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Apparent Fusion of the TOL Plasmid with the R91 Drug Resistance Plasmid in *Pseudomonas aeruginosa*

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Abstract

The TOL catabolic plasmid was shown to be compatible with the R91 drug resistance plasmid. However, the TOL plasmid was extremely unstable in mutant PA03 of *P. aeruginosa*. By selecting for stabilization of the TOL plasmid in PA03 harbouring R91, it was possible to isolate a strain in which markers from both R91 and TOL appeared to exist in a single recombinant plasmid. This plasmid, pND3, encoded resistance to carbenicillin, was able to transfer at the same frequency as the R91 plasmid and encoded the ability to grow on *m*-toluate, *p*-toluate, *m*-xylene, *p*-xylene and toluene. In addition, it was shown to be incompatible with the NAH catabolic plasmid and it could be transferred by transduction. The TOL plasmid could stabilize in PA03 harbouring R91 without fusion with R91, and could stabilize in PA03 in the absence of R91. PA03 harbouring either the recombinant plasmid or the stable TOL plasmid in the absence of R91 could promote bacterial chromosome transfer between mutant derivatives of *P. aeruginosa* strain PA0.

Introduction

The catabolic diversity encountered in the pseudomonads can be accounted for, in part, by the presence of transmissible plasmids which code for the degradation of unusual carbon sources (Chakrabarty 1972; Chakrabarty *et al.* 1973; Dunn and Gunsalus 1973; Rheinwald *et al.* 1973; Williams and Murray 1974; Wong and Dunn 1974; Williams and Worsey 1976). Although little has been reported on the transfer range of these plasmids, it is our experience that the catabolic plasmids have only a limited transfer range and are generally confined within the pseudomonads or closely related organisms. In view of the characteristics which are encoded by the catabolic plasmids, it is apparent that these plasmids could have potential in specific strain construction work. We therefore consider it desirable to increase the transfer frequency, but more particularly the transfer range of these catabolic plasmids. Although a number of approaches are available to achieve this end, we have chosen to attempt the *in vivo* fusion of the catabolic plasmids to drug resistance (R) plasmids. One of the R plasmids chosen for this study was R91 (Lowbury *et al.* 1969), which transfers at a high frequency within the strains of *Pseudomonas aeruginosa* and has been reported to transfer to a strain of *Shigella flexneri* (Chandler and Krishnapillai 1974*a*). In addition, this plasmid can promote chromosome transfer within mutant derivatives of *P. aeruginosa* strain PAT (Stanisich and Holloway 1971).

This paper reports an investigation of the interaction between the TOL catabolic plasmid, which encodes the degradation of the toluates (Williams and Murray 1974; Wong and Dunn 1974) and toluene and xylene (Worsey and Williams 1975), and

the R91 drug resistance plasmid. As a result of this study it has been possible to recombine these two plasmids.

Table 1. Bacterial strains, bacteriophages and plasmids[A]

Strain No.	Genotype	Derivation and/or reference
Bacterial strains		
PA01	prototroph, *chl*2, FP−	Holloway (1969)
PA03	*trp*54, FP−	Holloway (1969)
PA08	*met*28, *ilv*202, *str*1, FP−	Isaac and Holloway (1968)
PA038	*leu*38, *str*7, FP−	Stanisich and Holloway (1969)
PA0381	*leu*38, *str*7, (FP2+)	Stanisich and Holloway (1969)
PA067	*his*67, *ese*2, FP−	Stanisich and Holloway (1969)
PA01264	*trp*54, *str*, *chl*2, (FP39+)	Pemberton and Holloway (1973)
PP1-2	prototroph	Wong and Dunn (1974)
PP1-3	*ben*-1	Wong and Dunn (1974)
PP1-8	*met*-1	Wong and Dunn (1974)
PAr1-6(TOL)	*met*-1, *tra*-1, (*TOL*)	Wong and Dunn (1976)
PP2-4(NAH) (PpG379)	*leu*-1, *tra*-1, (*NAH*)	Dunn and Gunsalus (1973)
Shigella flexneri 4a 25 SM	*nic*, *str*	Chandler and Krishnapillai (1974*a*)

