhere.

Conjugations. Broth cultures of donor and recipient strains were grown overnight under selection for resident plasmids. Cells were washed in LB medium, and 100 μl of donor and 1 ml of recipient were deposited on a MicronSep cellulose filter (pore size, 0.22 μm; MSI, Westboro, Mass.) for *E. coli*. For matings with *P. aeruginosa*, 50 μl of donor and 500 μl of recipient were used. Recipients were in at least fivefold excess of donors for all matings. Filters were placed on solid medium and incubated for the appropriate time at 37°C. Controls consisted of mock matings containing either donor or recipient alone. After incubation, filters were placed in 1 ml of liquid medium and vortexed to remove cells from filters. Serial dilutions were plated on appropriate selective media.

RESULTS

Use of VEX to construct an *oriT*-defective mutant of RK2*lac*. The origin of transfer (*oriT*) is a *cis*-acting element essential for the conjugal transfer of a plasmid (20). For the IncP plasmid RK2, DNA transfer is initiated by endonucleolytic cleavage of a single strand at the *nic* sequence within *oriT* by the TraI protein (29), which is recruited by TraJ protein bound to *oriT* (Fig. 1) (51). Studies with cloned RK2 *oriT* segments have identified nucleotides critical for the nicking and transfer of *oriT*-containing plasmids (48). Because *oriT* is within a region important for the expression of two divergent transfer operons on RK2 (28), we considered it crucial to construct point mutations that inactivate *oriT* in the context of RK2 without affecting the expression of any transfer genes.

We previously described VEX system for the construction of precise deletion-substitution mutations in large DNA molecules, such as RK2 (2). Here we show that a simple modification of the VEX strategy permits the alteration of individual nucleotides directly in a large plasmid. For these experiments,

