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Evolution and Utility of a *Pseudomonas aeruginosa* Drug Resistance Factor

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We describe the addition to the *Pseudomonas aeruginosa* sex factor, FP2, of carbenicillin resistance encoded by the RP1 plasmid. This occurred in a stepwise manner as detected by variations in the characteristics of the FP2-RP1 plasmid aggregate. The addition of the carbenicillin resistance marker to FP2 facilitates estimates of FP2 transfer. Transfer frequencies for the presumed cointegrate plasmid, using carbenicillin selection, approached 10^{-1} per donor bacterium. The chromosomal mobilization properties of the derived plasmid, designated pR0271, resembled those of the progenitor plasmid FP2. Plasmid pR0271 was also observed to mobilize a nontransmissible drug resistance plasmid sharing genetic homology at frequencies corresponding to those observed for chromosomal markers proximal to the origin of transfer.

Conjugal fertility for *Pseudomonas aeruginosa* has been studied extensively and shown to be sex factor dependent. The *Pseudomonas* sex factor FP2 has been used to map a considerable region of the *Pseudomonas* chromosome (for reviews, see references 4, 5, and 8). FP2 also encodes resistance to mercuric ions in addition to mobilizing the *P. aeruginosa* strain PAO or strain PAT chromosome (11). However, estimates of the bacterial host range for FP2 are dependent on the expression of its mercury-resistant phenotype by exconjugants containing FP2. Our experience has been that selection for mercury resistance leads to low recovery of FP2 in exconjugants, approximating results for the scoring of nonselected mercury resistance among chromosomal recombinants. Accordingly, we envisaged the possibility of adding another convenient selective marker to FP2, which would facilitate a more accurate estimate of FP2 transfer independent of chromosomal mobilization. This report describes the process by which this was accomplished and confirms the retention of phenotypic properties formerly associated with FP2. In addition, we suggest the utility of the derived sex factor now encoding carbenicillin resistance to further studies on *Pseudomonas* conjugal fertility.

MATERIALS AND METHODS

Bacterial and phage strains. The bacterial and phage strains used are listed in Table 1. Their relevant characteristics and sources are also listed.

Media. Complex medium TN contained (per liter): tryptone, 5 g; dextrose, 1 g; yeast extract, 2.5 g; and NaCl, 8.5 g. TN medium was solidified by the addition of 20 g of agar per liter. When nutritional selection against auxotrophic donors was done, the minimal medium described previously (12) was used. Amino acid, purine, or pyrimidine requirements of recipients were satisfied by the addition of these components to 20 μg/ml. Antibiotic supplements are as described in the tables.

Mating and testing of exoconjugants. Conjugal pairing of donor and recipient suspensions was essentially as detailed previously (13), except that a 2-h mating prior to plating on selective medium was used. Exceptions to this procedure are described in the text. Purification of exconjugants was done on solid medium identical to that used for their primary isolation. Colonies were picked into liquid suspension and streaked out for single-colony isolation. Incompatibility and entry exclusion were determined using appropriate selective medium as described earlier (13).

Transductional analysis. Phage was grown on bacterial isolates to be tested in TN soft-agar overlays containing 7.5 g of agar per liter. Phage was harvested from emulsified overlays and purified by centrifugation of debris followed by filtration and titering on appropriate indicator bacteria. For transduction, exponential-phase cells were suspended in buffer to a density of approximately 10^8 cells per ml and mixed with an equal volume of phage dilution, resulting in a multiplicity of input of 0.1 to 1.0 viable phage per bacterium. The mixture was incubated for 15 min at 37 C followed by cooling in an ice bath, centrifugation and suspension of the cell pellet in buffer, and plating on selective medium.