Designation	Description	Reference
Bacteriophages		
G101	Temperate transducing phage of *P. aeruginosa*	Fargie and Holloway (1965)
F116	Temperate transducing phage of *P. aeruginosa*	Holloway and van de Putte (1968)
pf16	Virulent transducing phage of *P. putida*	Gunsalus *et al.* (1968)

Plasmid No.	Alternate designation	Phenotypic markers	Reference
Plasmids			
R91	R9169	Cb^R	Stanisich and Holloway (1971)
TOL	BEN/TOL,M1	mTol$^+$, pTol$^+$, mXyl$^+$, pXyl$^+$, Tel$^+$	Wong and Dunn (1974, 1976)
pND2		mTol$^+$, pTol$^+$, mXyl$^+$, pXyl$^+$, Tel$^+$, Stb	This paper
pND3		Cb^R, mTol$^+$, pTol$^+$, mXyl$^+$, pXyl$^+$, Tel$^+$	This paper
NAH		Nah$^+$, Sal$^+$	Dunn and Gunsalus (1973)

[A] Abbreviations: ben, benzoate; Cb^R resistance to carbenicillin; chl, chloramphenicol; ese, resistance to phage E79; FP−, absence of any known *Pseudomonas* sex factor; FP2+, presence of FP2 sex factor; FP39+, presence of FP39 sex factor; his, histidine; ilv, isoleucine/valine; leu, leucine; met, methionine; Nah, naphthalene; nic, nicotinic acid; PA0, prefix for strains derived from *P. aeruginosa* strain 1; PP1, prefix for strains derived from the *P. putida* prototroph PP1-2; PP2, prefix for strains derived from the *P. putida* prototroph which originally harboured the NAH plasmid (PpG7); PAr1, prefix for strains derived from the *P. arvilla* mt-2 strain which originally harboured the TOL plasmid; Sal, salicylate; Stb, acquired stability; str, streptomycin; Tel, toluene; Tol, toluate; tra, mutation leading to increased transfer frequency of the resident plasmid; trp, tryptophan; Xyl, xylene.

Materials and Methods

The bacterial strains, bacteriophages and plasmids used in this study are listed in Table 1.

References to general procedures can be obtained from a previous paper (Wong and Dunn 1976). References to specific media and techniques are as follows: PAS media (Chakrabarty 1972); VB media (Vogel and Bonner 1956); the technique of mitomycin C curing (Dunn and Gunsalus 1973);

bacterial chromosome transfer by plate mating using auxotrophic contraselection (Stanisich and Holloway 1969) and transduction using the bacteriophages F116 and G101 (Fargie and Holloway 1965; Holloway and van de Putte 1968). Transduction with the bacteriophage pf16 was carried out using a modification of the technique of Gunsalus *et al.* (1968) in that the transducing preparation was irradiated for only 60 s.

Plasmid Transfer by the Plate-mating Method

Both donor and recipient cultures were grown with vigorous aeration, in Luria broth, to titres of approximately 1×10^9 and 5×10^9 cells/ml respectively. Prior to mating, cultures were centrifuged and resuspended in either saline or saline + 10% Luria broth, then 0·1 ml of both the donor (appropriately diluted) and recipient were spread together onto the selection medium.

Plasmid Transfer by the Tube-mating Method

Donors and recipients were grown to late log phase in Luria broth with vigorous aeration to approximately 5×10^9 cells/ml. They were then mixed in the ratio of 1 : 5 donors to recipients, incubated in a stationary position for 2·5 h, vortexed for 1 min, diluted and plated to give between 100 and 300 transconjugants on the selection medium.

Selection Media and Incubation Temperatures for Plasmid Transfer Experiments

When selecting for transfer of plasmids encoding the ability to degrade the toluates all donor strains used were auxotrophs, thus permitting auxotrophic contraselection against the donors. If transferring to prototrophic strains the selection medium was PAS + 10 mM *m*-toluate or 10 mM *p*-toluate. If the recipient was also auxotrophic then that auxotrophic requirement was added to the selection medium. The same principles were applied when selecting for transfer of plasmids encoding resistance to carbenicillin, where the basic selection medium consisted of VB + 250 μg/ml carbenicillin. This carbenicillin concentration was suitable for plasmid transfer within *P. aeruginosa* and to *Sh. flexneri*. However, due to the high natural resistance to carbenicillin of PP1 derivatives, transfer by conjugation or transduction within this strain was difficult. Growth tests, but not plasmid transfer experiments, could be readily conducted with PP1 derivatives, to determine whether they carried plasmid-encoded carbenicillin resistance, by testing on VB plates supplemented with 1 mg/ml carbenicillin.

