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Evolution of *Pseudomonas* R-Plasmids: Consequences of Tn*1* Insertion and Resultant Partial Diploidy to Chromosome and Tra⁻ R-Plasmid Mobilization

RONALD H. OLSEN

Department of Microbiology, University of Michigan Medical School, Ann Arbor, Michigan 48104

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Tn*1* transposes from pRO161, a Tra⁻ derivative of RP1, to *Pseudomonas aeruginosa* sex factor FP2. The acquisition of Tn*1* by FP2 results in its ability to mobilize pRO161 to other bacteria. Genetic evidence presented here suggests two sequential mechanisms. Initially, transposition of Tn*1* results in trans-diploidy for the Tra⁺ and Tra⁻ plasmids. This subsequently allows mobilization of the Tra⁻ R-plasmid dependent on a host recombination mechanism. Transconjugants from this mating contain either stable cointegrate R-plasmids or aggregates resulting from dissociation of the cointegrates into a Tra⁺ and Tra⁻ plasmid. These aggregates have lost at least part of Tn*1* from their parent FP2::Tn*1* component, but now they mobilize the Tra⁻ R-plasmid from a recombination-deficient (Rec⁻) genetic background as well as from Rec⁺ donor strains. Transconjugants from these retransfer matings are aggregates. These results suggest a contribution of transposons to R-plasmid evolution and dissemination beyond the mere acquisition of resistance to a given antibiotic.

R-plasmids have been designated class 1 (1) or cointegrate (3) if they exist as a single replicon. Other R-plasmids have been shown to exist as separate components, one bearing the R-determinants specifying resistance to antibiotics and the other encoding for transfer functions. These have been designated class 2 (1) or aggregate (3) R-plasmids. Several reports have focused on the genetic behavior of aggregate R-plasmids to determine the ubiquity of the ability of sex factors to mobilize nonconjugative R-plasmids. Smith and Heller (16) tested F and the transfer factor for colicin I for their ability to mobilize several nonconjugative R-plasmids. These authors observed lack of specificity for mobilization of the nonconjugative plasmids by F or the transfer factor for colicin I. However, these sex factors were not equivalent in their plasmid donor ability (Pda). In any case, though, their results clearly indicate possible evolutionary relatedness between conjugative (Tra⁺) and nonconjugative R-plasmids which extends beyond incompatibility group. A similar study by van Embden and Cohen (17), using a three-component mating system, has also demonstrated the ability of different conjugative plasmids to mobilize a small nonconjugative R-plasmid which encodes resistance to tetracycline (Tcʳ). It is therefore apparent, from such studies and from numerous others, that some conjugative plasmids can mobilize nonconjugative plasmids although unrelated to them by present criteria for plasmid classification.

We noted previously the mobilization of pRO161, a transfer-defective (Tra⁻) variant of the P incompatibility group R-plasmid RP1 (9, 15). The mobilization of pRO161 was mediated by the unrelated Tra⁺ R-plasmids FP2 or R388, accompanied by their acquisition of carbenicillin resistance (Cbʳ) from the Tra⁻ R-plasmid pRO161. These Tra⁺/Tra⁻ doubles were found to be diploid for the R-determinant which specifies Cbʳ (5). Accordingly, as previously proposed (9, 15), it seems likely that the acquisition of Tn*1* (Cbʳ) by either R388, an incompatibility group W R-plasmid, or FP2, a *Pseudomonas aeruginosa* sex factor described by Holloway (6), reflects transposition of Tn*1* to these Tra⁺ R-plasmids. Multicopies of pRO161, then, would allow for the availability of a remaining pRO161 copy for mobilization promoted by shared Tn*1* homology with the transposed Tra⁺ companion plasmid.

