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Pseudomonas Streptomycin Resistance Transposon Associated with R-Plasmid Mobilization

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Plasmid pMG1 encodes resistance to gentamicin, streptomycin, sulfonamides, and mercuric ions and also mobilizes pRO161, a transfer-deficient plasmid derived from RP1. Upon mobilization, pRO161 acquires streptomycin resistance (Sm^r) and can subsequently be remobilized by pMG1 at significantly higher frequencies than pRO161 itself. Both the initial acquisition of Sm^r and the subsequent mobilization of the transfer-deficient plasmid are *recA* independent: thus, the Sm^r determinant appears to be located on a transposon, designated Tn*904*. Tn*904* transposes to a variety of other plasmids, including RP1, FP2, R388, K, pRO1600, and pBR322, and in some cases the acquisition of this transposon accompanied deletions in the target plasmid. When no deletion occurred, target plasmids gained 5.2 kilobase pairs of DNA and new restriction endonuclease cleavage sites for *Ava*I, *Bgl*II, *Pst*I, *Sma*I, and *Sst*I. Physical analysis of such plasmids showed that the Tn*904* termini are inverted repeat DNA sequences of approximately 124 base pairs. After cloning into vector pRO1723, a single site for restriction endonuclease *Ava*I was identified within the Sm^r determinant of Tn*904*. In *Escherichia coli*, but not in *Pseudomonas aeruginosa*, Tn*904* shows a gene dosage-dependent expression of streptomycin resistance.

Many R-plasmids exist as a single replicon, whereas other R-plasmids have been shown to exist as separate components, one bearing the R-determinants specifying resistance to antibiotics and the other encoding transfer functions. These multicomponent R-plasmids are called aggregates; another example of an aggregate R-plasmid is the combination of a transferable and a nontransferable plasmid, which may transfer together in bacterial matings when selecting for the acquisition of the transfer-defective plasmid (30).

We reported previously the mobilization of pRO161, a nontransferable plasmid, when Tn*1* transposed from pRO161 to a sex factor maintained by the same host bacterium (30, 32, 40). In retrospect, this activity of Tn*1* may have been associated with the formation of Tn*1*-mediated cointegrates during conjugation, which resolved into separate plasmids in the newly formed transconjugants (10, 15, 37). In our work, transposition of Tn*1* from pRO161 to either *Pseudomonas aeruginosa* sex factor FP2 or R-plasmid R388 subsequently facilitated the mobilization of pRO161 by FP2::Tn*1* (pRO271) or R388::Tn*1* (e.g., pRO231). Thus, plasmid mobilization ability (Pma) was presumably due to transdiploid homology between the transfer-proficient and -defective components of the plasmid aggregate that occurred when both plasmids had a copy of Tn*1*.

In the present study, we constructed a bacterial strain which contained plasmids pRO161 and pMG1, which are, respectively, defective and proficient in self-transfer. We expected that pMG1, an IncP-2 plasmid which transfers at a high frequency (19), would acquire transposon Tn*1* and thereby show Pma toward pRO161. However, contrary to our expectation, a pMG1 R-determinant encoding streptomycin resistance (Sm^r) was in every instance added to pRO161 concurrent with its mobilization by pMG1. Thus, Sm^r appears to be located on a transposon, which we have designated Tn*904*. In this report, we show the association of Pma with Tn*904* transposition and also the transposition of Tn*904* to several disparate plasmids. When Tn*904* transposed into plasmid pBR322 (3) or pRO1600 (31), the size of these plasmids was increased by approximately 5.2 kilobases (kb). We have mapped restriction endonuclease cleavage sites within Tn*904*, the location of the

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region which encodes streptomycin resistance, and have found inverted repeat termini of about 124 base pairs (bp). Tn*904* resembles, in its size, organization, and ability to delete target plasmids, the class II (Tn*3* family) transposons as described in the review by Kleckner (23).

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 (33). Minimal medium was supplemented with amino acids to a final concentration of 0.5 mM, except isoleucine and valine (each at 0.25 mM) and tyrosine (0.1% final concentration).

When antibiotics were used for the selection or characterization of *Escherichia coli* transconjugants, unless otherwise specified in the text, the media were supplemented as follows (micrograms per milliliter): carbenicillin, 500; streptomycin, 25; kanamycin sulfate, 30; trimethoprim, 100; and tetracycline, 25. For the selection of *P. aeruginosa*, antibiotics were added to the following concentrations (micrograms per milliliter): carbenicillin, 500; tetracycline 50; streptomycin, 250; trimethoprim, 500; tobramycin, 5; and gentamicin, 10.

