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Properties of an R Factor from *Pseudomonas aeruginosa*

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An R factor from *Pseudomonas aeruginosa*, which confers resistance to penicillins, kanamycin, and tetracycline, was studied in *Escherichia coli* K-12. The R factor could coexist with F-like or I-like plasmids and therefore constituted a novel compatibility group. The R factor was transferable from *E. coli* to bacterial genera outside the *Enterobacteriaceae* (*Pseudomonas* and members of the *Rhizobiaceae*) to which transfer of F-like and I-like plasmids could not be demonstrated.

R factors are subdivided into two classes, fertility inhibition$^+$ (*fi*$^+$) and *fi*$^-$ (25). The *fi*$^+$ factors inhibit F-mediated conjugation; *fi*$^-$ ones do not. The *fi*$^+$ group of R factors are related to F in that they determine the synthesis of sex pili similar to those of F (7). These R factors mutually exclude one another, and do not coexist stably in *Escherichia coli* K-12 (25), but, despite their similar pili, they are not excluded by F and stably coexist with F. Thus, they constitute a compatibility group distinct from that including F.

Among *fi*$^-$ R factors, one group has been distinguished which produces sex pili similar to those of the transmissible colicine factor I (I pili). These factors, and the ColI plasmid, show mutual exclusion and will not coexist in *E. coli* K-12. Among a group of R factors derived from *E. coli* and *Salmonella* sp., a minority of the *fi*$^-$ plasmids examined did not appear to produce either F or I pili (15).

R factors have been derived from strains of *Pseudomonas aeruginosa* highly resistant to carbenicillin (12, 16, 23). We have studied an apparently similar R factor, RP4, which came from *P. aeruginosa* strain S8 (3). This R factor, which is *fi*$^-$, gave no evidence of production of I pili despite repeated attempts to demonstrate multiplication of phage If1 in R$^+$ cultures.

The results presented here show that RP4 belongs to a class distinct from F-like and I-like factors in respect of superinfection immunity and coexistence in K-12. We propose to call this class P since the first example was derived from *P. aeruginosa*.

MATERIALS AND METHODS

Bacteria, plasmids, and bacteriophages. Bacteria, plasmids, and bacteriophages used are listed in Table 1.

Media. Nutrient broth, nutrient agar, and minimal agar were as specified in Clowes and Hayes (6). Soft agar for phage assay was nutrient agar containing only 0.3% Davis New Zealand agar. MacConkey agar was Oxoid code no. CM 7b. Selection for genetically distinct bacteria from mixed cultures was by supplementation of minimal salts medium with appropriate nutrients or antibiotics, or both.

Antibiotics. To demonstrate carbenicillin resistance in *P. aeruginosa*, carbenicillin (1,000 μg/ml) was used in solid media. When the same resistance genes (determining production of penicillinase) were transferred to *E. coli*, ampicillin (50 μg/ml) was used instead of carbenicillin. Other antibiotics were used as freshly prepared solutions added to media at the following final concentrations: streptomycin, 15 μg/ml; tetracycline, 10 μg/ml; chloramphenicol, 25 μg/ml; kanamycin, 25 μg/ml; sulfonamide, 100 μg/ml. Resistance patterns of strains were determined by the use of Multodisks (Oxoid) and confirmed by their ability to grow normally on media containing drugs at the above concentrations.

Conjugation. Donors were late log-phase, standing-broth cultures containing about 2×10^8 bacteria/ml. Recipients were late log-phase, shaken-broth cultures containing about 5×10^8 bacteria/ml. One part of donor was mixed with 4.5 parts of recipient with 4.5 parts of fresh broth. Mixtures were incubated for 1 hr at 37 C and 0.1-ml volumes were plated on selective media except where specified otherwise. The frequency of transfer was always calculated relative to the number of donors (i.e., as transfer per donor cell).

Transfer into genera outside the *Enterobacteriaceae* was as described above, except that the recipients were grown at 27 C and shallow mixed cultures were gently shaken, also at 27 C, for 2 to 3 hr before plating.