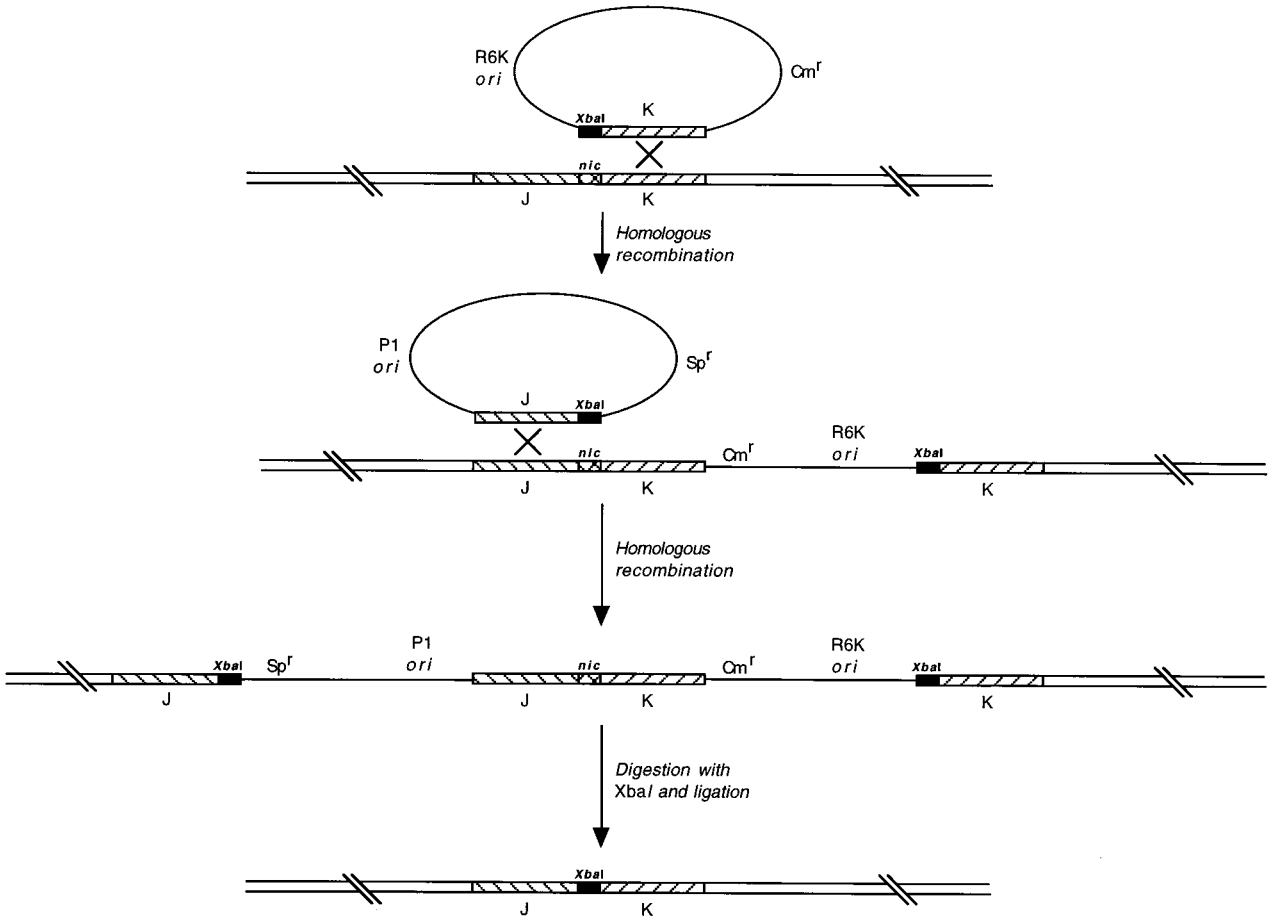

FIG. 1. Construction of the *oriT1* mutation by VEX. Four bases of the *nic* site of the RK2 *oriT* determinant were altered to create an *Xba*I site and the *oriT1* mutant allele. A 443-bp DNA fragment (K) with homology to part of *traK* and the *traK*-proximal region of *oriT* and terminating with an *Xba*I site was generated by PCR (Fig. 2) and cloned into the Cm^r vector pVEX2211, as described in Materials and Methods. A 206-bp fragment (J) with homology to part of *traJ* and the *traJ*-proximal region of *oriT* and terminating with an *Xba*I site was likewise generated by PCR and cloned into the Sp^r vector pVEX1212. Recombinants between the $oriT^+$ RK2 derivative (straight line) and the two pVEX clones (circles) were selected by sequential conjugal transfers. The double cointegrate (second line from bottom) was digested with *Xba*I and religated to delete $oriT^+$ and generate *oriT1*. R6K *ori* and P1 *ori*, the R6K and P1 origins of replication present in pVEX2211 and pVEX1212, respectively. Hatched lines labelled J and K show regions of homology to *traJ* and *traK*, respectively. The box labelled *nic* represents a 6-bp sequence of the *oriT* region that contains the site for endonucleolytic cleavage by the RK2 TraI protein. In *oriT1* this sequence is replaced by an *Xba*I cleavage site (filled box).

we chose to alter four nucleotides in RK2*lac* (pRK2526) predicted to inactivate the *nic* site of *oriT* (Fig. 1 and 2). This allele has been named *oriT1*. To verify the mutations in *oriT1*, the external PCR primers, K2 and J1 (Fig. 2), were used to amplify the *oriT* region from the RK2*lac oriT1* mutant (pRK21761) and wild-type RK2*lac* $oriT^+$ (pRK2526). The 643-bp PCR products were cloned and sequenced to confirm the $oriT^+$ and *oriT1* structures.

Properties of the cloned $oriT^+$ and *oriT1* regions. The self-transmissible RK2*lac* plasmid is able to supply in *trans* the functions necessary to mobilize a plasmid carrying the RK2 *oriT* region. We used RK2*lac* to compare the mobilization of small plasmid vectors carrying the 634-bp $oriT^+$ and *oriT1* fragments. The plasmid carrying the wild-type $oriT^+$ fragment, pRK21765, was mobilized at a frequency comparable to the self-transfer of RK2*lac* (Table 2), indicating that this cloned fragment contains a fully functional origin of transfer. In contrast, the *oriT1* mutant derivative, pRK21764, was not mobilized ($<2.5 \times 10^{-7}$ transconjugants per donor) even after 5 hours of mating. Thus, the four point mutations at the *nic* site of *oriT1* completely abolished the function of *oriT* in mobilization, as expected.

Self-transfer and mobilization properties of RK2*lac oriT1*. RK2*lac oriT1* was tested for self-transfer and, surprisingly, gave rise to transconjugants at a frequency of 10^{-4} of that of the wild type (Table 2). The plasmids that transferred were not revertants or pseudorevertants of RK2*lac oriT1* because they displayed the same transfer frequency in subsequent matings (data not shown). RK2*lac oriT1* was indistinguishable from RK2*lac* $oriT^+$ in its complete inability to mobilize a plasmid with the cloned *oriT1* region (pRK21764; Table 2). This result indicates that RK2*lac oriT1* had not acquired a compensatory mutation that allows low-level recognition of the mutant site. We do not know how the RK2*lac oriT1* plasmid is transferred. The transfer functions may recognize a secondary site in RK2*lac* that is similar to the *nic* site in *oriT*, or they may be able to act at low-level efficiency on the mutant *nic* site in the context of the entire RK2*lac* plasmid.

In contrast to its self-transfer defect, RK2*lac oriT1* showed no loss of ability to mobilize plasmid pRK21765, which carries the RK2 $oriT^+$ fragment. After 1 h of mating, the frequency of pRK21765 mobilization by RK2*lac oriT1* was comparable to the frequency of mobilization by wild-type RK2*lac* $oriT^+$ (Table 2). However, the results at later time points revealed that