Bacterial matings. To facilitate selection of transconjugants from matings which occur at a low frequency, donor and recipient bacteria were grown overnight on the surface of TN agar as mixed cultures. The cells were then harvested from the surface of the plates, suspended in buffer, and plated onto selective medium. Other bacterial matings were as described previously (40).

MIC determinations. Minimal inhibitory concentration (MIC) determinations were done essentially as described in the *Manual of Clinical Microbiology* (44). Streptomycin was diluted into TN broth from a stock solution of freshly rehydrated antibiotic. Cultures to be tested were freshly grown on TN agar medium, suspended in TN broth, and inoculated into TN broth with streptomycin to 10^5 cells per ml. After mixing, the tubes were incubated at 37°C for approximately 17 h. Visible turbidity in a tube indicates resistance to the concentration of antibiotic contained in the growth medium.

Preparation, electrophoresis, and transformation of plasmid DNA. Plasmid DNA was prepared using a modification of the method of Guerry et al. (11) described by us previously (31). For rapid screening of transconjugants or transformed plasmids, a miniaturized version of the procedure of Hansen and Olsen (12) was used (C. Gonzalez, A. Vidavir, F. Layher, and R. H. Olsen, manuscript in preparation). For physical mapping of the plasmids, DNA was cleaved with restriction endonucleases as recommended by the supplier. Plasmid DNA or *Pseudomonas* chromosomal fragments were cloned as reported previously (31). Cleaved or uncleaved plasmid DNA was electrophoresed as described previously (12, 28). *P. aeruginosa* was transformed with plasmid DNA by a previously published modification (31) of the procedure of Mercer and Loutit (27). *E. coli* was transformed by a modification of the procedure of Davis et al. (7) previously described by us (31).

Electron microscopy. Heteroduplex analysis of plasmid DNA followed the procedure of Davis et al. (8). Plasmid pBR322, converted to the open circular form by repeated freezing and thawing, was added to the hyperphase to serve as an internal size standard for the estimation of the size of the heteroduplex figures. Grids were viewed on a Zeiss EM10 electron microscope, and representative molecules were photographed. All molecules used for quantitation were photographed at the same magnification. All pBR322 size standards analyzed were intact circles. The lengths of the molecules were measured using a Numonics Digitizer. Each molecule was measured three times, and the average of these determinations was used as the length value. The 4,362-bp plasmid pBR322 served as a size standard (41).

RESULTS

Mobilization of pRO161. The mating behavior of the pMG1/pRO161 R-plasmid aggregate changed subsequent to its formation and serial transfer. The IncP-2 incompatibility group R-plasmid pMG1 (19) was the transfer-proficient (Tra$^+$) component of this aggregate. R-plasmid pMG1 is a large (12), limited-host-range *Pseudomonas* plasmid with multiple antibiotic resistance markers (19; Table 1). R-plasmid pRO161 is a Tra$^-$ deletion mutant of RP1 (40). Table 2 shows Pma of pMG1 toward pRO161. The first mating listed illustrates the behavior of a newly constructed aggregate: selection of transconjugants for their acquisition of tetracycline resistance (Tc^r) indicates the mobilization of pRO161. Selection for the acquisition of gentamicin resistance (Gm^r) in this and subsequent matings indicates the independent transfer of pMG1. The second mating listed in Table 2 shows, however, that Pma of pMG1 toward pRO161 (but not the transfer frequency of pMG1 itself) significantly increased after an initial mobilization of the aggregate. A transconjugant from the first mating designated PAO2(pMG1/pRO161)A1 was used as the donor for retransfer and now showed higher Pma toward pRO161 than the first mating listed in Table 2. Seven other transconjugants derived from the first mating listed in Table 2 were also tested and showed a similar enhancement of Pma (data not shown). Based on our previous work (30, 32), we expected that this enhancement of Pma after one mobilization reflected the transposition of Tn*1* from pRO161 to pMG1.

To determine the nature of a possible Pma-associated change in the aggregate, we did incompatibility tests. Recipients of the IncP-1 incompatibility group plasmid R751 (21), used to displace the IncP-1 plasmid pRO161, were tested for the maintenance of antibiotic resistance markers. The IncP-2 plasmid pMG5 (19) was similarly used to displace the IncP-2 plasmid