In this report, further changes occurring in the Tra⁺/Tra⁻ aggregates after serial retransfers are described. It appears that Tn*1* may contribute to the evolution of conjugative R-plasmids beyond the mere acquisition of resistance to ampicillin and related antibiotics. This study focuses on genetic evidence for the sequential changes in aggregates which occur during and after the acquisition of Pda. The results suggest

that, initially, intermolecular recombination (following transposition of Cb^r) accounts for the mobilization of pRO161. This is followed in transconjugants by either the maintenance of a cointegrate or its dissociation into separate Tra$^+$ and Tra$^-$ components. Dissociation, in turn, is accompanied by the loss of the determinant Cb^r from the Tra$^+$ component of the aggregate complex. Interestingly, the Tra$^-$ plasmid component of the aggregate is now mobilized independently of host bacterium recombination activity.

MATERIALS AND METHODS

Bacterial strains and plasmids. The relevant properties of the bacterial strains and plasmids used in this study are listed in Table 1.

Media. Minimal medium (VBG) and complex medium (TN) were prepared as described previously (9, 10). When complex medium with brain heart infusion was used for the determination of resistance to mercuric chloride (Hg^r), it contained (per liter) 36 g of brain heart infusion (Difco Laboratories, Detroit, Mich.) and 30 μg of Hg per ml. Brain heart infusion medium was solidified by the addition of 20 g of agar per liter. When nutritional selection against auxotrophic donors was done, amino acid requirements were satisfied by the addition of these components to a final concentration of 20 μg/ml. Antibiotic supplements were as described in the tables.

Mating and testing of exconjugants. All matings were done in TN broth medium. For this, TN broth medium was inoculated with overnight growth from TN agar which contained appropriate antibiotic or Hg for the strain containing a plasmid. These broth cultures were incubated for 3 h with agitation at 37°C. Inoculation was adjusted to result in approximately 10^8 cells per ml of TN broth culture after 3 h of growth. Donor and recipient cells were mixed 1:1 and incubated at 37°C for 2 h. Mating mixtures were centrifuged at ambient temperature, and cell pellets were suspended to 1/10 the original volume of 0.01 M phosphate buffer (pH 7.0). Cell suspensions were diluted and plated onto VBG medium supplemented with the nutrients required by the recipient and with one of the antibiotics to which the plasmid confers resistance in the donor. Plates were incubated for 48 h at 37°C. Transconjugants were purified by picking colonies into liquid suspension, followed by streaking out for single-colony isolation on solid medium identical to that used for their primary isolation. Incompatibility was determined with appropriate selective medium as described previously (10).

TABLE 1. *Bacteria and plasmids used*

Bacterial strain or plasmid[a]	Relevant characteristic
P. aeruginosa	
PAO2	*ser-3*
PAO38	*leu-38*
PAO170	*leu-38* (FP2)
PAO2003	*arg-32 rec-2* (2)
Plasmid	
pRO271	*bla mer* (9)
pRO161	Cb^r Tc^r Tra$^-$ IncP, derived from RP1 (15)[b]
FP2	*mer* (6)
R751	Tp^r IncP (8)

[a] The sources of these bacterial strains and plasmids have been given in previous publications (9, 10, 15).

[b] RP1 from our laboratory is indistinguishable from RP4 (11). Hence, the transposable genetic element that specifies Cb^r is probably the same. In view of this, we have designated the Cb^r transposon used here as Tn*1*, as suggested by Cohen (4). R-plasmid pRO161 is a deletant of RP1 (15).

RESULTS

Mobilization by *Pseudomonas* sex factor FP2. Of immediate concern in this work and our prior report (9) is the possibility that pRO161 may be mobilized by a cryptic plasmid contained in *P. aeruginosa* PAO and its auxotrophic derivatives, PAO2, PAO38, and PAO2003. Therefore, as a control for this work, pRO161 was transduced to these strains. None of them showed transfer of either Cb^r or pRO161 (i.e., transfer of Cb^r and Tc^r) to other *P. aeruginosa* strains. Furthermore, conjugal recipient bacteria that contained FP2 showed entry exclusion, as measured by lower frequencies of transconjugant formation, towards all the donor strains containing plasmids used in this study. These observations, then, diminish or preclude the likelihood of a cryptic plasmid being responsible for the observations noted below. The strains used are isogenic except for auxotrophic markers or maintenance of FP2. Physical characterization by Pemberton and Clark (13) has also failed to reveal the presence in these strains of a cryptic plasmid of sufficient molecular size to encode for transfer functions that might promote pRO161 mobilization to other bacteria.