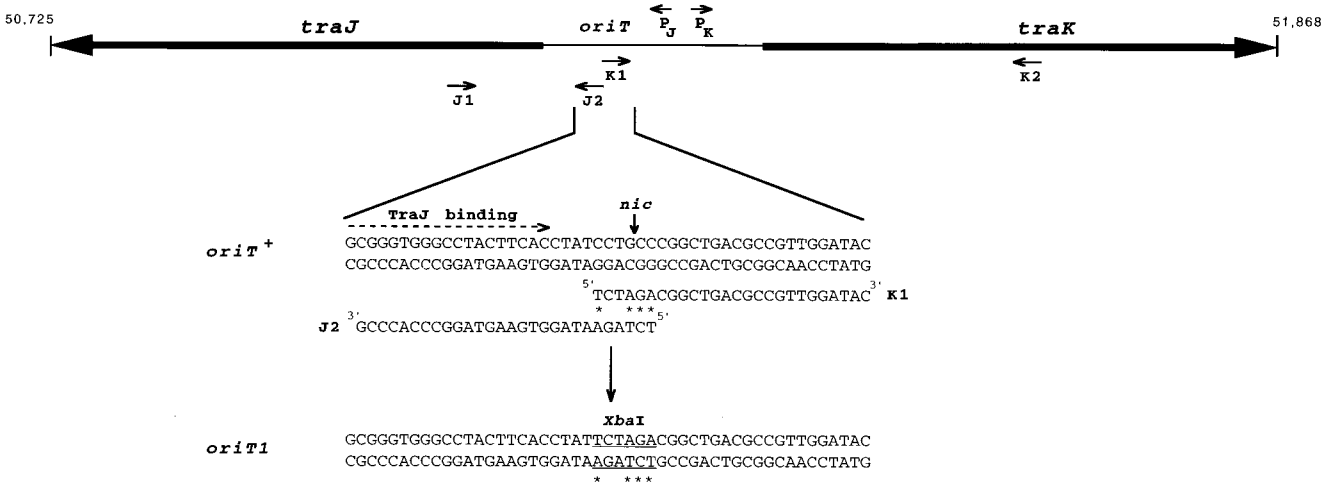

FIG. 2. Details and nucleotide sequence of the *oriT1* allele. The top line shows the segment of RK2 containing *oriT* and the *traJ* and *traK* genes. The numbers at the ends of this line indicate RK2 nucleotide coordinates (28). Bold arrows on this line show the directions of the *traJ* and *traK* coding sequences. P_J and P_K indicate the locations of the *traJ* and *traK* promoters, respectively, with arrows pointing in the directions of transcription. Below this line are the locations of the four PCR primers; arrows indicate the 5′-to-3′ direction. J1 and J2 were used to generate the 206-bp J fragment (Fig. 1); K1 and K2 were used to amplify the 443-bp K fragment. The 643-bp *oriT*$^+$ and *oriT1* fragments were generated from RK2*lac oriT*$^+$ (pRK2526) and RK2*lac oriT1* (pRK21761) by J1 and K2. The nucleotide sequence of the region surrounding the *nic* site of *oriT*$^+$ is expanded below this line. The TraJ binding site (51) is noted by a dashed arrow, and the downward arrow shows the site of endonucleolytic cleavage by TraI (29). The nucleotide sequences of the complementary and overlapping 5′ ends of the J2 and K1 primers are shown, with differences from that of the wild-type *nic* region indicated by asterisks. The bottom line shows the nucleotide sequence of the *oriT1* allele. Differences from the wild-type *nic* sequence are noted by asterisks, and the *Xba*I cleavage site is underlined.

the kinetics of pRK21765 mobilization by RK2*lac oriT1* are different from those of RK2*lac oriT*$^+$ (Fig. 3). For wild-type RK2*lac oriT*$^+$, the transconjugant-to-donor ratio rises sharply for 1 h and then levels off. The mutant RK2*lac oriT1* plasmid showed an equivalent rise in transconjugants per donor for 1 h, but the ratio dropped at 5 h before leveling off. This reproducible difference is partly due to the fact that during the mobilization of pRK21765 by RK2*lac oriT*$^+$, nearly all transconjugants become new donors because they acquire not only pRK21765 but also the self-transmissible RK2*lac oriT*$^+$. In contrast, the mobilization of pRK21765 by RK2*lac oriT1* yields transconjugants that contain only pRK21765. These transconjugants cannot engage in additional mating, so there is no further accumulation of transconjugants. The unexpected decline in the transconjugant-to-donor ratio at 5 h reflects the growth of the donor population during the mating period without concomitant growth of transconjugants (data not shown).

TABLE 2. Effect of the *oriT1* allele on mobilization and self-transfer

Test plasmid in donor[a] for:		Transconjugants/donor[b]	
Self-transfer	Mobilization	Self-transfer	Mobilization
pRK2526 (*oriT*$^+$)	pEKA33 (vector)	1.3	$<2.5 \times 10^{-7}$
pRK2526 (*oriT*$^+$)	pRK21765 (*oriT*$^+$)	5.0	3.0
pRK2526 (*oriT*$^+$)	pRK21764 (*oriT1*)	1.7	$<2.5 \times 10^{-7}$
pRK21761 (*oriT1*)	pEKA33 (vector)	1.1×10^{-4}	$<2.5 \times 10^{-7}$
pRK21761 (*oriT1*)	pRK21765 (*oriT*$^+$)	5.3×10^{-6}	1.8
pRK21761 (*oriT1*)	pRK21764 (*oriT1*)	4.5×10^{-5}	$<2.5 \times 10^{-7}$
pRK2526 (*oriT*$^+$)	pBK1 (IncQ Mob$^+$)	1.0	6.3×10^{-1}
pRK21761 (*oriT1*)	pBK1 (IncQ Mob$^+$)	2.3×10^{-5}	1.6

[a] *E. coli* DH5α was the donor host.
[b] *E. coli* DF4063 was the recipient. Conjugations were done for 1 h, as described in Materials and Methods. Selection for self-transfer was Km^r Nal^r; selection for mobilization was Cm^r Nal^r.

This result raises the possibility that nascent transconjugants are arrested for growth.

IncQ plasmids are incapable of self-transfer, but they specify their own origin of transfer (*oriT*$_Q$) and mobilization (Mob) functions that allow them to be efficiently mobilized by IncP plasmids (9, 11). The mobilization of IncQ plasmid pBK1 by RK2*lac oriT1* was efficient (Table 2) and showed kinetics comparable to those of IncP *oriT* plasmid pRK21765 (data not shown).