TABLE 1. Bacteria and plasmids

Bacterial strain or plasmid	Relevant characteristics[a]	Reference or source
P. aeruginosa		
PAO2	*ser-3*	38
PAO38	*leu-38*	32
PAO2003	*argH, recA*	5
P. putida AC541	Met$^-$, contains K-plasmid	—[b]
E. coli		
ED8654	Met$^-$ *hsdR, hsdM*	31
ROE531	Met$^-$	35
J53	Met$^-$, Pro$^-$	35
AE85	*aroA*, His$^-$, Leu$^-$, Trp$^-$, *recA*	—[c]
V517	Contains standard plasmids	26
Plasmid		
RP1	Cb^r, Tc^r, Km^r, Tra$^+$	33, 34
pRO161	Cb^r, Tc^r, Tra$^-$	40
pRO165	Cb^r, Tc^r, Sm^r, Tra$^-$, pRO161::Tn*904*	This study
pRO169	Cb^r, Tc^r, Km^r, Sm^r, Tra$^+$, RP1::Tn*904*	This study
pRO1600	Cryptic	31
pRO1614	Cb^r, Tc^r	31
pRO1723	Deleted pRO1614, Cb^r	This study
pRO1742	Sm^r, pRO1600::Tn*904*	This study
pRO1743	Sm^r, pRO1600::Tn*904*	This study
pBR322	Cb^r, Tc^r	3
pRO1744	Cb^r, Tc^s, Sm^r, pBR322::Tn*904*	This study
pRO1745	Cb^s, Tc^r, Sm^r, pBR322::Tn*904*	This study
pRO1746	Cb^r, Tc^r, Sm^r, pBR322::Tn*904*	This study
pRO1747	Deleted pRO1744, Cb^r, Tc^s, Sm^r	This study
pRO1748	pRO1723:pRO1747, recombinant, Cb^r, Sm^r	This study
FP2	Hg^r, sex factor	18
pRO272	Hg^r, Sm^r, FP2::Tn*904*, sex factor	This study
R388	Tp^r	40
pRO232	Tp^r, Sm^r, R388::Tn*904*	This study
K	Cryptic, sex factor	—[a]
pRO302	Sm^r, K::Tn*904*, sex factor	This study
pMG1	Gm^r, Sm^r, Su^r, Hg^r	19
pMG5	Km^r, Su^r, Tm^r, Hg^r	19
R751	Tp^r	21

[a] Marker abbreviations: bacterial strains—*arg*, arginine; *aro*, aromatic amino acid; His, histidine; *hsdR*, K-12 restriction; *hsdM*, K-12 modification; *leu*, leucine; Met, methionine; Pro, proline; *rec*, recombination; *ser*, serine; and Trp, tryptophan; plasmids—Cb^r, carbenicillin resistance; Tc^r, tetracycline resistance; Km^r, kanamycin resistance; Sm^r, streptomycin resistance; Gm^r, gentamicin resistance; Tm^r, tobramycin resistance; Su^r, sulfonamide resistance; Tp^r, trimethoprim resistance; Hg^r, mercuric ion resistance; Tra, conjugal transfer.
[b] Received from A. Chakrabarty, University of Illinois Medical Center.
[c] Received from D. Oxender, University of Michigan.

pMG1. When pRO161 was displaced by R751, carbenicillin resistance (Cb^r) encoded by pRO161 was lost. Therefore, Tn*1* had not transposed to pMG1 and, thus, was not responsible for the facilitated mobilization of pRO161, unlike our previous observations of pRO161 mobilization by FP2 (30). However, when pMG1 was displaced by pMG5, streptomycin resistance was retained, indicating that pRO161 had Sm^r as a consequence of its mobilization by pMG1. To test whether the Sm^r determinant was now part of pRO161, we did transduction tests as described previously (40). *P. aeruginosa* bacteriophage F116 (18) was grown on PAO38(pMG1/pRO161)A1 and three other aggregates similarly used for the serial retransfer experiment described in Table 2. Regardless of whether F116 transductants were selected for Cb^r, Tc^r, or Sm^r, each of the other two independent R-determinant markers were found to be cotransduced. This result suggested that the pMG1 Sm^r determinant had been added to pRO161 concurrent with its mobilization by pMG1. One such transductant plasmid was designated pRO165.

TABLE 2. Transfer and retransfer of plasmid pMG1 aggregates

Mating	Donor	Recipient	Selection[a]	Transconjugants per donor
1	PAO38(pMG1/pRO161)	PAO2	Gm^r	8×10^{-2}
			Tc^r	9×10^{-5}
2	PAO2(pMG1/pRO161)A1	PAO38	Gm^r	5×10^{-2}
			Tc^r	4×10^{-3}
3	PAO2003(pMG1/pRO161)[b]	PAO2	Gm^r	3×10^{-2}
			Tc^r	3×10^{-5}
4	PAO2003(pMG1/pRO165)[c]	PAO2	Gm^r	1×10^{-2}
			Tc^r	1×10^{-4}

[a] Selection was on minimal medium supplemented with antibiotic (see the text).
[b] This strain was constructed by phage F116 transduction of pRO161 to PAO2003(pMG1).
[c] This strain was constructed by phage F116 transduction of pRO165 to PAO2003(pMG1).