We have previously described the sequence of events leading to the acquisition of the Cb^r determinant by *Pseudomonas* sex factor FP2 (9). Data shown in Table 2 now point up the frequency of this event and confirm previous phenotypic properties reported for the FP2 Cb^r plasmid pRO271. A comparison of the FP2$^+$ and FP2$^+$/pRO161$^+$ donors shows that they yield similar numbers of *ser-3*$^+$ transconjugants and, hence, that pRO161 does not affect the chromosome donor ability (Cda) of FP2. FP2 does not mobilize pRO161 at a detectable frequency ($<1 \times 10^{-8}$ Tc^r transconjugants) but can acquire its Cb^r determinant and hence transfer it at a frequency of about 4×10^{-6} per donor. This frequency is similar to that obtained from the recombination-deficient donor PAO2003

TABLE 2. *Chromosome and pRO161 transfer by sex factor FP2 and its derivative, pRO271*

Donor	Selection for[a]:	Transconjugants per donor
PAO170(FP2)	*ser*$^+$	1×10^{-5}
PAO170(FP2/pRO161)	*ser*$^+$	1×10^{-5}
	Cb^r	4×10^{-6}
	Tc^r	$<1 \times 10^{-8}$
PAO38(pRO271)	*ser*$^+$	7×10^{-5}
	Cb^r	2×10^{-2}
PAO38(pRO271/pRO161)	*ser*$^+$	6×10^{-5}
	Cb^r	2×10^{-2}
	Tc^r	3×10^{-5}

[a] Selection was on minimal medium or amino acid-supplemented minimal medium containing antibiotic, 500 μg of Cb per ml or 50 μg of Tc per ml.

(FP2/pRO161) (data not shown), suggesting that the acquisition of the Cb^r determinant is due to transposition of Tn*1* from pRO161 to FP2. The remaining matings in Table 2 show that pRO271, the Tn*1*$^+$ derivative of FP2, retains the Cda$^+$ phenotype of its parent but has now acquired the ability to mobilize pRO161; that is, it is Pda$^+$.

The data in Table 2 describe the behavior of a newly constructed strain maintaining two plasmids that have not been previously cotransferred. To determine whether any alteration occurred in the mating properties of the donor after several serial transfers of the pRO271/pRO161 plasmid aggregate originating in PAO38, Tc^r PAO2 transconjugants from the last mating in Table 2 were purified and tested for resistance to Hg, Cb, or Tc. The plasmids from 16 of these isolates were then retransferred back to PAO38 with selection for the acquisition of the Tc^r phenotype. These isolates were designated PAO38(pRO271/pRO161)B. After their purification and the confirmation of the presence of all the R-determinants, these isolates were again mated with PAO2. Based on the ratio of Tc^r to Cb^r transfer, these 16 isolates formed two groups typified by strains designated B2.5 and B3.12 (Table 3). PAO38(pRO271/pRO161) was included as a control to indicate the behavior of the parent strain, and it clearly differs from that of the B2.5 and B3.12 strains. The recovery of Cb^r transconjugants from strain B2.5 is 100-fold lower than from the parent strain or from pRO271. It now resembles that observed for Tc^r, either from strain B2.5 or from the parent newly constructed double. Therefore, this result resembles that expected for a derivative of pRO271 that has lost the Cb^r determinant but retained Pda.

Strain B3.12, on the other hand, shows transfer of Tc^r at a frequency resembling that of Cb^r from pRO271. This result would be appropriate for a donor containing a cointegrate of the pRO271 and pRO161 plasmids that now transferred at a frequency characteristic of the Tra$^+$ pRO271. To detect other possible alterations in the pRO271 derivative carried by the B2.5 strain, and to establish the cointegracy of plasmids carried by B3.12, transductional analysis and incompatibility tests were done.