Plasmid interaction. To test for the *fi* character of R factors, they were transferred to *E. coli* K-12 58-161 (F$^+$), which was then tested for sensitivity to phage MS2.

Relative frequencies of R transfer by conjugation (in mixtures incubated for 1 hr) into a strain of K-12, with and without a second plasmid under test, were determined. This gave a measure of exclusion (17). Recipient colonies, carrying both plasmids, were purified on nonselective medium and tested, by replica plating, for the presence of characters of each plasmid to give a measure of compatibility. The physical independence of the two plasmids was tested by transfer by conjugation

TABLE 1. *Bacteria, plasmids, and bacteriophages used*

Bacteria		Plasmids				Bacteriophage		
Organism	Reference	No.	fi	Resistance pattern[a]	Reference	No.	Receptor	Reference
Escherichia coli K-12 J5-3 F−	7	RP4	−	ATK	−	MS2	F pili	8
E. coli K-12 J6-2 F−	7	R1	+	ASCKSu	15	If1	I pili	19
E. coli K-12 58-161 F+	6	R1-19*drd*	−	ASCKSu	15			
E. coli K-12 803 F−	2	R136	+	T	15			
Pseudomonas aeruginosa PAO 2 (FP−)	14	R136-8*drd*	+	T	15			
		R192-7*drd*	+	STCSu	15			
		R538-1	+	SCSu	21			
Chromobacterium violaceum D254	22	R538-1*drd*	+	SCSu	21			
		R64	−	ST	15			
Agrobacterium tumefaciens B6	9	R64-11*drd*	−	ST	15			
		R144-3*drd*	−	K	15			
		R163	−	STK	15			
Rhizobium trifolii 24G	26	R163-7*drd*	−	STK	15			
R. meliloti 1.5	10	colB-K98	+		11			
		F	−		5, 13			
		I-16*drd*	−		18			

[a] Abbreviations: A, ampicillin-carbenicillin resistant; S, streptomycin resistant; T, tetracycline resistant; C, chloramphenicol resistant; K, kanamycin-neomycin resistant; Su, sulfonamide resistant.

to another K-12 strain, selecting for each character separately.

Bacteriophage increase. Sex pili are receptors for sex-specific phages. With most wild-type transmissible plasmids, pilus production is repressed and there is no visible lysis by sex-specific phages. The presence of receptors is indicated by the ability of the phage to multiply in the culture (15). To show multiplication of phage If1, bacteria from 5-ml, late log-phase broth cultures of J5-3, with or without the factors under test, were concentrated by centrifugation into approximately 0.1 ml; 2×10^{10} plaque-forming units of If1 were added and left at room temperature for 10 min. An excess of antiphage serum was added and left for 5 min. Prewarmed broth (100 ml) was added. This dilution was sufficient to render the antiserum ineffective. Phage titration was carried out immediately. The indicator was *E. coli* K-12 803(R64-11) (2). Titration was in agar layer at 25 C. The broths were incubated for 24 hr at 37 C, samples were centrifuged to deposit the bacteria, and the supernatant fluids were titrated for If1. To test the phage sensitivity of newly infected cells, a fresh culture of J5-3 (R^-) was mixed with a sample of each broth culture, diluted 20 times in fresh broth, incubated overnight, and titrated again.

RESULTS

Superinfection immunity. RP4 was transferred from J6-2 to J5-3 with and without plasmids of each of four compatibility groups, i.e., F, R136 (an F-like R factor), R64 (an I-like R factor), and colB-K98 (an *fi*$^+$ plasmid compatible with *fi*$^+$ R factors). No reduction in frequency of transfer was observed with any recipient (Table 2).

Coexistence. 58-161(F)(RP4) was sensitive to the male-specific phage MS2 showing that the two plasmids could coexist and that F piliation was not inhibited. RP4 also coexisted stably with F-like or I-like R factors (Table 3).