In a *recA* mutant of *P. aeruginosa*, PAO2003, which resembles similar mutants described previously for *E. coli* (5), both the acquisition of Sm^r by PRO161 and its subsequent mobilization still occurred (Table 2, matings 3 and 4), although in the recombination-deficient genetic background, enhanced Pma from a *recA* donor for a serially retransferred aggregate was less than that observed in Rec$^+$ donors (cf. matings 2 and 4). The acquisition of Sm^r by pRO161 in the aggregate plasmids maintained in a *recA* genetic background suggested the behavior of a transposon. These results also suggested that the recombination-independent mobilization occurred as a function of transdiploid transposon homology as reported previously for Tn*1* (30).

Tn*904* transposition. We designated the putative Sm^r transposon as Tn*904*. Tn*904* was able to transpose to plasmids besides pRO161, even in the *recA* bacterial strain PAO2003 (Table 3). Mating 1 in Table 3 shows the strategy used for the selection of an RP1::Tn*904* plasmid (later designated pRO169). Since plasmid RP1 freely transfers from pseudomonads to *E. coli* (33), whereas pMG1 does not (35), transfer of the Sm^r determinant to an *E. coli* recipient reflects the transposition of Tn*904* to RP1 in a *recA* background. In the next three matings, plasmid pRO161::Tn*904* (pRO165, described earlier) was used as a transposon donor to *P. aeruginosa* sex factor FP2 (18), to a broad-host-range R-plasmid, R388 (40), and to *Pseudomonas putida* sex factor K (18). Since pRO165 is Tra$^-$, the mobilization of Sm^r could reflect either the transposition of Tn*904* into the Tra$^+$ companion plasmid of the aggregate or the mobilization of pRO165 by the Tra$^+$ plasmid. The latter possibility was ruled out by the failure to cotransfer all of the pRO165 markers (i.e., Cb^r, Tc^r, and Sm^r): in the four matings in Table 3, transconjugants only acquired the Sm^r marker when selecting for that resistance. Therefore, Tn*904* transposed to plasmids unrelated to RP1. With transposition to FP2 (mating 2 in Table 3), selection for Cb^r was done also as an internal control to compare the transposition frequency of Tn*1*, located on plasmid pRO165, with that of Tn*904*: they were equivalent. In mating 4 in Table 3, it was necessary to do a triparental mating since *P. putida* sex factor K encodes no selectable markers. Typical transconjugants were selected from the four matings in Table 3, and the transconjugant plasmids were designated pRO272 (FP2::Tn*904*), pRO232 (R388::Tn*904*), and pRO302 (K::Tn*904*). These plasmids freely retransferred in serial matings, and, in the cases of pRO272 and pRO302, their chromosome mobilization abilities were unimpaired (data not shown).

We also transposed Tn*904* to pRO1600, a small multicopy cryptic plasmid (31). For this, pRO169 (RP1::Tn*904*) was mated into a *P. aeruginosa* strain containing pRO1600. DNA was then prepared from the aggregate-plasmid-containing strain and used to transform strain PAO2 with selection for the acquisition of Sm^r. Most of the transformants obtained were only Sm^r and thus putatively contained pRO1600::Tn*904*. Two pRO1600::Tn*904* transformants designated pRO1742 and pRO1743 were used for physical mapping of the transposon (see below).

In analogous experiments, the *recA*-independent transposition of Tn*904* was shown by introducing both plasmids pRO169 and pBR322 into the *recA E. coli* strain AE85. DNA was then prepared for transformation and selection (Sm^r) of pBR322::Tn*904*. Along with Sm^r, transformants also acquired either Cb^r or Tc^r alone or both Cb^r and Tc^r together. Thus, for some transformants, Tn*904* had inserted into regions of pBR322 encoding either Cb^r or Tc^r. Three such transformants were designated AE85(pRO1744) (Sm^r,

TABLE 3. Transposition of Tn*904* to other plasmids

Mating	Aggregate plasmid donor	Recipient	Selection[a]	Transfer frequency per donor[b]
1	PAO2003(RP1/pMG1)	J53	Sm^r (Tn*904*)	10^{-7}
2	PAO2003(FP2/pRO165)	PAO2	Cb^r (Tn*1*)	10^{-7}
			Sm^r (Tn*904*)	10^{-7}
3	ED8654(R388/pRO165)[c]	ROE531	Sm^r (Tn*904*)	10^{-7}
4	Triparental mating: AC541 × PAO2003(pRO165) × PAO2	PAO2	Sm^r (Tn*904*)	Unknown

[a] See the text.

[b] This transfer frequency reflects the product of the transposition frequency and the transfer frequency of the target, transfer-proficient plasmids.