RESULTS

Formation of the hybrid plasmid. We noted previously that *Pseudomonas* sex factor FP2 apparently decreased the donor frequency for

TABLE 1. *Bacteria and phages used*

Strain	Characteristic
P. aeruginosa[a]	
PAO170	*leu-38*, FP2+
PAO2	*ser-2*
PAO38	*leu-38*
PAO67	*his-67*
PAO222	*met-28, trp-6, lys-12, his-4, pro-82, ilv-225*
PAO222.1	Same as PAO222 but resistant to 500 μg of streptomycin per ml
PAO833.1	*pur-66, his-151, pyr-21, thi-1, lys-53, ese-14;* received as PAO833, made resistant to 500 μg of streptomycin per ml
Pu21 (pMG1)[b]	*ilvB112, leu-1, str-1*, RmR, pMG1 plasmid donor
P. fluorescens[c]	
PFO151	Prototroph
PFO154 (RP1)	*met-4*, RP1 plasmid donor
P. putida[c]	
PPO13	Prototroph, ATCC 12633
A. calcoaceticus[c]	
ACJ1	*try-1*
Phage[c]	
PRR1	P plasmid incompatibility group-specific ribonucleic acid phage (15)
PRD1	P, N, W plasmid incompatibility group-specific deoxyribonucleic acid phage (14).

[a] *P. aeruginosa* PAO strains were provided by Bruce Holloway, Monash University, Clayton, Victoria Australia.

[b] *P. aeruginosa* Pu21 (pMG1) was provided by George Jacoby, Massachusetts General Hospital, Boston, Mass. It is chromosomally resistant to streptomycin (SmR) and rifamycin (RmR).

[c] These bacterical strains and plasmid-specific phages were described previously (12, 14, 15).

RP1 from FP2/RP1 double-plasmid-containing strains (RP1 was formerly designated R1822 by our laboratory [12, 14]). Subsequently, some of the manifest diminution of apparent RP1 donor activity has been shown to reflect loss of RP1 antibiotic resistance markers used for selection of RP1 exconjugants as influenced by the presence of FP2 (unpublished data).

In view of the possible instability of RP1 determinants in the presence of FP2, we considered that occasional recombination might be detected between FP2 and RP1. We tested this possibility in *P. aeruginosa* strain PAO, rather than the PAT strain used previously (12), in view of the collection of chromosomal mutants available of the PAO series. For this work, we mated RP1 from *P. fluorescens* PF0154(RP1) into PAO170 recipients, which contained FP2. PAO170 exconjugants containing RP1 were selected for the acquisition of carbenicillin resistance (CbR) encoded by RP1. All exconjugants from this mating retained the resistance to $HgCl_2$ specified by FP2. However, some exconjugants were found to be insensitive to the RP1 pilus phage, PRR1 (15), and to PRD1, the RP1-encoded somatic receptor-adsorbing phage (14). One of these isolates, designated PAO170-(RP1.3), was found unable to act as a conjugal donor for RP1 in 2-h mating tests but was normal for FP2 chromosomal mobilization. On this basis we presumed that RP1.3 was transfer defective (Tra^- phenotype).

We next determined whether FP2 mobilization of RP1.3 could occur during overnight growth of a culture in TN broth. For this, broth was appropriately inoculated with PAO170-(RP1.3) and PAO67 and grown to early stationary phase after static incubation overnight at 37 C. The culture was then sampled and plated on minimal medium containing histidine and carbenicillin (500 μg/ml). Three histidine-requiring, carbenicillin-, tetracycline-, and kanamycin-resistant colonies were obtained and found to be mercury resistant. These were designated PAO67(FP2/RP1.31, -.32, -.33). We tentatively viewed these as FP2-mobilized RP1.3 plasmid-containing exconjugants and accordingly tested them for their RP1 plasmid donor activity using PAO2 recipient bacteria. The results of this mating are shown in Table 2. The FP2/RP1.31 donor strain clearly shows mobilization of carbenicillin resistance in a 2-h mating, unlike the PAO170(RP1.3) isolate described previously. It was also normal for the

mobilization of the chromosomal determinant *ser-2*. The FP2/RP1.32 donor, on the other hand, did not show detectable RP1.3 mobilization (less than 10^{-8} per donor) although it also was normal for *ser-2* mobilization. The third donor strain, FP2/RP1.33, showed mobilization of carbenicillin resistance but a reduced mobilization frequency for the chromosomal determinant *ser-2*. From the behavior of these three donors it is apparent that the properties of FP2 and/or resistance determinants derived from RP1 may be altered in the process of mobilization attendant to strain construction.