Incompatibility and transductional analysis. The P incompatibility group R-plasmid R751 (8) was mated into the donor strains listed in Table 3. From each mating, 20 trimethoprim-resistant (Tp^r) transconjugants were selected, serially streaked for single colonies twice, and then tested for the unselected maintenance of pRO271 and pRO161 markers (i.e., Hg^r Cb^r and Cb^r Tc^r, respectively; Table 4). The parent strain gave the result expected for the separate maintenance of pRO271 and its companion IncP plasmid, pRO161. Loss of Tc^r indicates displacement of pRO161 by R751. The result for the retransferred aggregate designated B2.5 supports the possibility that pRO271 had lost Cb^r while maintaining Pda. With this strain, R751 has displaced the Cb^r and Tc^r determinants associated with

TABLE 3. *Mating of retransferred pRO271-pRO161 aggregate R-plasmids to PAO2*

Donor	Selection for[a]:	Transconjugants per donor
PAO38(pRO271/pRO161)[b]	Cb^r	5×10^{-2}
	Tc^r	4×10^{-5}
PAO38(pRO271/pRO161)B2.5	Cb^r	4×10^{-4}
	Tc^r	1×10^{-4}
PAO38(pRO271/pRO161) B3.12	Cb^r	6×10^{-2}
	Tc^r	4×10^{-2}

[a] Selection was as described in Table 2, footnote *a*.
[b] This donor was a newly constructed "double" that had not previously been transferred.

TABLE 4. *Incompatability tests for pRO271/pRO161 strains*

Recipient of R751[b]	Unselected resistance phenotype[a]		
	Hg^r	Cb^r	Tc^r
PAO38(pRO271/pRO161)[c]	20	20	0
PAO38(pRO271/pRO161)B2.5	20	0	0
PAO38(pRO271/pRO161)B3.12	20	0	0

[a] Number of isolates resistant per 20 tested.
[b] Tp^r transconjugants were selected on the appropriately supplemented minimal medium, which contained 500 μg of Tp per ml.
[c] Strain described in Table 3, footnote *b*.

pRO161, leaving Hg^r presumably located on the altered pRO271 plasmid.

When R751 was transferred to isolate B3.12, the purified transconjugants also lost Cb^r and Tc^r but retained Hg^r. Thus, if B3.12 is a cointegrate plasmid, as suggested by data in Table 3, only the IncP component derived from pRO161 is affected by the entry of R751. Another interpretation of the results with B2.5 or B3.12 is possible. The Hg^r encoded by FP2 may have been transposed to R751, but this is unlikely on the basis of data to be discussed later.

The donor strains (Table 3) were also tested for their aggregate or cointegrate characteristic employing *Pseudomonas* phage F116 (7), as described previously (9). Briefly, this procedure allows transduction of pRO161 but not of FP2 or pRO271. This presumably reflects the inability of F116 to transduce plasmids larger than pRO161 because of their size (Table 5). The first two strains listed, a newly constructed double and strain B2.5, clearly showed transduction of R-determinants characteristic of pRO161. Accordingly, it seems likely that pRO161 is maintained in these strains as part of an aggregate complex similar to a pRO271/pRO161 aggregate double described previously (9). No transductants were obtained when phage F116 was grown on isolate B3.12. This would be the expected result for a cointegrate plasmid larger than the transductional capacity of F116, as observed previously for pRO271 (9). Therefore, assuming that isolate B3.12 carries a cointegrate plasmid composed of pRO271 and pRO161, the incompatibility test with R751 in Table 4 had an unexpected result. Incompatibility seemed to affect only the pRO161 component and not the FP2 component incorporated into the pRO271 parent.