[c] This strain was constructed by transforming pRO165 into ED8654 (31) and subsequently adding plasmid R388 by conjugal mating.

Tc^s, Cb^r), AE85(pRO1745) (Sm^r, Tc^r, Cb^s), and AE85(pRO1746) (Sm^r, Tc^r, Cb^r). Plasmid DNA of pBR322::Tn*904* derivatives is shown in Fig. 1. Lane A shows DNA prepared from the plasmid aggregate, pRO169/pBR322, used later to select pRO1744 and pRO1745 transformants. The plasmid profile included not only plasmid pBR322 (bottom of the lane) and plasmid pRO169 (top of the lane), but also a band intermediate to the transposon donor, pRO169, and the target plasmid, pBR322, whose size corresponded to plasmids pRO1744 and pRO1745 (lanes B and D). The intermediate-size plasmid in lane A was associated with the maintenance of the bacteria on medium which contained streptomycin (25 μg/ml) (data not shown).

Expression of streptomycin resistance. Expression of streptomycin resistance by Tn*904* appeared to vary with gene dosage in *E. coli* but not in *P. aeruginosa*. It has been shown previously that RP1 (identical to RK2 [4]) is maintained as a low-copy-number plasmid (9). Plasmid pBR322 (replicator derived from plasmid ColE1 [3]), on the other hand, is a high-copy-number plasmid, with 28 to 40 copies per chromosome (9). Thus, copy number effects can be determined when comparing pRO169 (RP1::Tn*904*) and pBR322::Tn*904* plasmids. The results of MIC of antibiotic determinations on bacterial strains with and without Tn*904*-plasmids are shown in Table 4. There was no influence of plasmid pBR322 on the intrinsic resistance of strain AE85 to streptomycin. However, there was less expression of Sm^r for AE85(pRO169) than for PAO2(pRO169). Plasmid pRO169 (RP1::Tn*904*), a low-copy-number plasmid, conferred only a low level of Sm^r to *E. coli* AE85, whereas high-copy-number plasmid pRO1744 (pBR322::Tn*904*) conferred a significantly higher level of streptomycin resistance to AE85. Thus, the expression of resistance correlated with the plasmid copy number per cell (i.e., gene dosage). In *P. aeruginosa*, however, the level of Sm^r expression for limited-copy-number plasmid pMG1 or PRO169 was indistinguishable from multicopy plasmid pRO1742 (pRO1600::Tn*904*). Therefore, growth of *E. coli* AE85 on streptomycin selected for pBR322::Tn*904*-containing cells which had greater Sm^r, and this explains the harvest of large numbers of pBR322::Tn*904* molecules in DNA from an *E. coli* strain with pRO169 (the transposon donor) and pBR322 (the transposon multicopy target plasmid) grown on streptomycin medium (Fig. 1).

Inverted repeat sequences of Tn*904*. Heteroduplex analysis showed that inverted repeat sequences existed at the termini of Tn*904*. This heteroduplex analysis was facilitated by finding that Tn*904* had no cleavage sites for restriction endonuclease *Eco*RI, *Hin*dIII, *Sal*I, *Pvu*II, or *Bam*HI (data not shown). For heteroduplex studies, plasmid pRO1746 (pBR322::Tn*904*) was cleaved with restriction endonuclease *Bam*HI

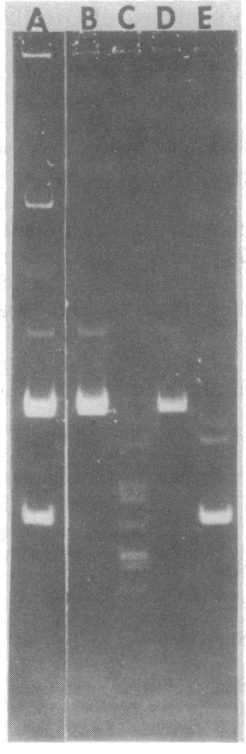

FIG. 1. Agarose gel electrophoresis of Tn*904* plasmids. DNA was prepared and electrophoresed as described in the text. Lane A, Plasmid aggregate pRO169/pBR322; lane B, pRO1744; lane C, multiple-size-standard plasmids of *E. coli* V517; lane D, pRO1745; lane E, pBR322.