From these results, we conclude that RK2*lac oriT1* is defective in self-transfer but normal for the mobilization of IncQ or IncP *oriT*-containing plasmids in *trans.*

Does retrotransfer require transfer of the conjugative plasmid? Retrotransfer of an IncQ plasmid from the recipient cell to the donor cell was observed in matings in which wild-type RK2*lac oriT*$^+$ was present in the donor (Table 3 and Fig. 3). In these experiments, the Trp$^+$ Nal^s donor strain contained RK2*lac oriT*$^+$ (Km^r) and the mobilizable IncP *oriT*$^+$ plasmid pRK21765 (Cm^r). The Trp$^-$ Nal^r recipient strain carried the Tc^r IncQ Mob$^+$ plasmid pEKA28. The differentially marked plasmids and strains allowed us to track the movement of all plasmids between strains. We observed self-transfer of RK2*lac oriT*$^+$ and mobilization of pRK21765 to recipient cells at high-level frequencies, even in matings as short as 20 min. The frequencies of transfer and mobilization were higher at 1 h and reached a maximum by 5 h. We also observed retrotransfer of the IncQ plasmid (pEKA28) from recipient to donor. The retrotransfer of pEKA28 was initially 10^5-fold lower than self-transfer of RK2*lac oriT*$^+$ and mobilization of pRK21765 during matings of 20 min and 1 h. Whereas self-transfer and mobilization leveled off after 5 h of mating, retrotransfer continued. The retrotransfer frequency relative to that of self-transfer increased to about 10^{-4} after 5 h of mating and to greater than 10^{-3} after 24 h.

We then asked if retrotransfer can occur when RK2*lac oriT1* is in the donor. The two-way conduction model for retrotransfer holds that establishing a conjugation bridge is sufficient for

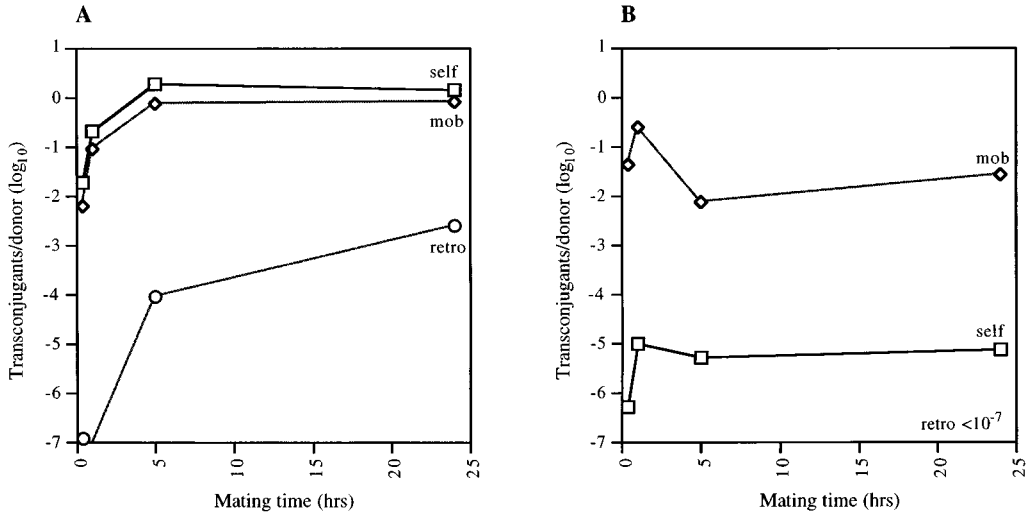

FIG. 3. Kinetics of self-transfer, mobilization, and retrotransfer by RK2*lac oriT*$^+$ (pRK2526; A) and RK2*lac oriT1* (pRK21761; B). Conjugations were done for different times, as described in the text and Table 3. Squares, self-transfer (self) of the RK2*lac* derivative; diamonds, mobilization (mob) of a coresident *oriT*$^+$ plasmid (pRK21765) in the donor; circles, retrotransfer (retro) of IncQ plasmid pEKA28 from recipient to donor.

the transfer of DNA from recipient to donor. This model predicts that retrotransfer of the IncQ plasmid from recipient to donor will be unaffected by nontransmissible RK2*lac oriT1* in the donor. The one-way conduction model requires prior transfer of the self-transmissible plasmid to the recipient and the establishment of a new conjugation bridge before DNA can be mobilized back to the donor. This model predicts that retrotransfer will be severely reduced by the use of the RK2*lac oriT1* plasmid in the donor.

The high-level frequency of mobilization of the coresident *oriT*$^+$ plasmid pRK21765 from RK2*lac oriT1* donor to recipient demonstrated that mating pairs were formed efficiently in these experiments, as expected, despite the low-level frequencies of RK2*lac oriT1* self-transfer (Table 3 and Fig. 3). Nevertheless, retrotransconjugants were extremely rare, occurring at a frequency of less than 2.5×10^{-7} and appearing only after 24 h. Late retrotransconjugants were likely to have arisen from low-level RK2*lac oriT1* self-transfer. Similar results were observed in experiments with *E. coli* donors and *P. aeruginosa* recipients (Table 3). We conclude that retrotransfer requires the recipient to acquire the self-transmissible plasmid from the donor before retrotransfer can occur.