Eight PAO2 Ser$^+$ exconjugants were purified on selective medium and further tested for the RP1-encoded nonselected markers CbR, tetracycline resistance (TcR), and kanamycin resistance (KmR) and FP2-encoded mercury resistance (MeR), as well as their ability to mobilize chromosome into another PAO female recipient, PAO833.1. The results (Table 3) show that not all PAO2 Ser$^+$ exconjugants had received all of the FP2 or RP1.3 donor markers. Isolates 4, 6, and 8 were only chromosomal recombinants and had not acquired plasmid determinants. Isolate 7 received only RP1-encoded KmR but not other RP1 determinants or FP2. Isolates 1, 2 and 5, on the other hand, received FP2-encoded MeR and one or more, but not all, of the RP1 determinants originally present in PAO67(FP2/RP1.31). Their FP2-mediated sex factor activity was also confirmed in subsequent donor testing wherein mobilization of *pyr-21*, *his-151*, and *lys-53* chromosomal determinants was demonstrated. The variable mobilization of RP1.3 antibiotic resistance determinants into PAO2 raised the possibility that the previous donor population, PAO67(FP2/RP1.31), either contained a heterogenous population of co-integrate FP2-RP1-derived determinants or contained separate FP2 and RP1.3 plasmids with variable FP2 mobilization of the individual RP1.3 determinants.

TABLE 2. *FP2 mobilization of chromosomal and plasmid markers*

Mating	Exconjugants/donor[a]	
	ser-2$^+$	*ser-2*$^+$ CbR
PAO67 (FP2/RP1.31) × PAO2	3×10^{-5}	7×10^{-6}
PAO67 (FP2/RP1.32) × PAO2	1×10^{-5}	0
PAO67 (FP2/RP1.33) × PAO2	4×10^{-7}	2×10^{-7}

[a] Nutritional selection against the donor was done on appropriately supplemented minimal medium used previously (12). Zero denotes less than 10^{-8} exconjugants per donor.

To gain a further estimate of the co-integrate or aggregate nature (2) of the PAO67(FP2/RP1.31) plasmid markers, we prepared a phage F116-transducing lysate (6) on this strain and selected for the transduction of RP1 drug resistance determinants. We observed RP1.31 transduction but no cotransduction of RP1 markers with the mercury resistance encoded by FP2. Therefore, it seems likely that PAO67(FP2/RP1.31) contains separate FP2- and RP1-derived plasmids. It follows from this, then, that some homology between the two plasmids may exist, accounting for the relatively enhanced mobilization from PAO67 (FP2/RP1.31) when compared with PAO170(RP1.3). The establishment of homology between FP2 and RP1.3 may have occurred during mobilization of RP1.3 from PAO170(RP1.3) to PAO67.

We next attempted to obtain from PAO67(FP2/RP1.31) a co-integrate plasmid complex exhibiting enhanced mobilization fre-

TABLE 3. *Properties of PAO2 ser-2*$^+$ *exconjugants (from RP1.31 mating)*

Isolate no.	Plasmid marker[a]				Donor test × PAO833.1 with selection for:[b]	
	CbR	TcR	KmR	MeR	*pyr-21*$^+$	*his-151*$^+$ *lys-53*$^+$
1	+	−	+	+	1×10^{-6}	2.1×10^{-5}
2	+	−	−	+	1×10^{-6}	2.1×10^{-5}
3	−	−	+	−	0	0
4	−	−	−	−	0	0
5	+	−	−	+	1×10^{-6}	2.3×10^{-5}
6	−	−	−	−	0	0
7	−	−	+	−	0	0
8	−	−	−	−	0	0

[a] Scored here and in other tests as the ability to form colonies on solid medium supplemented with antibiotics as follows: TN containing 500 μg of carbenicillin per ml; TN containing 50 μg of tetracycline per ml; minimal medium containing 0.5 % Casamino Acids (Difco) and 200 μg of kanamycin per ml. MeR was scored as the ability to grow on solid brain heart infusion medium (Difco) containing 60 μg of $HgCl_2$ per ml.