The optimum growth temperature of PP1 derivatives is 30°C whereas that of PA0 derivatives is 37°C. All plasmid transfer experiments within PP1 or within PA0 strains were carried out at the appropriate optimum temperatures. When selecting for transfer between these two strains all incubation of mating bacteria was performed at 32°C.

Prior to plasmid transfer to PA0 derivatives from non-PA0 strains, the strain-specific restriction was removed by growth of the recipient at 43°C (Holloway 1965).

Unless specifically stated, the plate-mating method was used when selecting for transfer of the ability to grow on the toluates, whereas the tube-mating method was used when selecting for transfer of resistance to carbenicillin.

Spot Test for Plasmid-encoded Catechol meta-*Cleavage Pathway*

Strains were grown on PAS + *m*-toluate or *p*-toluate, then a colony was taken and patched in a 1-cm square onto an agar plate containing 0·2 ml of 0·25 M catechol spread over the plate surface. If the catechol was convered to a yellow intermediate then this was indicative of a functional catechol 2,3-dioxygenase, the initial enzyme of the catechol *meta*-cleavage pathway.

Results

Plasmid Stability in PA03

P. aeruginosa (PA03) grows readily on benzoate, and this substrate is degraded through the catechol *ortho*-cleavage pathway. This strain cannot utilize either *m*-toluate or *p*-toluate as a carbon source; however, a black coloration is observed in the medium if this contains *m*-toluate. This coloration is due, presumably, to the conversion of *m*-toluate to 3-methylcatechol by benzoate 1,2-dioxygenase and subsequent chemical oxidation. The TOL plasmid can therefore be studied in this strain by selecting for growth on either *m*- or *p*-toluate. These compounds are degraded

by the plasmid-encoded catechol *meta*-cleavage pathway (Williams and Murray 1974; Wong and Dunn 1974). The R plasmid R91 expresses resistance to carbenicillin in *P. aeruginosa*, and transconjugants can be selected in this strain by selection for resistance to carbenicillin (Chandler and Krishnapillai 1974*a*).

To study TOL in PA03 the plasmid was transferred, by the plate-mating method, from PAr1-6 (TOL) to PA03. Transconjugants were obtained at a frequency of 5×10^{-5} per donor cell provided the recipient had been grown at 43°C prior to the conjugation. No transconjugants were obtained if the recipient was not grown at 43°C, implying that the TOL plasmid is susceptible to the restriction system of PA03. Twenty transconjugants were purified by single-colony isolation on the selection media, and all were found to grow on *m*-toluate and *p*-toluate, were tryptophan-requiring, were sensitive to the PA0-specific phage, could transfer to other PA0 strains and carried the plasmid-encoded catechol *meta*-cleavage pathway as indicated by detection of catechol 2,3-dioxygenase. One of the transconjugants was chosen for additional studies and called PA03 (TOL).

Preliminary experiments were conducted to determine the stability of TOL in PA03. A single colony from an *m*-toluate-containing plate was inoculated into Luria broth and grown overnight, then plated for single colonies on VB + tryptophan. One hundred and twenty clones were then streaked twice for single colonies on the same medium. Under these conditions there had been no selection for maintenance of TOL. Upon testing, after this procedure, it was found that 57% of the clones had lost the ability to grow on *m*-toluate, illustrating that the plasmid is unstable in PA03. Independently to this experiment, it was noted that during growth and storage of PA03(TOL) on various carbon sources the TOL plasmid could stabilize and thus no longer be readily lost. One of these plasmids was named pND2 and a strain of PA03(pND2) retained for further study.

R91 was transferred from PA08(R91) to PA03 and transconjugants were obtained at a frequency of 3×10^{-1} per donor cell. By testing stability using a technique analogous to that used for TOL, it was determined that all 120 clones had retained the R plasmid. These results demonstrated that R91, unlike TOL, was stable in PA03.