IncQ plasmids encode their own DNA processing (Mob) functions (9). Their mobilization requires only one RK2-encoded protein, TraG, that is not required for mating pair formation (8, 23). TraG is thought to act as the link between the $oriT_Q$-Mob protein complex and the RK2 conjugation pore (23). We tested the possibility that the original conjugation

TABLE 3. Effect of RK2*lac oriT1* on retrotransfer

Test plasmid in donor[a] for:		Recipient[b]	Test plasmid in recipient for retrotransfer	Mating time (h)	Transconjugants/donor[c]		
Self-transfer	Mobilization				Self-transfer	Mobilization	Retrotransfer
	pRK21765 (*oriT*$^+$)	*E. coli*	pEKA28 (IncQ Mob$^+$)	0.33	NA[d]	$<2.5 \times 10^{-7}$	$<2.5 \times 10^{-7}$
	pRK21765 (*oriT*$^+$)	*E. coli*	pEKA28 (IncQ Mob$^+$)	1	NA	$<2.5 \times 10^{-7}$	$<2.5 \times 10^{-7}$
	pRK21765 (*oriT*$^+$)	*E. coli*	pEKA28 (IncQ Mob$^+$)	5	NA	$<2.5 \times 10^{-7}$	$<2.5 \times 10^{-7}$
	pRK21765 (*oriT*$^+$)	*E. coli*	pEKA28 (IncQ Mob$^+$)	24	NA	$<2.5 \times 10^{-7}$	$<2.5 \times 10^{-7}$
pRK2526 (*oriT*$^+$)	pRK21765 (*oriT*$^+$)	*E. coli*	pEKA28 (IncQ Mob$^+$)	0.33	1.3×10^{-1}	4.6×10^{-2}	1.6×10^{-6}
pRK2526 (*oriT*$^+$)	pRK21765 (*oriT*$^+$)	*E. coli*	pEKA28 (IncQ Mob$^+$)	1	4.1×10^{-1}	3.3×10^{-1}	1.2×10^{-6}
pRK2526 (*oriT*$^+$)	pRK21765 (*oriT*$^+$)	*E. coli*	pEKA28 (IncQ Mob$^+$)	5	1.3	5.6×10^{-1}	6.4×10^{-4}
pRK2526 (*oriT*$^+$)	pRK21765 (*oriT*$^+$)	*E. coli*	pEKA28 (IncQ Mob$^+$)	24	9.8×10^{-1}	5.3×10^{-1}	4.2×10^{-3}
pRK21761 (*oriT1*)	pRK21765 (*oriT*$^+$)	*E. coli*	pEKA28 (IncQ Mob$^+$)	0.33	2.4×10^{-5}	2.5×10^{-2}	$<2.5 \times 10^{-7}$
pRK21761 (*oriT1*)	pRK21765 (*oriT*$^+$)	*E. coli*	pEKA28 (IncQ Mob$^+$)	1	8.1×10^{-6}	2.7×10^{-1}	$<2.5 \times 10^{-7}$
pRK21761 (*oriT1*)	pRK21765 (*oriT*$^+$)	*E. coli*	pEKA28 (IncQ Mob$^+$)	5	8.0×10^{-6}	2.0×10^{-2}	$<2.5 \times 10^{-7}$
pRK21761 (*oriT1*)	pRK21765 (*oriT*$^+$)	*E. coli*	pEKA28 (IncQ Mob$^+$)	24	1.9×10^{-5}	1.4×10^{-2}	$<2.5 \times 10^{-7}$
pRK2526 (*oriT*$^+$)		*P. aeruginosa*	pEKA28 (IncQ Mob$^+$)	24	1.0×10^{-2}	NA	4.2×10^{-5}
pRK21761 (*oriT1*)		*P. aeruginosa*	pEKA28 (IncQ Mob$^+$)	24	3.0×10^{-6}	NA	$<2.5 \times 10^{-7}$

[a] *E. coli* DH5α was the donor host for all *E. coli* × *E. coli* conjugations; *E. coli* EKA76 was the donor host for *E. coli* × *P. aeruginosa* conjugations.
[b] The *E. coli* recipient was DF4063; the *P. aeruginosa* recipient was PAC452.
[c] Selection for self-transfer was done on LB-kanamycin-nalidixic acid for *E. coli* and M9-CAA-kanamycin for *P. aeruginosa*; selection for mobilization was done on LB-chloramphenicol-nalidixic acid; selection for retrotransfer was done on M9-CAA-tetracycline for *E. coli* and LB-rifampin-tetracycline for *P. aeruginosa.*
[d] NA, not applicable.

bridge established between donor and recipient could be used for retrotransfer if RK2 TraG was provided in the recipient along with the IncQ Mob functions. Matings were done as described above, except that recipients also contained Cm^r plasmid pVW8703 (47), which carries and expresses the RK2 *traG* gene (confirmed by its ability to complement an RK2 *traG* mutant). The presence of *traG* in the recipient did not allow retrotransfer of the IncQ plasmid in matings with donors carrying RK2*lac oriT1*, nor did it increase the frequency of retrotransfer mediated by RK2*lac* $oriT^+$ (data not shown).

DISCUSSION

Retrotransfer is the transmission of chromosomal or plasmid DNA from recipient cell to donor cell during bacterial conjugation (27). This phenomenon raises interesting possibilities about the structure and function of the poorly understood conjugation bridge, and it has important implications with respect to bacterial evolution, ecology, and biocontainment. Essentially any bacterial cell has the potential to transfer DNA when it encounters another cell carrying a self-transmissible plasmid.

To determine if retrotransfer requires transfer of the conjugative plasmid, we constructed an *oriT* mutation (*oriT1*) in the otherwise wild-type RK2*lac* plasmid by a modification of the VEX strategy for the mutagenesis of large genomes. The RK2*lac oriT1* plasmid is defective in self-transfer but equivalent to wild-type RK2*lac* in the mobilization of IncQ plasmids and plasmids containing the cloned IncP $oriT^+$ region. RK2*lac oriT1* allowed us to test directly whether prior plasmid transfer to the recipient is required for retrotransfer. Our results show unequivocally that retrotransfer of an IncQ plasmid from recipient to donor is sensitive to the presence of a functional *oriT* on the RK2*lac* plasmid in the donor. These results prove that retrotransfer requires the transfer of conjugation genes to the recipient. Thus, retrotransfer involves two DNA transfer events.