TABLE 5. *Transductional analysis of pRO271/pRO161 strains*

Phage F116 donor	Selection for[b]:	Transductants per phage	Resistance phenotype[a]		
			Cb	Tc	Hg^r
PAO38(pRO271/pRO161)[c]	Tc^r Mer	2×10^{-6} $<1 \times 10^{-8}$	r	r	s
PAO38(pRO271/pRO161)B2.5	Tc^r Mer	2×10^{-6} $<1 \times 10^{-8}$	r	r	s
PAO38(pRO271/pRO161)B3.12	Tc^r Mer	$<1 \times 10^{-8}$ $<1 \times 10^{-8}$			

[a] Determined by streaking transductants on media described below or in Table 2, footnote *a*. r, Resistant; s, sensitive..

[b] Selection was on TNA medium containing 50 μg of Tc per ml or brain heart infusion agar (Difco) containing 30 μg of Hg per ml.

[c] Strain described in Table 3, footnote *b*.

Behavior of FP2 derived from doubles. In view of apparent alterations in pRO271 after serial mobilization of pRO161, it now seemed pertinent to determine whether these changes in pRO271 had affected the Cda phenotype. Accordingly, Cda for isolates B2.5 and B3.12 was determined (Table 6). Results for the first two strains listed again showed no alteration of Cda occurring with the addition of Tn*1* to FP2, nor did the presence of pRO161 in a newly constructed donor, as in the second strain listed, have an effect on Cda. Strains B2.5 and B3.12, on the other hand, clearly lost Cda, as shown here, for the transfer of *ser-3*$^+$. This result has been confirmed for other chromosomal auxotrophic markers (data not shown). Therefore, the presumed deletion of Cb^r from pRO271 in the case of strain B2.5 seems to have been accompanied by loss of Cda, although Pda directed towards pRO161 was retained (Table 3).

The behavior of isolate B3.12 also shows the loss of Cda. This result may reflect the deletion of part of FP2 that accompanies the incorporation of pRO161. These data, therefore, clearly indicate further alterations in aggregate plasmid properties that occur after several transfers employed for the isolation and characterization of isolates B2.5 and B3.12.

Isolates obtained from the incompatibility test, which had lost pRO161 by virtue of the addition of R751, were now used as transductional recipients for the readdition of pRO161. This transduction displaced R751 and resulted in the reestablishment of their former phenotype. These derivatives could then be tested for the maintenance and location of Pda. In the case of isolate B2.5, it was considered that Pda might reflect either residual Tn*1* sequences borne by the Cb^s variant of pRO271, or, alternatively, the acquisition by pRO161(B2.5) of FP2 sequences. In Table 7, the first strain listed was constructed by introducing pRO161 (derived from strain B2.5) into an FP2$^+$ strain. The deriv-

TABLE 6. *Chromosome transfer mediated by FP2 and its derivatives to PAO2*

Donor	Selected marker[a]	Transconjugants per donor	Cda phenotype[b]
PAO170	*ser*$^+$	1×10^{-5}	+
PAO38(pRO271/pRO161)[c]	*ser*$^+$	7×10^{-5}	+
PAO38(pRO271/pRO161)B2.5	*ser*$^+$	$<1 \times 10^{-8}$	−
PAO38(pRO271/pRO161)B3.12	*ser*$^+$	$<1 \times 10^{-8}$	−

[a] Selection was as described in Table 2, footnote *a*.

[b] +, Chromosome mobilization; −, no detectable chromosome mobilization.

[c] Strain described in Table 3, footnote *b*.

TABLE 7. *Mating to PAO2 of reconstructed R-plasmid doubles*

Donor	Selection for[a]:	Transconjugants per donor	Mating phenotype	
			Cda	Pda
PAO170(FP2/pRO161B2.5)	*ser*$^+$	2×10^{-5}	+	
	Cb^r	9×10^{-6}		
	Tc^r	$<1 \times 10^{-8}$		−
PAO38(MerB2.5/pRO161)[b]	*ser*$^+$	$<1 \times 10^{-8}$	−	
	Cb^r	6×10^{-4}		
	Tc^r	1×10^{-4}		+
PAO38(Mer3.12/pRO161)[b]	*ser*$^+$	$<1 \times 10^{-8}$	−	
	Cb^r	2×10^{-4}		
	Tc^r	2×10^{-4}		+

[a] Selection was as described in Table 2, footnote *a*.