Interactions between TOL and R91

R91 was transferred from PA08(R91) into PA03(TOL) and transconjugants were obtained at a frequency of 6×10^{-1} per donor cell. One hundred and twenty transconjugants were purified by streaking twice for single colonies on the selection medium VB + tryptophan + carbenicillin. Following this procedure it was found that 47% had lost TOL, therefore selection for maintenance of R91 did not appear to markedly influence segregation of TOL.

Two clones harbouring both R91 and TOL, PA03(R91,TOL), were grown in *m*-toluate selecting for maintenance of TOL. This duplicate experiment was carried out in liquid media where viable counts were maintained between 1×10^5 and 1×10^8 cells/ml. Following growth for approximately 50 generations, aliquots of the cultures were plated for single colonies on Luria agar plates. After incubation overnight at 37°C the colonies were replicated onto VB + tryptophan + carbenicillin and onto PAS + *m*-toluate + tryptophan + carbenicillin. Approximately 1000 clones from each experiment were tested and all were found to be resistant to carbenicillin; they had thus retained R91. Therefore selection for maintenance of the catabolic plasmid had not forced the segregation of R91.

An interesting observation was made on the *m*-toluate + carbenicillin test plates. Those clones arising on the plates which had retained TOL could be separated into two distinct colony types. One (*L*-type) formed large colonies while the other (*S*-type) grew more slowly and appeared similar to PA03(TOL). The frequency at which each colony type arose in the duplicate experiments was as follows:

Expt	Colony type (%)	
No.	*L*-type	*S*-type
1	45	55
2	25	75

The results showed considerable variation between duplicates; however, from the nature of the experiment this was not unexpected.

To determine the stability of TOL in the *L*- and *S*-type clones, 12 of each type were tested as follows: all clones were streaked three times selecting for maintenance of only R91, then 10 colonies of each clone from the final streak plate were tested for the presence of TOL. All colonies of the *L*-type retained the ability to grow on *m*-toluate, whereas a degree of segregation of TOL had occurred with all of the *S*-type colonies tested. Additional testing of the *S*-type colonies revealed that they were very similar, and probably the same as the starting culture of PA03(R91,TOL). The *L*-type strains were of considerable interest because of the apparent stabilization of the catabolic plasmid.

One possible explanation for the stability of TOL was that the catabolic plasmid had recombined with R91. If this was the case then it might be possible to obtain evidence for recombination by comparing plasmid transfer and cotransfer frequencies. To test transfer frequencies, six of the *L*-type strains were used as donors and mated with PA038, selecting for transfer of carbenicillin resistance. Ten transconjugants from each cross were purified by streaking twice for single colonies on the selection media, then 10 clones of each transconjugant were tested for coinheritance of the ability to utilize *m*-toluate. The frequency of R91 transfer from PA03(R91) and from PA03(R91,TOL) was also determined. In addition, crosses were performed selecting for transfer of TOL (*m*-toluate utilization) from PA03(TOL) and PA03 (R91,TOL). When selecting for R plasmid transfer the tube-mating method was employed, whereas the plate-mating method was used when selecting for transfer of the catabolic plasmid. The results of these crosses are given in Table 2 and illustrate that independent transfer of TOL is much lower in frequency than transfer of R91. Transfer of resistance to carbenicillin from the six *L*-type clones was similar to that usually obtained for R91 transfer. Since R91 transfer was much more efficient than transfer of TOL, the majority of recipients inheriting R91 would not be expected to coinherit TOL by a mechanism of independent transfer. However, for two of the *L*-type strains, 100% coinheritance of the ability to grow on *m*-toluate and resistance to carbenicillin was observed with each transconjugant tested. To determine the exact nature of TOL stability and coinheritance, one of the cotransferring plasmids was named pND3 and retained for detailed examination.

Characterization of pND3

In this section pND2, the TOL plasmid which had stabilized independently of R91, was used as a control in most experiments. It was not considered worthwhile to use PA03(TOL) because of both the instability of TOL and the frequency at which it stabilized naturally.

(i) *Phenotypic properties of pND3*

PA03(pND3) was able to grow on *m*-toluate, *p*-toluate, *m*-xylene, *p*-xylene and toluene. These are the growth phenotypes which are known to be carried by the parent TOL plasmid. In addition, the pND3 plasmid encoded resistance to carbenicillin, from the R91 parent. On transfer to other PA0 recipients, for example PA038, all these phenotypes were cotransferred.