The transfer genes of RK2 are involved in two distinct functions, mating pair formation and DNA processing (28). Of all the transfer functions needed for RK2 mobilization of IncQ plasmids, only the *traG* product is not involved in mating pair formation. TraG is thought to be important for the interaction of the IncQ mobilization complex with the RK2 conjugation bridge (23). We found that RK2*lac* transfer is required to provide more than the *traG* product. This result demonstrates that retrotransfer requires the expression in the recipient of at least one function involved in the formation, maintenance, or function of the conjugation bridge. We conclude that retrotransfer involves either (i) the establishment of a second conjugation bridge for the transfer of DNA from recipient to donor or (ii) modification and repolarization of the original conjugation bridge for use by the recipient cell.

Previous work has supported the one-way conduction model. Genetic experiments by Blanco et al. (3) suggested the need for physical association of the conjugative plasmid and the recipient chromosome prior to retrotransfer of chromosomal markers in *Azotobacter vinelandii*. Strong additional support comes from the work of Heinemann and Ankenbauer (14) showing that retrotransfer requires protein synthesis in the recipient. In addition, UV-treated maxicells, whose chromosomes are largely degraded, are able to act as recipients and promote retrotransfer, indicating that the genes which need to be expressed in the recipient are those of the self-transmissible plasmid (15). Top et al. (45) have studied the kinetics of retrotransfer in mathematical mass action models. Surprisingly, their experimental data show that the number of retrotransconjugants is linearly related to the number of donor cells, suggesting that retrotransfer results from a single encounter of donor and recipient cells.

Our results show clearly that retrotransfer requires prior transfer of the conjugative plasmid. We suggest that the linear relationship of retrotransconjugants to donors holds for the one-way conduction model if there is a preference for retrotransfer to occur in the same mating pair as the original transfer event. It seems reasonable that prior formation of a mating pair would establish conditions which are favorable to retrotransfer. This modification of the one-way conduction model assumes that the mating pair does not necessarily separate upon transfer of the self-transmissible plasmid to the recipient cell. Such a mechanism would explain why retrotransconjugants have been found to appear earlier than do ultimate transconjugants in a triparental mating (33). It would also explain why the conjugative plasmid does not need to be capable of replication in the recipient for retrotransfer to occur (27, 30). Furthermore, the ability of some mating pairs to remain associated after the initial transfer event may serve to bypass the surface exclusion barrier that normally exists between two cells harboring the same self-transmissible plasmid (50). This model has additional implications with respect to the transfer functions needed for retrotransfer. If the mating pair has already formed, then retrotransfer may require only a subset of the transfer functions normally needed for conjugal transfer of DNA between cells. We are testing this possibility.

Because *oriT* occurs in a region that contains two divergent promoters for transfer genes, we considered it important to avoid deletions or insertions that might perturb the structure of the region and affect gene expression. The mutant *oriT1* allele we chose to construct consists of four nucleotide changes in the *nic* site of the RK2 *oriT* region that convert it to an *Xba*I cleavage site. From previous genetic and biochemical analyses of the cloned *oriT* region (48), these changes were expected to inactivate *oriT* completely. Each of the two nucleotide substitutions to the left of the *nic* site (Fig. 2) has been shown to reduce *oriT* function in vivo by a factor of 10^6 or more and to inactivate the ability of TraI to nick *oriT* in vitro (48). Consistent with these results, the cloned *oriT1* fragment was completely defective in mobilization activity in the presence of wild-type RK2 transfer functions. However, the RK2*lac oriT1* plasmid was not completely defective in self-transfer. Transconjugants were detected at a frequency of 10^{-4} of that of the wild type, and the plasmids in these transconjugants were unchanged. It is possible that *oriT1* can be nicked inefficiently when present in the larger RK2*lac* plasmid. Perhaps the difference in activity between the cloned *oriT1* allele and the *oriT1* present in RK2*lac* reflects a difference in superhelicity or local topology in these two plasmids. Higher superhelicity of the *oriT* region in RK2*lac*, for example, might allow the mutant *nic* site to melt sufficiently to expose single strands to the RK2 TraI relaxase. Alternatively, the transfer of RK2*lac oriT1* may initiate from a secondary site on the plasmid. The low-level frequency of transfer by RK2*lac oriT1* appears to involve host functions because it was not observed with *P. aeruginosa* donor cells (unpublished results).

The RK2*lac oriT1* plasmid and derivatives with other markers (41) will also be useful tools for bacterial genetics. While largely incapable of self-transfer, RK2 *oriT1* plasmids can be used for efficient mobilization of IncQ plasmids or suicide vectors for transposon mutagenesis and allele replacement in gram-negative bacteria. Any cotransfer of an RK2 *oriT1* plasmid is easily detectable by its marker and the presence of plasmid DNA. RK2 *oriT1* plasmids may offer an advantage over the integrated RP4::Mu strains, SM10 and S17-1 (31, 42),

that are routinely used for the mobilization of suicide vectors for transposon mutagenesis. The low levels of RK2*lac oriT1* self-transfer make it convenient to construct a mobilizing host in any genetic background. In addition, it has recently been shown that integrated RP4::Mu strains are capable of mobilizing a functional Mu to the recipient cell (49). Some transconjugants carrying insertions of the selected transposon also harbor a Mu genome at another site in the chromosome. This complication is eliminated by the use of an RK2 *oriT1* plasmid.