[b] Mer2.5 and Mer3.12 are the provisional designations assigned to the mercury resistances remaining after pRO161 displacement by R751. This mercury resistance could be transferred independently of R751 from the doubles produced by incompatibility tests with the addition of plasmid R751. These Mer transconjugants were resistant to IncP plasmid phages (11, 12), and hence are considered to be FP2 derivatives.

ative showed no difference in FP2-mediated Cda, indicating that pRO161(B2.5) does not affect this property. In this same mating, the transposition of Tn*1* (transfer of Cb^r) was again observed, as shown earlier in Table 2. However, no mobilization of Tc^r, indicative of pRO161 transfer, was obtained. From this result it seems unlikely that Pda for the parent B2.5 reflects the acquisition by pRO161(B2.5) of any new properties. The second donor strain listed in Table 7 was constructed by introducing the wild-type pRO161 into a strain carrying the sex factor component of the B2.5 aggregate complex (designated here MerB2.5). This newly formed double was still Cda$^-$. However, MerB2.5 retained the ability to acquire Tn*1*, as indicated by the frequency of Cb^r transfer. More importantly, Tc^r was also transferred, which presumably indicates pRO161 mobilization by MerB2.5. Therefore, these data, when considered with those obtained from the first mating, show that Pda is a property associated with MerB2.5, the plasmid resulting from the loss of Cb^r from pRO271. Consequently, it is concluded that the full or partial deletion of Cb^r from pRO271(B2.5) (i.e., MerB2.5) was accompanied by loss of FP2 genes concerned with Cda. Furthermore, the maintenance of Pda by the Tra$^+$ MerB2.5 plasmid suggests that the origin of the deletion may be internal to Tn*1*, allowing for the maintenance of Pda but extending into the parent FP2 region of pRO271. Similar behavior consistent with this interpretation was observed for the MerB3.12 isolate shown in Table 7, although before challenge with R751, a plasmid incompatible with pRO161, this double was presumed to exist as a cointegrate. These data indicate that the MerB2.5 and MerB3.12 are indistinguishable. The loss of Cda occurred as a consequence of recombination between the two plasmids. Serial retransfers of the aggregate complex MerB3.12/pRO161 have not resulted in the isolation of plasmid derivatives that can again transfer Tc^r at the high frequency characteristic of pRO271. Instead, they continue to behave as observed for the B2.5 strain. Therefore, the formation of recombinants seems to be inhibited or diminished by the prior mating experience of aggregates.

Rec functions required for Pda. Chandler and Krishnapillai (2) have described mutant *P. aeruginosa* PAO strains bearing a recombination deficiency mutation analogous in its phenotypic properties to the *recA* mutation described for *Escherichia coli*. The availability of this strain allowed testing of the Rec dependency of the Pda phenotype (Table 8). For this, a donor was constructed by the conjugal addition of pRO271 to the Rec$^-$ strain PAO2003, followed by the transductional addition of pRO161. PAO2003 derivatives containing the plasmid complexes designated B2.5 or B3.12 were obtained by conjugal transfer from reference PAO38 strains.