Table 2. Transfer frequency of R91, and cotransfer of TOL, from the TOL-stabilized L-type strains, using PA038 as recipient

R plasmid transfer experiments were carried out using the tube-mating technique, whereas transconjugants utilizing *m*-toluate were selected using the plate-mating method. Cb^R, selection for transfer of resistance to carbenicillin; $mTol^+$, selection for transfer of the ability to degrade *m*-toluate; NA, not applicable; NT, not tested

Donor	Selection for transfer of (transconjugants/donor cell)		Cotransfer of $mTol^+$ (%)
	Cb^R	$mTol^+$	
PA03(R91)	4×10^{-1}	NA	NA
PA03(TOL)	NA	4×10^{-6}	NA
PA03(R91,TOL)			
S-type	5×10^{-2}	8×10^{-6}	0
L-type, 1	4×10^{-2}	NT	100
2	2×10^{-2}	NT	100
3	1×10^{-1}	NT	0
4	7×10^{-2}	NT	0
5	8×10^{-2}	NT	0
6	2×10^{-1}	NT	0

(ii) *Frequency of plasmid transfer*

From results presented in Table 2 it is clear that the pND3 is transmissible. However the tube-mating method was used to select for transfer of resistance to carbenicillin and the plate-mating method was used to select for transfer of the ability to utilize *m*-toluate. To demonstrate more clearly the nature of the transfer system of pND3, the two mating techniques were compared using PA01 as recipient. The transfer frequency to *Sh. flexneri* 4a 25 SM was also determined. For control purposes, PA03(pND2) and PA03(R91) were used as donor strains. The results are given in Table 3. It is apparent that the frequency of R91 transfer to PA01 is much higher than that of pND2 transfer. When using PA03(pND3) as donor the transfer frequencies are comparable to those for R91, whether selecting for growth on *m*-toluate or selecting for resistance to carbenicillin. It seems that the transfer system of R91 is responsible for transfer of pND3. It also appears that it is not the duration of the mating which is responsible for the poorer transfer ability of pND2. The R91 plasmid transferred readily to *Sh. flexneri* 4a 25 SM, as expected (Chandler and Krishnapillai 1974*a*). When selecting for transfer of resistance to carbenicillin from PA03(pND3), transconjugants were also readily obtained. However, when selecting for growth on *m*-toluate, no transconjugants were obtained with either PA03(pND2) or PA03(pND3) as the donor strains; *m*-toluate (5 and 10 mM) incorporated into

nutrient agar medium did not inhibit growth of the *Sh. flexneri* strain. Therefore it is possible that genetic information for the utilization of toluate could not have been expressed or that the host strain did not carry the appropriate information to allow expression of the toluate growth phenotype. Purified transconjugants from the PA03(pND3) donor, from the carbenicillin selection plates, also failed to grow on *m*-toluate. Attempts to transfer this plasmid back to either *P. aeruginosa* or *P. putida* to test for the presence of the *m*-toluate growth genotype were unsuccessful. Similar inability of R91 to transfer out of a *Shigella* strain had been previously reported by Chandler and Krishnapillai (1974*a*).

Table 3. Plasmid transfer frequencies to PA01 comparing the tube-mating and plate-mating techniques, and transfer to *Shigella flexneri* 4a 25 SM

Cb^R, selection made for transfer of resistance to carbenicillin; *m*Tol^+, selection made for transfer of the ability to grow on *m*-toluate; NT, not tested. Values given for recipients are numbers of transconjugants per donor cell

Donor	Mating method	Selection	Recipient	
			PA01	*Sh. flexneri*
PA03(pND3)	Plate	*m*Tol^+	1×10^{-3}	NT
	2·5 h tube	*m*Tol^+	7×10^{-3}	$<1 \times 10^{-8}$
	2·5 h tube	Cb^R	4×10^{-3}	1×10^{-4}
	5 h tube	*m*Tol^+	2×10^{-2}	NT
	5 h tube	Cb^R	1×10^{-2}	NT
PA03(pND2)	Plate	*m*Tol^+	4×10^{-6}	NT
	2·5 h tube	*m*Tol^+	2×10^{-6}	$<1 \times 10^{-8}$
	5 h tube	*m*Tol^+	3×10^{-7}	NT
PA03(R91)	2·5 h tube	Cb^R	1×10^{-3}	6×10^{-6}
	5 h tube	Cb^R	1×10^{-2}	NT

(iii) *Segregation of R91, pND2 and pND3*

A mitomycin C curing experiment was carried out on the various plasmid-containing strains to determine plasmid stability. No spontaneous or mitomycin-C-induced curing was observed of R91, pND2 or pND3 from PA03. Of the clones tested, 55% had lost TOL from PA03(TOL) spontaneously and 100% had lost the plasmid following growth in the presence of mitomycin C (15 μg/ml).