[b] Selection was on minimal medium containing appropriate supplements. Zero exconjugants denotes mating frequencies of less than 10^{-8} per donor.

quencies for either chromosomal determinants or a drug resistance marker. This seemed feasible in view of the variable behavior exhibited for donor strains shown in Table 2. We used PAO67(FP2/RP1.31) as the donor of a possible co-integrate plasmid and PAO38 as the recipient. In this experiment, donor and recipient cultures were paired for 2 h and plated on leucine-supplemented minimal medium containing carbenicillin. This resulted in the recovery of carbenicillin-resistant PAO38 exconjugants at a frequency of 7×10^{-2} per donor. This mating frequency, using selection for the acquisition of carbenicillin resistance, was higher than observed earlier, and from this we conclude that periodic transfer of the donor strain on selective medium containing carbenicillin resulted in the donor frequency improvement.

Sixteen carbenicillin-resistant PAO38 exconjugants were purified by serial restreaking and single-colony isolation on selective medium and finally tested for the TcR, KmR, and MeR determinants shown by the donor PAO67(FP2/RP1.31). All sixteen isolates were mercury resistant but tetracycline and kanamycin sensitive. This result is consistent with the transposition of the CbR determinant from RP1 to the *Pseudomonas* sex factor, FP2, in a manner analogous to the behavior of the CbR determinant of RP4 reported by Hedges and Jacob (3).

Chromosomal mobilization. One of the foregoing mercury- and carbenicillin-resistant exconjugants was designated PAO38(pRO271), and its sex factor activity was compared with FP2 (Table 4). These results show that pRO271 gave one order of magnitude greater transfer frequency of the *his-67* or MeR determinants. Histidine-independent PAO67 exconjugants were purified and tested for the acquisition of sex factor-linked mercury resistance; 9 of 30 exconjugants derived from PAO170 donors and 10 of 30 derived from pRO271 donors were mercury resistant. Thus, the frequency of sex factor transfer per chromosomal recombinant was similar for FP2 and pRO271. In addition, all exconjugants, when pRO271 was the donor, either selected for mercury resistance or selected for histidine independence and found to be mercury resistant, were also carbenicillin resistant. Similarly, when CbR exconjugants from the pRO271 mating were tested, all were HgR. However, the apparent donor frequency for the CbR determinant is significantly greater than that observed when mercury selection is used. We consider this variance to reflect the rigor of combined mercury-streptomycin selection or, alternatively, delayed expression in exconjugants of mercury resistance. In any case, we observed complete correspondence between the transfer of CbR and MeR determinants, independent of the selection used. We consider this linkage indicative of the co-integrate nature of the pRO271 sex factor-CbR complex.

We next attempted to further establish this possibility by transductional analysis, using *Pseudomonas* phage F116. We were unable to recover either MeR or CbR transductants, although the same phage preparation was normal with respect to the transduction of a chromosomal determinant. This negative result may reflect the large size of the FP2-CbR co-integrate (pRO271) and the inability of phage F116 to accomplish transductional shortening, unlike that shown previously for phage P22 and RP1 plasmid in *Salmonella typhimurium* LT2 (16). This negative result contrasts with transduction of surviving RP1 determinants in the presumed aggregate complex discussed earlier in the initial transductional analysis of drug resistance markers carried by PAO67(FP2/RP1.31).