We have previously described the VEX system and demonstrated its simplicity, precision, and utility in constructing deletion-substitution mutations in large plasmids such as RK2 (2) and RK2*lac* (40). The simple modification of VEX used in this study permitted us to alter specific nucleotides directly in RK2*lac* to inactivate *oriT*. This general strategy will be particularly useful for generating point mutations at other locations on RK2. However, while VEX is a powerful tool for genetic studies of RK2, we emphasize that the convenience and precision of VEX are applicable to any gene on any large plasmid or cosmid.

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REFERENCES

1. **Amabile-Cuevas, C. F., and M. E. Chicurel.** 1992. Bacterial plasmids and gene flux. Cell **70:**189–199.
2. **Ayres, E. K., V. J. Thomson, G. Merino, D. Balderes, and D. H. Figurski.** 1993. Precise deletions in large prokaryotic genomes by vector-mediated excision (VEX): the *trfA* gene of promiscuous plasmid RK2 is essential for replication in several gram-negative hosts. J. Mol. Biol. **230:**174–185.
3. **Blanco, G., F. Ramos, J. R. Medina, J. C. Gutierrez, and M. Tortolero.** 1991. Conjugational retrotransfer of chromosomal markers in *Azotobacter vinelandii*. Curr. Microbiol. **22:**241–246.
4. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
5. **Cohen, S. N., A. C. Y. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA **69:**2110–2114.
6. **Diels, L., D. Springael, N. van der Lelie, E. Top, and M. Mergeay.** 1993. Use of DNA probes and plasmid capture in a search for new interesting environmental genes. Sci. Total Environ. **139–140:**471–478.
7. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
8. **Fong, S. T., and V. A. Stanisich.** 1993. Identification and characterization of RP1 Tra1 cistrons involved in pilus function and plasmid mobilization. J. Bacteriol. **175:**448–456.
9. **Frey, J., and M. Bagdasarian.** 1989. The molecular biology of IncQ plasmids, p. 79–94. *In* C. M. Thomas (ed.), Promicuous plasmids of gram-negative bacteria. Academic Press, New York.
10. **Fürste, J. P., W. Pansegrau, R. Frank, H. Blocker, P. Scholz, M. Bagdasarian, and E. Lanka.** 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. Gene **48:**119–131.
11. **Guiney, D. G.** 1993. Broad host-range conjugation and mobilizable plasmids in gram-negative bacteria, p. 75–103. *In* D. B. Clewell (ed.), Bacterial conjugation. Plenum Publishing, New York.
12. **Hayes, W.** 1953. Observations on a transmissible agent determining sexual differentiation in *Bacterium coli*. J. Gen. Microbiol. **8:**72–88.
13. **Heinemann, J. A.** 1991. Genetics of gene transfer between species. Trends Genet. **7:**181–185.
14. **Heinemann, J. A., and R. G. Ankenbauer.** 1993. Retrotransfer in *Escherichia coli* conjugation: bidirectional exchange or de novo mating? J. Bacteriol. **175:** 583–8.
15. **Heinemann, J. A., and R. G. Ankenbauer.** 1993. Retrotransfer of IncP plasmid R751 from *Escherichia coli* maxicells: evidence for the genetic sufficiency of self-transferable plasmids for bacterial conjugation. Mol. Microbiol. **10:** 57–62.
16. **Hershfield, V., H. W. Boyer, C. Yanofsky, M. A. Lovett, and D. R. Helinski.** 1974. Plasmid ColE1 as a molecular vehicle for cloning and amplification of DNA. Proc. Natl. Acad. Sci. USA **71:**3455–3459.
17. **Hill, K. E., J. C. Fry, M. J. Weightman, D. J. Bradley, and B. Cousland.** 1995. Retrotransfer of IncP1-like plasmids from aquatic bacteria. Lett. Appl. Microbiol. **20:**317–322.
18. **Kahn, M., R. Kolter, C. Thomas, D. Figurski, R. Meyer, E. Remaut, and D. R. Helinski.** 1979. Plasmid cloning vehicles derived from plasmids ColE1, F, R6K, and RK2. Methods Enzymol. **68:**268–280.
19. **Kletsova, L. V., and Y. D. Tsygankov.** 1990. Mapping of *pgi* and *gpd* genes involved in C-1 assimilation in the obligate methylotroph *Methylobacillus flagellatum*. Arch. Microbiol. **153:**139–145.
20. **Lanka, E., and B. M. Wilkins.** 1995. DNA processing reactions in bacterial conjugation. Annu. Rev. Biochem. **64:**141–169.
21. **Larsen, M. H., and D. H. Figurski.** Unpublished results.
22. **Lederberg, J., and E. Tatum.** 1946. Gene recombination in *E. coli*. Nature (London) **158:**558.
23. **Lessl, M., D. Balzer, K. Weyrauch, and E. Lanka.** 1993. The mating pair formation system of plasmid RP4 defined by RSF1010 mobilization and donor-specific phage propagation. J. Bacteriol. **175:**6415–6425.
24. **Levy, S. B.** 1986. Ecology of antibiotic resistance determinants, p. 17–30. *In* S. B. Levy and R. N. Novick (ed.), Antibiotic resistance genes: ecology, transfer, and expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Mazodier, P., and J. Davies.** 1991. Gene transfer between distantly related bacteria. Annu. Rev. Genet. **25:**147–171.
27. **Mergeay, M., P. Lejeune, A. Sadouk, J. Gerits, and L. Fabry.** 1987. Shuttle transfer (or retrotransfer) of chromosomal markers mediated by plasmid pULB113. Mol. Gen. Genet. **209:**61–70.
28. **Pansegrau, W., E. Lanka, P. T. Barth, D. H. Figurski, D. G. Guiney, D. Haas, D. R. Helinski, H. Schwab, V. A. Stanisich, and C. M. Thomas.** 1994. Complete nucleotide sequence of Birmingham IncPα plasmids: compilation and comparative analysis. J. Mol. Biol. **239:**623–663.
29. **Pansegrau, W., G. Ziegelin, and E. Lanka.** 1990. Covalent association of the *traI* gene product of plasmid RP4 with the 5′-terminal nucleotide of the relaxation site. J. Biol. Chem. **265:**10637–10644.
30. **Powell, B., M. Mergeay, and N. Christofi.** 1989. Transfer of broad host range plasmids to sulphate-reducing bacteria. FEMS Microbiol. Lett. **59:**269–274.
31. **Priefer, U. B., H. J. Burkardt, W. Klipp, and A. Pühler.** 1981. IS*R1*: an insertion element isolated from the soil bacterium *Rhizobium lupini*. Cold Spring Harbor Symp. Quant. Biol. **45:**87–91.
32. **Prince, A. S., and T. Barlam.** 1985. Isolation of a DNA fragment containing replication functions from IncP2 megaplasmid pMG2. J. Bacteriol. **161:** 792–794.
33. **Ramos-González, M.-I., M.-A. Ramos-Díaz, and J. L. Ramos.** 1994. Chromosomal gene capture mediated by the *Pseudomonas putida* TOL catabolic plasmid. J. Bacteriol. **176:**4635–4641.
34. **Reimmann, C., and D. Haas.** 1993. Mobilization of chromosomes and nonconjugative plasmids by cointegrate mechanisms, p. 137–188. *In* D. B. Clewell (ed.), Bacterial conjugation. Plenum Publishing, New York.
35. **Rose, R. E.** 1988. The nucleotide sequence of pACYC184. Nucleic Acids Res. **16:**355.
36. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. I. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:** 487–491.
37. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
38. **Schoonejans, E., and A. Toussaint.** 1983. Utilization of plasmid pULB113 (RP4::mini-Mu) to construct a linkage map of *Erwinia carotovora* subsp. *chrysanthemi*. J. Bacteriol. **154:**1489–1492.
39. **Shafferman, A., and D. R. Helinski.** 1985. Transcription signals in a region essential for replication of plasmid R6K. Plasmid **13:**51–58.
40. **Sia, E. A., R. C. Roberts, C. Easter, D. R. Helinski, and D. H. Figurski.** 1995. Different relative importances of the *par* operons and the effect of conjugal transfer on the maintenance of intact promiscuous plasmid RK2. J. Bacteriol. **177:**2789–2797.
41. **Sia, E. A., J. W. Wilson, and D. H. Figurski.** Unpublished results.
42. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–791.
43. **Thomas, C. M., and C. A. Smith.** 1987. Incompatibility group P plasmids: genetics, evolution, and use in genetic manipulation. Annu. Rev. Microbiol. **41:**77–101.