Since it was not possible to cure the stabilized plasmids with mitomycin C, attempts were made to force segregation of pND3 using an incompatible plasmid. In comparative studies on the TOL and NAH catabolic plasmids, Austen and Dunn (1977) demonstrated that these two plasmids were incompatible. It is likely that pND3 may also be incompatible with NAH, and so segregation of the stabilized plasmid may be achieved. Since NAH could not be transferred to PA0 strains, this experiment was carried out in PP1-3. The plasmid pND3 was transferred from PA03 (pND3) to PP1-3, selecting for growth on *p*-toluate, and transconjugants were obtained at a frequency of 1×10^{-3} per donor cell. One of the transconjugants was purified and was found to carry all phenotypes associated with pND3. The NAH plasmid was then introduced into PP1-3 and PP1-3(pND3) from PP2-4(NAH), selecting for growth on naphthalene. In each case transconjugants were obtained at

a frequency of approximately 5×10^{-6} per donor cell. Thirty transconjugants from the PP1-3(pND3) recipient were purified by streaking twice for single colonies on naphthalene. Upon testing it was found that 29 clones had lost all the pND3-specified phenotypes. No segregation was observed after growth of PPI-3(pND3) for an equivalent number of generations without selection for maintenance of pND3. A similar experiment was conducted which showed that NAH was also incompatible with pND2.

(iv) *Transfer of pND3 by Transduction*

All results reported to this point suggest that the catabolic plasmid was recombined with the drug resistance plasmid, resulting in pND3. If it were possible to cotransduce all plasmid-encoded markers, this would illustrate the expected very close linkage between all markers.

Table 4. Chromosome transfer mediated by R91, pND2, pND3, FP2 and FP39
Selected markers were: PA067, his+; PA08, ilv+; PA038, leu+. NA, not applicable. Values given for recipients are numbers of conjugants per donor cell

Donor	Recipient		
	PA067	PA08	PA038
PA0381(FP2)	1×10^{-4}	4×10^{-5}	NA
PA01264(FP39)	1×10^{-5}	2×10^{-7}	NA
PA03(R91)	1×10^{-6}	1×10^{-5}	2×10^{-5}
PA03(pND2)	2×10^{-5}	2×10^{-5}	4×10^{-5}
PA03(pND3)	3×10^{-5}	2×10^{-5}	5×10^{-5}

Initial attempts to transduce the plasmid in strain PA0 using the bacteriophages F116 and G101 were unsuccessful. However, neither of these phages was able to transduce TOL or pND2 alone within PA0. Transduction of TOL using pf16 has been reported in *P. putida* (Wong and Dunn 1974), so pND3 was transferred to PP1-2 from PA03 (pND3), selecting for growth on *p*-tuluate. One of the transconjugants was purified and tested, then a transducing phage culture, pf16.PP1-2 (pND3), was prepared. By selecting for growth on *m*-toluate, transductants were obtained in PP1-8 at a frequency of approximately 10^{-9} per phage particle. On testing, transductants were obtained which had inherited all known characteristics of pND3. They grew on *m*-toluate, *p*-toluate, *m*-xylene, *p*-xylene and toluene, were resistant to carbenicillin, and were able to transfer by conjugation.

(v) *Mediation of Chromosome Transfer by pND2 and pND3 in PA0 Strains*

Stanisich and Holloway (1971) have demonstrated that R91 can promote chromosome transfer within *P. aeruginosa* strain PAT. Chromosome transfer in that system was subject to derepression. An experiment was performed to determine whether pND2 and pND3 could promote chromosome transfer in strain PA0. This was compared to chromosome transfer mediated by R91, without use of a derepressing mixed-culture technique or u.v.-irridiation treatment, and to transfer promoted by the sex factors FP2 and FP39. These results are given in Table 4. Very similar transfer frequencies were obtained with R91, pND2 or pND3 as the donor strains. Mobilization of chromosome markers by pND3 may have been expected, but transfer

promoted by pND2 was quite unexpected. Chromosome transfer promoted by the catabolic plasmid is presently being studied in more detail.