The first mating (Table 8) indicates the inability of pRO271 to mobilize pRO161 from the Rec$^-$ strain, as evidenced by the lack of Tc^r transconjugants. This result was reproduced with five such donors of independent origin. From this, it is concluded that the first mobilization that occurs after establishment of diploidy for Cb^r between the Tra$^+$ plasmid FP2 Cb^r and a companion Tra$^-$ R-plasmid is dependent

TABLE 8. *Mobilization to PAO2 of pRO161 R-plasmid complexes from Rec$^-$ P. aeruginosa PAO2003*

Donor	Selection for[b]:	Transconjugants per donor	Resistance phenotype[a]		
			Cb^r	Tc^r	Hg^r
PAO2003(pRO-271/pRO161)[c]	Cb^r	2×10^{-3}	16	0	16
	Tc^r	$<1 \times 10^{-8}$			
PAO2003(pRO-271/pRO161)-B2.5	Cb^r	7×10^{-5}	16	0	16
	Tc^r	2×10^{-5}	16	16	16
PAO2003(pRO-171/pRO161)-B3.12	Cb^r	4×10^{-3}	16	16	16
	Tc^r	3×10^{-3}	16	16	16
PAO2003(pRO271)	Cb^r	4×10^{-3}	16		

[a] Determined as described in Table 5, footnote *a*, per 16 clones tested.

[b] Selection was as described in Table 2, footnote *a*.

[c] This donor was a newly constructed "double" that was constructed as described in the text.

on host Rec functions. The behavior of a serially retransferred aggregate is shown in the second mating listed. In this instance, transfer of Tc^r and, hence, pRO161 was observed. Therefore, the sex factor present in this aggregate derived from pRO271 has acquired the ability to mobilize pRO161 from Rec^- hosts. Furthermore, selection for the transfer of Cb^r and characterization of the phenotype for the transconjugants provides an estimate of the transposition frequency of Tn*1* from pRO161 to the Cb^s variant of pRO271 in a Rec^- genetic background. Independent transfer of the re-formed pRO271 sex factor exclusive of pRO161-linked Tc^r also indicates lack of a cointegrate in this donor.

The mating frequency observed for the B3.12 strain from the Rec^- donor resembles that shown previously for transfer from Rec^+ donors. Therefore, the stability of the cointegrate is not dependent on the Rec functions that had been mutated in strain PAO2003.

The results in this section and the foregoing suggest two pathways for the mobilization of Tra^- plasmids. The recombination-dependent pathway results from the establishment of transdiploidy associated with the insertion of a transposon. The recombination-independent pathway results from the prior mating experience of the plasmid aggregate. The extent of homology, if any, between strain B2.5 Tra^+ and Tra^- plasmids is indeterminate, but it is clear from the results in Table 8 that Rec-independent Pda has evolved.

DISCUSSION

Tn*1* is a transposable genetic element encoding type TEM β-lactamase that confers resistance to ampicillin, Cb, and related antibiotics. It is a segment of DNA of $3.2 \times 10^6 \pm 0.3 \times 10^6$ daltons, whose distal regions are complementary (albeit inverted) sequences of approximately 140 base pairs. Rubens et al. (14) have recently shown that its insertion is indistinguishable in Rec^+ or Rec^- bacterial hosts with regard to frequency of insertion or the distribution of insertion sites. Tn*1* is found widely distributed in nature, and its salient biological features have recently been summarized by Cohen (4). Data now presented in this report suggest another way in which Tn*1* may contribute to the evolution of R-plasmids. By one mechanism, the location of Tn*1* on Tra^+ and Tra^- plasmids promotes recombination between them, allowing mobilization to another host. After such transfer, the Tra^+/Tra^- complex may be maintained as a cointegrate plasmid or may dissociate into separate Tra^+ and Tra^- plasmids. However, when this dissociation occurs, the Tra^+ plasmid loses the Cb^r determinant. This may not reflect deletion of the entire Tn*1* but, more likely, a partial deletion which also extends into a contiguous region of the parent plasmid. Such an event could lead to an observable change in the phenotype of the parent plasmid. In the present study, this was observed as the loss of Cda. The resulting phenotype thus corresponds to that observed for naturally occurring Cda^- R-plasmids.