43a. **Thomson, V. J., O. S. Jovanovic, R. F. Pohlman, C.-H. Chang, and D. H. Figurski.** 1993. Structure, function, and regulation of the *kihB* locus of promiscuous plasmid RK2. J. Bacteriol. **175:**2423–2435.

44. **Top, E., M. Mergeay, D. Springael, and W. Verstraete.** 1990. Gene escape model: transfer of heavy metal resistance genes from *Escherichia coli* to *Alcaligenes eutrophus* on agar plates and in soil samples. Appl. Environ. Microbiol. **56:**2471–2479.

45. **Top, E., P. Vanrolleghem, M. Mergeay, and W. Verstraete.** 1992. Determination of the mechanism of retrotransfer by mechanistic mathematical modeling. J. Bacteriol. **174:**5953–5960.
46. **Top, E. M., W. E. Holben, and L. J. Forney.** 1995. Characterization of diverse 2,4-dichlorophenoxyacetic acid-degradative plasmids isolated from soil by complementation. Appl. Environ. Microbiol. **61:**1691–1698.
47. **Waters, V., and D. G. Guiney.** Unpublished results.
48. **Waters, V. L., K. H. Hirata, W. Pansegrau, E. Lanka, and D. G. Guiney.** 1991. Sequence identity in the nick regions of IncP plasmid transfer origins and T-DNA borders of *Agrobacterium* Ti plasmids. Proc. Natl. Acad. Sci. USA **88:**1456–1460.
49. **Wiater, L. A., A. Marra, and H. A. Shuman.** 1994. *Escherichia coli* F plasmid transfers to and replicates within *Legionella pneumophila*: an alternative to using an RP4-based system for gene delivery. Plasmid **32:**280–294.
50. **Willetts, N., and R. Skurray.** 1987. Structure and function of the F factor and mechanism of conjugation, p. 1110–1133. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
51. **Ziegelin, G., J. P. Fürste, and E. Lanka.** 1989. TraJ protein of plasmid RP4 binds to a 19-base pair invert sequence repetition within the transfer origin. J. Biol. Chem. **264:**11989–11994.