TABLE 4. MIC determinations for plasmids with Tn*904*

Bacterial strain	MIC per trial[a]: 1	2
E. coli		
AE85	<8	<8
AE85(pBR322)	<8	<8
AE85(pRO169)	32	64
AE85(pRO1744)	>1,000	>1,000
P. aeruginosa		
PAO2	32	32
PAO2(pMG1)	>1,000	>1,000
PAO2(pRO169)	>1,000	>1,000
PAO2(pRO1742)	>1,000	>1,000

[a] MICs (micrograms per milliliter) were determined as described in the text.

and heteroduplexed with *Bam*HI-cleaved pBR322. A typical heteroduplex figure, as observed with electron microscopy, is shown in Fig. 2. A number of molecules displaying stem-loop structures, as well as open circular, size standard molecules, were photographed and measured (Table 5). The stem structure of the heteroduplex consisted of a region of approximately 124 bp, and the stem resembled that expected for repeated sequences that are in reverse orientation (Table 5). Thus, Tn*904* is similar in this respect to transposons with short inverted repeat sequences, e.g., Tn*3* (23). Heteroduplex determinations also showed the location of the Tn*904* insertion on the pBR322 molecule, since the distance from the stem structure to the end of the molecule cleaved with *Bam*HI could also be measured (see below).

Location of the Tn*904* Sm^r determinant. A physical map of plasmid pRO1746, used for heteroduplex studies, is displayed at the top of Fig. 3. The plasmid encodes Cb^r, Tc^r, and Sm^r, and this phenotype is consistent with the site of the Tn*904* insertion determined by heteroduplex analysis. The termini of Tn*904*, mapped in relation to cleavage sites within the transposon, are shown in Fig. 3 as horizontal arrows. Plasmid pRO1744 (Fig. 3) shows a Tn*904* transposition into pBR322 in reverse orientation and a different location, compared with pRO1746, which has insertionally inactivated the Tc^r region of pBR322.

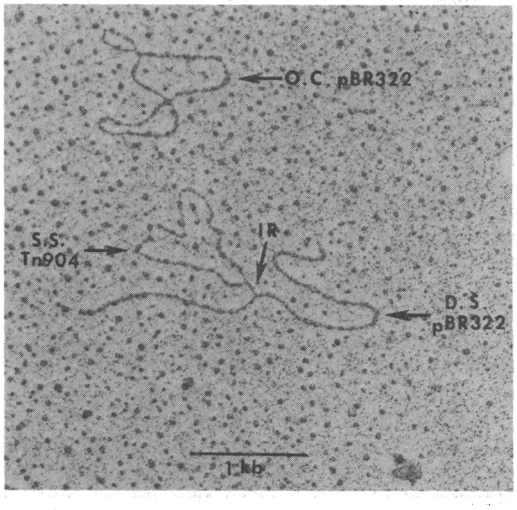

FIG. 2. Electron micrograph of a heteroduplex molecule formed between pBR322 and pRO1746. Samples were prepared and processed as described in the text. Designations: S.S. Tn*904*, single-stranded loop corresponding to Tn*904*; D.S. pBR322, double-stranded segments corresponding to pBR322; O.C. pBR322, open circular pBR322; IR, double-stranded, inverted repeat sequences at the point of insertion of Tn*904* into pBR322; kb, kilobase pairs.

TABLE 5. Measurement of heteroduplex molecules formed between pRO1746 and pBR322[a]

Molecule description	No. measured	Molecule size	
		Mean (bp)	±SD (bp)
pBR322	20	4,362	241
pBR322/pRO1746 heteroduplex stem	22	124	27
pBR322/pRO1746 shortest arm	16	1,530	110

[a] Preparation of the samples and measurements were done as described in the text.

To determine the region of Tn*904* associated with Sm^r, we deleted the 3.3-kb *Sst*I fragment of pRO1744: after restriction endonuclease digestion, the fragment was purified by agarose gel electrophoresis and electroelution and then ligated and transformed into ED8654. Transformant plasmids, typified by pRO1747 (Fig. 3), had deleted most of the internal region of Tn*904* and yet still encoded Sm^r, presumably within the left arm of Tn*904* (the right arm contained only 250 bp between the *Sst*I cleavage site and the right-hand inverted repeat).

An *Ava*I restriction endonuclease cleavage site was located in the left arm of *Sst*I-deleted Tn*904* (pRO1747, Fig. 3), perhaps within the structural gene for Sm^r. To construct a plasmid with only that single *Ava*I cleavage site, we first derived vector plasmid pRO1723 from our *Pseudomonas* cloning vector pRO1614 (31). Plasmid pRO1723 contained no *Ava*I sites (Fig. 4; the pBR322 replicator region and part of the Tc^r determinant of pBR322 were removed from pRO1614 by digestion with *Bgl*I). To clone the region of pRO1747 which contained the Sm^r determinant into vector pRO1723, both pRO1747 and pRO1723 were cleaved with *Bam*HI and *Pst*I. Fragments were annealed, ligated, and transformed into PA02. This strategy precluded the recovery of the parental Sm^r plasmid pRO1747, since its replicator (pBR322) is nonfunctional in *Pseudomonas* bacteria (31). One transformant plasmid from this experiment, designated pRO1748, is shown at the bottom of Fig. 4. In pRO1748, the *Bam*HI-*Pst*I region of pRO1723 was replaced with the *Bam*HI-*Pst*I region (Sm^r) from pRO1747.