We next did gradient-of-transmission analysis for chromosomal mobilization mediated by either FP2 in PAO170 or pRO271 in PAO38. We considered that similar gradients of transmission for chromosomal markers would indicate similar origin of transfer for FP2 or pRO271

TABLE 4. *Sex factor activity of FP2 and pRO271*

Mating	Selection[a]	Exconjugants per donor
PAO170 × PAO67	*his-67*$^+$	3×10^{-5}
PAO170 × PAO222.1	MeR	2×10^{-6}
PAO38(pRO271) × PAO67	*his-67*$^+$	1×10^{-4}
	CbR	3×10^{-2}
PAO38(pRO271) × PAO222.1	MeR	2×10^{-5}

[a] Selection for histidine independence was on minimal medium. Selection for mercury-resistant exconjugants (MeR) was on brain heart infusion medium containing 60 μg of $HgCl_2$ and 500 μg of streptomycin per ml. Acquisition of the CbR determinant was selected on minimal medium containing histidine and 500 μg of carbenicillin per ml.

TABLE 5. *Comparison of FP2 and pRO271 chromosomal mobilization*

Mating	Exconjugants/donor for marker[a]		
	his-4$^+$	*met-28*$^+$	*pro-82*$^+$
PAO170 × PAO222	1×10^{-5}	7×10^{-7}	2×10^{-7}
PAO38(pRO271) × PAO222	4×10^{-5}	2×10^{-6}	5×10^{-7}

[a] Time of entry for FP2-mediated conjugation for these markers is: *his-4*, 8 to 10 min; *met-28*, 27 to 30 min; *pro-82*, 45 min (Bruce Holloway, personal communication). Selection was on solid medium appropriately supplemented with amino acids.

chromosomal mobilization. The known entry times for selective markers and the frequencies of exconjugant formation are shown in Table 5. These data show similar frequencies for either FP2- or pRO271-mediated conjugation. When the histidine-, methionine-, or proline-independent exconjugants were tested for nonselected markers, the gradient of transmission shown in Table 6 was obtained. The results in Tables 5 and 6 show similar behavior for the FP2 and pRO271 sex factor activity. However, as indicated in these results and previously in Table 4, the donor activity for pRO271 exceeds that observed for FP2-mediated chromosomal transfer.

We next tested the ability of FP2 and pRO271 to mobilize pRO161, a nontransmissible antibiotic resistance plasmid derived from RP1 and identical to the RP1-S2 plasmid described previously (16). Presumably, pRO271 shares homology with pRO161 by virtue of the common origin of the CbR determinant from RP1. It was thought that shared CbR homology might facilitate mobilization of pRO161 by pRO271. These results are shown in Table 7. The data again indicate the inability of FP2 to mobilize RP1-derived resistance determinants (Table 2). On the other hand, pRO271 significantly mobilized pRO161, and the frequency of exconjugant formation (about 10^{-5} per donor) resembles that for pRO271-mobilized chromosomal determinants. However, although 30% of the exconjugants selected for the acquisition of chromosomal determinants usually contain sex factor FP2 or pRO271, none of 48 PAO222(pRO161) exconjugants showed the acquisition of sex factor as denoted by MeR. Also, when six of the PAO222(pRO161) exconjugants were tested for their donor activity, none was observed to transfer pRO161. This result argues against recombination between pRO271 and pRO161 with loss of the sex factor-linked determinant, MeR, attendant to the mobilization process.

TABLE 7. *Mobilization of the nontransferable RP1 derivative pRO161 by FP2 or pRO271*

Mating	Isolate no.	TcR exconjugants per donor[a]
PAO170(pRO161)[b] × PAO67	1	0
	2	0
	3	0
PAO67(pRO271/pRO161)[c] × PAO222	1	1.1×10^{-5}
	2	1.2×10^{-5}
	3	1.2×10^{-5}

[a] Selection of exconjugants was on amino acid-supplemented minimal medium containing 50 μg of tetracycline per ml.