The observation that this Cb^s Cda^- evolutionary product was still Pda^+ but could now mobilize pRO161 from a Rec^- as well as a Rec^+ strain was unexpected. The obvious difference between the Rec-dependent and Rec-independent variants of FP2 is the absence in the latter of at least part of Tn*1* and perhaps of adjacent FP2 DNA. It seems, therefore, that the Rec-independent mobilization process may be hindered in some way by the complete Tn*1*, or that internal deletion of Cb^r may have removed a regulatory gene that limits mobilization. Alternatively, the initial insertion of Tn*1* into FP2 may have resulted in the substitution of the distal Tn*1* terminus for a Cda control region. Thus, complete excision of Tn*1* as a consequence of Rec-dependent mobilization might be expected to effect removal of part of the Cda locus. By this model, reinsertion of Tn*1* at this site should result in the reacquisition of Cda. This has not been observed for 16 isolates tested (unpublished data). However, it may be that the ability of Tn*1* to insert at any one of a variety of sites in FP2 may bias against the isolation of a reconstructed FP2 Cb^r that has regained Cda.

The results from the incompatibility testing of the cointegrate class of pRO271/pRO161 R-plasmids (isolates designated B3.12) seem enigmatic. Although these are cointegrates, on the basis of transductional analysis and conjugational linkage, only their IncP constituent, pRO161, was invariably displaced by another IncP plasmid. This suggests that incompatibility functions here are directed specifically against the related IncP plasmid DNA region of the FP2/pRO161 cointegrate. In these experiments, no selection was imposed for the FP2 component of the cointegrate, yet it was maintained while losing its IncP pRO161 region. The remaining Mer plasmid was still Tra^+ and gave rise to transconjugants that were insensitive to the IncP plasmid-specific phages PRR1 and PRD1 (11, 12). This precludes the possibility of transposition of the *mer*$^+$ locus to the IncP plasmid (R751) used to displace the pRO161 component. A consequence of the formation of a stable cointegrate was the loss of Cda, and the dissociation product produced by incompatibility remained Cda^-. The *mer*$^+$ plasmid now behaved like the

altered Cda$^-$ Cb^s derivative of pRO271 observed for the aggregate complex (illustrated here by the isolate B2.5). These corresponding properties allow the following overview of the evolution of Pda by the Rec-dependent and later Rec-independent pathways, and also facilitates an extension and refinement of our previous model for the evolution of Pda.

These results suggest that the Rec-dependent pathway is preceded by the establishment of trans-diploidy for Tn*1* on the Tra$^+$ and Tra$^-$ plasmids. The Tra$^-$ plasmid now recombines with the Tra$^+$ plasmid prior or in response to mobilization by the Tra$^+$ sex factor. Newly formed transconjugants may now maintain cointegracy required for transfer and exist stably thereafter. These cointegrates have lost Cda as a consequence of recombination. Alternatively, the cointegrate may dissociate into a Tra$^-$ component, indistinguishable from its parent pRO161, and a unique Tra$^+$ component. The Tra$^+$ plasmid, presumably derived from pRO271, is unlike pRO271 in that it now lacks both Tn*1* and Cda. However, as a consequence of this, it is now able to mobilize either its dissociated pRO161 companion plasmid or a newly added pRO161 not previously mobilized by pRO271. This occurs in the absence of host Rec function(s). Therefore, it is proposed that a single mobilization event reflecting recombination between Tra$^+$ and Tra$^-$ plasmids in their Tn*1* loci results in an alteration of the Tra$^+$ plasmid which, upon dissociation in transconjugants, produces a Tra$^+$ derivative plasmid, MerB2.5. This variant Tra$^+$ plasmid now shows Pda independent of host recombination functions. It is unclear at this time whether the altered phenotype of MerB2.5 reflects the expression of residual Tn*1* functions on MerB2.5 or altered parental FP2 functions, either of which are now expressed as a consequence of a prior recombination and mobilization experience with pRO161. Interestingly, the evolved Rec independence of pRO161 mobilization reported here corresponds to a previous observation of Warkus and Cohen (17). They reported this property as a distinguishing characteristic of a naturally occurring sex factor with respect to its ability to mobilize a Tra$^-$ R-plasmid from a recombination-deficient *E. coli* genetic background.

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