Plasmid pRO1748 was used to clone fragments of *P. aeruginosa* chromosomal DNA; *Ava*I-cleaved chromosome and pRO1748 DNAs were mixed, ligated, and transformed into strain PA02 with selection for Cb^r. Of 300 such transformants, 25 were streptomycin sensitive (Sm^s) and thus presumably had chromosomal DNA incorporated into their *Ava*I cleavage site. Several of

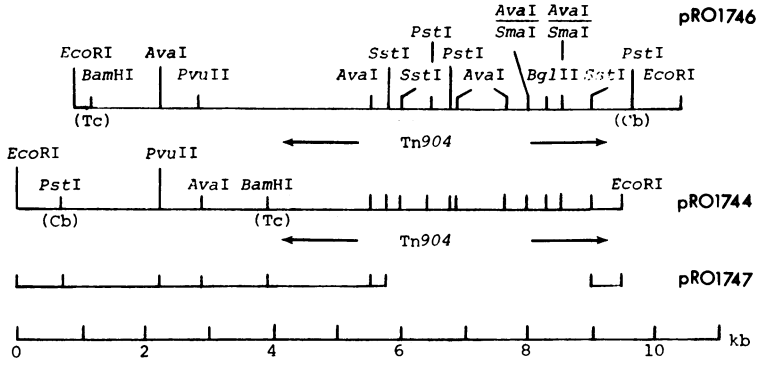

FIG. 3. Restriction endonuclease map of plasmids pRO1746, pRO1744, and pRO1747. The horizontal arrows point to the distal ends of Tn*904*.

the Sm^s transformants were examined and found to contain plasmid DNAs that were clearly larger than the vector (data not shown). When two such Sm^s transformant plasmids were cleaved with *Ava*I, ligated, and transformed into PA02 with selection for Cb^r, most transformants were now also Sm^r. Thus, after removal of the cloned chromosomal DNA from the *Ava*I cleavage site of the Tn*904*-derived fragment, Sm^r was reconstituted. Thus, the *Ava*I site is within Sm^r.

DISCUSSION

We have reported previously transposon-mediated plasmid donor ability (Pma) of a nonconjugal plasmid, pRO161 (30, 32). We showed the association of Pma with the transposition of Tn*1* from pRO161 to *P. aeruginosa* sex factor FP2; the resulting FP2::Tn*1* mobilized pRO161. In those reports, we described transconjugants from matings whose phenotypes resembled the result of analogous mobilizations with different plasmids as reported by Crisona et al. (6). These phenotypes included the occurrence of unstable conintegrate plasmids formed from the sex factor and the Tra$^-$ plasmid of the aggregate donor, as well as the dissociation products of the cointegrate, i.e., the sex factor and the Tra$^-$ plasmids. Our previous results, then, with Tn*1*-mediated Pma, resembled those of Crisona et al. with Tn*3* (6). In the present work, however, we have not observed cointegrate plasmids occurring as a consequence of Tn*904*-mediated Pma, although in this instance, Tn*904*-facilitated cointegration of the plasmids may be so unstable as to preclude their detection.

A recent report by Van Gijsegem and Toussaint described the formation of R-prime plasmids in members of the family *Enterobacteriaceae* which is facilitated by the transposable element mini-Mu (43). These authors showed the formation of RP4 primes, which include hetero-DNA (derived in this case from the chromosome) whose termini are defined by mini-Mu (43) in a direct repeat orientation. This structure may be analogous to the cointegrate intermediate proposed by us (30, 32) and others (6) for transposon-mediated Pma.

There have been other reports in which Tn*904* may have functioned in illegitimate recombination. Jacoby et al. observed that plasmids of the IncP-1 and IncP-2 groups formed recombinants in a recombination-deficient (*recA*) host, and one example that they cited was a recombination between plasmids RP1 and pMG1 resulting in the acquisition of Sm^r by RP1 (20). They suggested that a mechanism of insertion sequences on IncP-1 and IncP-2 plasmids was responsible (20). Sm^r, presumably including Tn*904*, was used for labeling the octopine-Ti plasmid of *Agrobacterium tumefaciens* (22, 36). Ooms et al. (36) found that the acquisition of Sm^r resulted in variable increases in the size of the target Ti plasmid. The reason(s) for this variable increase is not clear, although Ooms et al. suggest the possible amplification of the DNA sequence encoding Sm^r during selection for high levels of streptomycin resistance (2 mg/ml). We have not seen the amplification of Tn*904* per se and would instead expect that such variation in size is due to other causes; e.g., transposing DNA from their donor plasmid may have included a larger, unstable region than just Tn*904* itself.