[b] Plasmid pRO161 is identical to RP1-S2 described previously (16). It was mobilized by plasmid RWP1 (16) from *E. coli* J53(RWP1/pRO161) to *P. aeruginosa* PAO67. Plasmid pRO161 was then transduced from PAO67(pRO161) to PAO170 using phage F116 (17).

[c] Plasmid pRO161 was transformed into PAO67, as described by Lederberg and Cohen (10), using deoxyribonucleic acid prepared from *E. coli* J53(pRO161). pRO271 was then mated into PAO67(pRO161) from PAO38(pRO271).

Incompatibility and entry exclusion of FP2 and pRO271. The foregoing results have focused primarily on sex factor activity or the selective markers occurring on FP2 or RP1. Additionally, other properties intrinsic to plasmid composition might be altered as a result of recombination between FP2 and RP1. Specifically, the incompatibility property of pRO271 may have changed by inclusion of the P replicator. The property of entry exclusion toward related plasmids (in this case, FP2 or a P plasmid) may be different for pRO271 than its progenitor, FP2. Also possible is a perturbation of pRO271 fertility by unrelated *Pseudomonas*

TABLE 6. *Gradient of transmission analysis of FP2- or pRO271-mobilized chromosomal recombinants*

Donor	Selection[a]	Exconjugants (%) scored as:[b]					
		ilv-225$^+$	*his-4*$^+$	*lys-12*$^+$	*met-28*$^+$	*trp-6*$^+$	*pro-82*$^+$
PAO170	*his-4*$^+$	100	*100*	66	18	4	0
	met-28$^+$	40	44	24	*100*	20	8
	pro-82$^+$	60	44	56	36	80	*100*
PAO38(pRO271)	*his-4*$^+$	100	*100*	58	4	0	2
	met-28$^+$	68	48	48	*100*	32	20
	pro-82$^+$	32	24	16	20	44	*100*

[a] Selection was on amino acid-supplemented solid minimal medium.

[b] Exconjugants were purified by streaking for single colonies on the same medium used for primary selection. For testing, a single remote colony was picked onto a medium lacking the amino acid requirement listed. Time of entry for FP2-mediated conjugation for these markers is: *ilv-225*, 3 min; *lys-12*, 19 min; *trp-6*, 32 to 34 min (Bruce Holloway, personal communication). Other marker entry times are listed in Table 5.

plasmids in a manner analogous to our previous observations with the P, N, W, and X incompatibility group plasmids (13).

In an early report describing the *Pseudomonas* fertility system, Holloway and Jennings called attention to the low interfertility of strain L (7), later called strain PAT. In the light of present knowledge, this may reflect an FP2-encoded entry exclusion barrier to FP2 × FP2 mating, resulting in low frequency of conjugal pair formation. Accordingly, we compared pRO271 with FP2 for the detection of a possible alteration of entry exclusion reflecting the addition of the CbR determinant to FP2. These results are shown in Table 8. The matings using PAO170, which contains FP2, as the donor of the chromosomal marker *his-4* clearly indicate that pRO271 has not lost the entry exclusion property associated with FP2. In this case, unless pRO271 perturbs chromosomal recombination, the formation of conjugal pairs is apparently significantly inhibited when pRO271 is present in recipients. The transfer of pRO271 into the PAO170 strain, which contains FP2, is also diminished by three orders of magnitude. This result, however, may reflect the combined effects of entry exclusion on conjugal pairing and subsequent incompatibility in the newly formed exconjugant. Although selection is maintained for the donor plasmid pRO271, it is possible that apparent transfer frequencies are slightly diminished solely by the incompatibility encoded by the resident plasmid in the recipient. We previously reported a situation analogous to this for the P incompatibility group plasmid, RP1, and its derivative lacking entry exclusion, RP1-S2 (13). In any case, however, entry exclusion can be presumed to be expressed significantly by FP2 towards pRO271 as was observed in the reverse situation.