Discussion

From the evidence presented in this paper it appears that the TOL plasmid is compatible with the R91 drug resistance plasmid. This was demonstrated primarily by measuring the rate of segregation of TOL after introducing, and selecting for maintenance of, R91 as compared to the unselected segregation rate of TOL. When selecting for maintenance of TOL no segregation of R91 was detected. Since R91 transfers at such a high frequency, reinfection of cured strains could have occurred during this experiment. Therefore this R91 segregation data alone may not have been sufficient to suggest compatibility.

Recombinants of compatible plasmids have been shown, in the literature, to be somewhat difficult to isolate, using *in vivo* techniques. This has been due to the absence of ready methods for directly selecting for recombination. However, because of the instability of TOL in PA03, it is possible to select for stability in the presence of R91, anticipating that stability could arise by recombination with the non-segregating R91. Using this strategy an apparent recombined plasmid was obtained (pND3) which carried the catabolic activities of TOL and the resistance to carbenicillin of R91.

In addition to recombination between TOL and R91, it was demonstrated that TOL could stabilize in PA03 hosts which either carried or did not carry R91. This data suggested that there were at least two mechanisms leading to stabilization of TOL, one of which was independent of the presence of R91 and the other possibly dependent on the presence of R91. It is possible, however, that recombination with R91 is independent and secondary to initial stabilization of TOL.

Evidence for recombination in pND3 included cotransduction, conjugational cotransfer and incompatibility with NAH; in all cases all known properties were transferred or lost simultaneously. This evidence supports the view that recombination has occurred between TOL and R91. However, to understand the exact nature of pND3 more extensive studies would be required and should include plasmid isolation and physical characterization. Such studies should reveal whether fusion has been partial or complete. The incompatibility between NAH and pND3 may provide a means of selecting for recombination between these two plasmids.

Data on plasmid transfer shows clearly that pND3 can transfer at the same frequency as the parent R91 and much higher than that of either TOL or pND2. It is apparent that the R91 transfer system has been inherited; however, it has not been possible to determine whether the TOL transfer system has also been retained in pND3. Although the transfer frequency is improved considerably, the transfer range does not appear to have been markedly increased (White and Dunn, unpublished data). However, the R plasmid information encoded by pND3 may facilitate further recombination with the promiscuous and highly homologous (Ingram *et al.* 1973) R plasmids such as R68 and RP1 (Chandler and Krishnapillai 1974*b*).

Provided that the genetic material for catabolism of *m*-toluate is retained upon transfer of pND3 to *Sh. flexneri*, then this raises the question of certain host strains not permitting expression of the genetic material encoding catabolic pathways. Such characteristics would need to be considered if specific strain construction work was being attempted.

Of general interest is the observation that pND2 and pND3 can promote chromosome transfer. It is known that R91 can promote chromosome transfer in *P. aeruginosa* strain PAT2 (Stanisich and Holloway 1971). The chromosome transfer observed from pND3 could have been due to the transfer mechanism of R91. However, conjugational transfer frequencies similar to that observed for R91 and pND3 were observed when using PA03(pND2) as the donor strain. In this strain pND2 has stabilized in the absence of R91, and therefore must be capable of promoting chromosome transfer in the absence of any contributing influence from R91. In this experiment it was only the stabilized pND2 plasmid that was studied; the instability and the frequency of stabilization of TOL in PA03 made it impractical to study TOL. One other catabolic plasmid, the CAM plasmid, has been shown to promote low levels of chromosome transfer in *P. putida* (Shaham *et al.* 1973). Chakrabarty and Friello (1974) reported detection of a plasmid (K) in the strain which also harboured the OCT catabolic plasmid. The K plasmid was independent from OCT and could be used to promote chromosome transfer. The chromosome transfer promoted by pND2 and pND3 in *P. aeruginosa* occurs at quite a high frequency. It may be possible to make use of these plasmids to promote chromosome transfer within other pseudomonad strains.

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