Heteroduplex analysis of pRO1746 with pBR322 showed that Tn*904* is flanked by inverted repeat sequences of about 124 bp. However, Heffron et al. (17) suggest that this should be taken only as a maximum value since various estimates of the size of the Tn*A* inverted repeats gave results of 140 bp (17, 25), whereas DNA sequence analysis showed that the repeats were only 38 bp in length (16). However, our present studies do show that Tn*904* resembles the group of transposable elements which have short distal repeated DNA sequence termini.

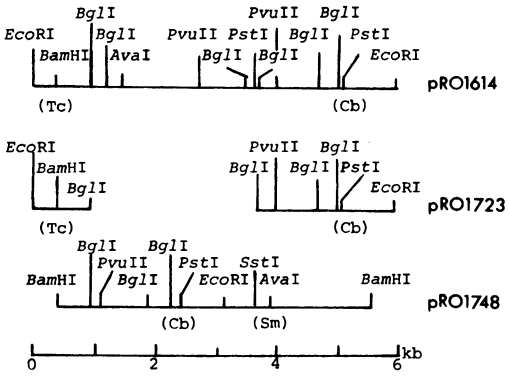

FIG. 4. Restriction endonuclease maps showing the derivation of cloning vector pRO1723 and recombinant plasmid pRO1748 with the Sm^r determinant of Tn*904*. DNA was prepared and digested as described in the text.

We were able to associate a region of Tn*904* with its Sm^r determinant. This was facilitated by the development of cloning vector pRO1723. When recombinant plasmids were constructed in vitro which contained the region of Tn*904* encoding Sm^r, hetero-DNA inserted into an *Ava*I restriction endonuclease cleavage site resulted in the loss of Sm^r. Removal of the inserted DNA resulted in the reconstitution of the Sm^r determinant. Thus, the Sm^r determinant from Tn*904* may have utility for the development of cloning vectors useful for studies with *Pseudomonas* bacteria which characteristically show high intrinsic resistances to other antibiotics such as ampicillin, carbenicillin, chloramphenicol, or kanamycin. Our estimate of the size of Tn*904* (5.2 kb) is derived from restriction endonuclease digests of the small plasmid pRO1742 and other pRO1600::Tn*904* isolates. A similar 5.2-kb size increase was also observed when pBR322::Tn*904* plasmids were examined. In some instances, we observed deletions in plasmids pRO1600, RP1, FP2, and R388 after their acquisition of Tn*904* (data not shown).

Two aminoglycoside-modifying enzymes, streptomycin-spectinomycin adenylyltransferase and streptomycin phosphotransferase, have been shown to be associated with Sm^r encoded by plasmids (2); only the latter has been shown for *Pseudomonas* bacteria (24). Examples of both types of activities have been associated with transposons. Barth et al. (1) have described Tn*7*, which encodes Sm^r, spectinomycin resistance (Sp^r) (i.e., adenylyltransferase activity [2]), and trimethoprim resistance. Kopecko and Cohen (25) described Tn*4* which also encodes Sm^r and Sp^r. Hedges et al. (13) have described the properties of a transposon conferring resistance to penicillins and streptomycin that they designated TAβ. This transposon, which encodes phosphotransferase (13), was derived from R938, a broad-host-range P incompatibility group plasmid (14). More recently, Rubens et al. (39) have described Tn*1696*, originally isolated in *Pseudomonas* bacteria, which encodes multiple resistances to aminoglycoside antibiotics but not Sp^r; therefore, its Sm^r also likely derives from phosphotransferase activity. The transposon described in our study, Tn*904*, also lacks Sp^r (unpublished data) and therefore likely encodes phosphotransferase activity (2). However, Tn*904* differs from Tn*1696* in that it only encodes Sm^r and not resistance to other antibiotics or mercuric ions.

Nordström et al. (29) and later Uhlin and Nordström (42) showed that there is a linear correlation between enzyme synthesis and the number of gene copies and also a linear correlation between resistance and enzyme production (29). This was demonstrated for copy number mutants of R-plasmid R1*drd*-19, where the frequency of conjugal transfer, resistance to antibiotics (ampicillin, chloramphenicol, and streptomycin), and the production of the corresponding enzymes were shown to correlate with the copy number. In our present work, a similar correlation was observed for *E. coli* strains when the MICs for Sm^r were compared for pRO169 (RP1::Tn*904*), a low-copy-number plasmid (9), and pRO1744 (pBR322::Tn*904*), a multicopy plasmid (3, 9). However, in contrast to *E. coli*, the relationship between the copy number and the level of Sm^r did not occur in *Pseudomonas* bacteria.

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