Pseudomonas plasmids RP1 (12) and pMG1 (9) were studied with regard to their entry exclusion and incompatibility properties towards FP2 or pRO271. No evidence was obtained for interactions between pMG1 or RP1 with FP2 or its derivative, pRO271. Evidently, the entry exclusion or incompatibility properties of RP1 were not transposed to FP2 along with the CbR determinant in the formation of the co-integrate, pRO271.

TABLE 8. *Effect of FP2 entry exclusion and incompatibility on P. aeruginosa mating*

Mating	Selection	Exconjugants per donor
PAO170 × PAO222.1	*his-4*+	7×10^{-5}
PAO170 × PAO222.1(pRO271)	*his-4*+	$<2 \times 10^{-8}$
PAO38(pRO271) × PAO222.1	CbR	9×10^{-2}
PAO222.1(pRO271) × PAO170	CbR	2×10^{-5}

[a] Selection was on amino acid-supplemented minimal medium containing 500 μg of carbenicillin per ml for the acquisition of the CbR determinant or appropriately supplemented minimal medium for chromosomal recombinants and 500 μg of streptomycin per ml.

The presence of the CbR selective marker on pRO271 allowed an estimate of host range by bacterial strains possibly unable to express MeR encoded by FP2. Accordingly, PAO38(pRO271) was mated into several genera of disparate properties previously used by us for RP1 host range determinations. These recipient strains included *P. fluorescens* PFO151, *P. putida* PP013, *Escherichia coli* J53, and *Acinetobacter calcoaceticus* ACJ1. All of these strains are capable of expressing RP1-encoded CbR. No transfer was observed. Therefore, one may surmise that FP2-encoded pili present for pRO271 donors do not recognize the cell surface of these strains or that the FP2 replicator is unsuitably matched with the membrane properties and supportive metabolism of these recipients. Alternatively, FP2 may be severely restricted in these hosts. From these results, using the advantage of CbR selection, we conclude that FP2 has a host range significantly narrower than that observed for RP1 (12) or the *Pseudomonas*-specific plasmid, pMG1, reported by Jacoby (9).

DISCUSSION

A situation has been described by Stanisich which parallels our results (18). She has reported the mobilization of MeR by an R-factor from *P. aeruginosa* PAT to PAO. Some of the MeR exconjugants showed cotransduction of R-determinants and MeR. Although in PAT strains a naturally occurring MeR genetic element is thought to exist independent of FP2, this result clearly shows the addition of MeR to an R-factor otherwise unaltered. Our results, on the other hand, appear to reflect the incorporation of an R-determinant into *Pseudomonas* sex factor.

Some features of the data included in our report may correspond to what might be surmised for stages in the evolution of a conjugally transmissable drug resistance factor. Unlike the abrupt inclusion of a new drug resistance determinant into a preexisting R-factor, the development of various plasmids described herein, including pRO271, has been observed to occur in stages with distinctive properties, based on genetic evidence, pertinent to intermediate stages of development. A comment concerning the evolutionary aspects of our findings seems appropriate in view of the correspondence between the observations cited in

this report and those on clinical *Pseudomonas* isolates. These clinical isolates have rarely demonstrated the comobilization of all significant antibiotic resistances irrespective of the selective antibiotic used (unpublished data). In this regard, we usually observe variations in the linkage groups transferred depending on the selection. This and related observations are the subject of another report (in preparation).

The stepwise evolution of plasmids studied in this report seems to have occurred in the following manner. Step one, might be envisaged as a rare recombinational event between FP2 and the transfer-deficient, drug resistance linkage group derived from RP1 which occurred in PAO170. The result of this may have been a transient association between FP2 and the R-determinants, enabling their mobilization into PAO67 during overnight growth. In PA067, however, dissociation of FP2 and the R-determinants may have occurred as indicated by transductional analysis of these exconjugants. Furthermore, this presumed dissociation probably was accompanied by gene duplication resulting in the establishment of genetic homology between the sex factor element, FP2, and the R-determinant plasmid in two of the three isolates studied. Pursuant to this possibility, then, one might expect an improved mobilization frequency for the R-determinants in subsequent matings. This possibility is supported by the data shown in Table 2 for isolates designated PAO67(FP2/RP1.31) and PAO67(FP2/RP1.33). Isolate PAO67(FP2/RP1.32), on the other hand, may have dissociated to the parental genetic elements present in PAO170(RP1.3). At this stage of development, then, the presumed behavior of PAO67(FP2/RP1.31) and PAO67(RP1.33) is analogous to properties reported by Anderson and Natkin (1) for class 2 R-factors of the Δ transfer system whereby, on the basis of P1*kc* transductional analysis, class 2 R-factors are characterized by the location of transfer and resistance determinants on distinct plasmids.

Step two, as it pertains to this report, is illustrated by the behavior and properties of PAO2(FP2/RP1.31) exconjugants derived from the mating in Table 2 and characterized by the data in Table 3. In this instance, the randomly selected PAO2 exconjugants (isolates 1 through 8) showed variations with regard to their sex factor activity and drug resistance phenotype. This may reflect a heterogeneous population of PAO67(FP2/RP1.31) donors used for this mating, or segregation and loss of determinants in the newly formed exconjugants. In either case, however, this result points up the apparent instability of the drug resistance linkage group at this time. The transience of this instability is evidenced by the fact that, when pRO271 was later (9 weeks) isolated from the PAO67(FP2/RP1.31) strain serially transferred during that time, the variation in exconjugants was not seen. This instability also corresponds to our previous report concerning the behavior of the nontransmissible RP1 variant, RP1-S2, in *Salmonella* bacteria attendant to mobilization by the W incompatibility group plasmid, R388 (16). Interestingly, that result and the present observation both showed the loss of the TcR determinant with concurrent mobilization by another plasmid.

Step three seems to be the stable incorporation of surviving drug resistance genes (in this case carbenicillin resistance) into the mobilizing plasmid. This step, then, might be regarded as the final formation by recombination of a stable plasmid with the nonselected maintenance of all its phenotypic traits.

The impact of homology on mobilization is clear from the data shown in Table 7, wherein the mobilization frequency of pRO161 by pRO271 sharing homology for the CbR determinant allowed transfer frequencies approaching those attainable for early chromosomal markers under identical mating conditions (e.g., *his-4* shown in Table 5). This transfer frequency of 10^{-5} for heteroplasmid or chromosomal mobilization is much less than that of pRO271 alone (e.g., 9×10^{-2} in Table 8). The high frequency for the self-transfer of pRO271 may indicate the optimum mating frequency in this system as limited by conjugal pair formation. The lower heteroplasmid or chromosome mobilization frequency may be due to infrequent pairing of the sex factor with the mobilized sequence. From these observations one may also conclude that the disparity between *Pseudomonas* sex factor transfer alone versus the mobilization of other genetic elements would be obviated in *Pseudomonas* by the development of a chromosomally integrated sex factor analogous to *E. coli* Hfr bacteria. Furthermore, if this were accomplished, the potential donor frequency for a *Pseudomonas* "Hfr," then, might be on the order of 10^{-1} per donor for genes proximal to the origin of transfer, as indicated in this report for independent plasmid transfer when selection for the sex factor-encoded CbR determinant was used.

In summary, the addition of transfer-defective plasmids such as pRO161 or the RP1.3 variant of RP1 to undefined genetic backgrounds may, in the future, facilitate the detection and description of sex factor plasmids not

known to encode for assayable functions. This procedure may lead to the occurrence of sex factor-selective marker co-integrate plasmids whose behavior can subsequently be described more conveniently. The efficacy of this approach, however, would depend on the ability of the gene in question to be transposed to sex factor. Some aspects of this report may also be appropriate to the detection of genetic homology between known and uncharacterized drug resistance genes on the basis of mobilization tests. This possibility is illustrated by the mobilization behavior of pRO271 with respect to pRO161 and the requirement for homology between their CbR determinants.

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