Physical independence. Transfer of resistances from double R^+ strains indicated that, with one exception, the plasmids had not recombined (Table 4). From J5-3(RP4)(R538-1), each plasmid was transferred independently and at normal frequency. From J5-3(RP4)(R64), each plasmid was transferred independently, but the frequency of transfer of RP4 was reduced 10- to 100-fold. The mutant of R64, R64-11*drd*, with constitutive pilus production, reduced the transfer frequency of RP4 as much as did the repressed wild type. Recipients with RP4 were also R64-11$^+$; this probably only reflected the high efficiency of transfer of the derepressed R factor.

Recombination. As noted above, in most bacteria carrying two plasmids we found no evidence for recombination between them (Table 5). There was one exception. RP4 and R64 occasionally underwent recombination. A strain of J6-2 from a mating with J5-3(RP4)(R64-11*drd*), which received all markers of both factors, was used as donor. R64-11*drd* was transferred independently at high frequency (>100%); selection for RP4 was made with either ampicillin or kanamycin. Ampicillin resistance was transferred to J5-3 at a frequency of 5×10^{-3}; kanamycin re-

sistance was at 2×10^{-5}. The colonies on ampicillin selection plates contained a recombinant R factor; they were resistant to ampicillin-streptomycin-tetracycline, sensitive to kanamycin, and lysed by Ifl phage. When used as donors, they transferred all three resistance markers and Ifl sensitivity with 100% efficiency. In another experiment (not recorded in Table 4), recombination between RP4 and R64 occurred. Here too, the recombinant had the characteristics of R64 with the penicillinase gene of RP4.

TABLE 2. *Tests for exclusion*

Donor	Recipient	Frequency of transfer
J6-2(RP4)	J5-3	1.5×10^{-4}
J6-2(RP4)	J5-3(R136)	1.2×10^{-4}
J6-2(RP4)	J5-3(R538-1)	1.6×10^{-4}
J6-2(RP4)	J5-3(colB-K98)	1.6×10^{-4}
J6-2(RP4)	58-161(F)	3.2×10^{-4}
J6-2(RP4)	J5-3(R64)	1.7×10^{-4}
J6-2(RP4)	J5-3(R163)	1.6×10^{-4}

A search for reciprocal recombinants (i.e., factors resembling RP4, but without ampicillin resistance) failed. J5-3(RP4)(R64) was mated with J6-2 and selection was made on kanamycin. Approximately 10,000 colonies were replicated to medium containing ampicillin (1,000 μg/ml); all grew.

Pilus synthesis. We tried to derepress the conjugation mechanism phenotypically. Broths were inoculated with mixtures of R^+ and R^- bacteria and incubated to allow spread of R through the recipient population, but spread was limited and satisfactory HFT preparations were never ob-

TABLE 3. *Coexistence of pairs of plasmids in Escherichia coli K-12*

Culture	Resistance pattern[a]	No. of colonies on master plate	No. of colonies missing from replica plates containing:[b]		
			K	C	S
J5-3(RP4)	ATK	217	0	NT	NT
J5-3(RP4)(R538-1)	ATKSCSu	248	0	0	NT
J5-3(RP4)(R64)	ATKS	1356	0	NT	0

[a] See footnote to Table 1.

[b] Growth on K (kanamycin) indicates presence of RP4; on C (chloramphenicol), presence of R538-1; on S (streptomycin), presence of R64; NT, not tested.

TABLE 4. *Transfer of resistance from double R^+ strains*[a]

Mating	Donor	Recipient	RP4		Other	
			Selection[b]	Frequency	Selection[b]	Frequency
1	J5-3(RP4)	J6-2	K	8.6×10^{-4}		
			A			
2	J5-3(R538-1)	J6-2			C	2×10^{-1}
3	J5-3(R64)	J6-2			S	1.5×10^{-3}
4	J5-3(R64-11*drd*)	J6-2			S	>1
5	J5-3(RP4)(R538-1)	J6-2	K	8.3×10^{-4}	C	2.8×10^{-1}
6	J5-3(RP4)(R64)	J6-2	K	9×10^{-6}	S	2.5×10^{-3}
7	J5-3(RP4)(R64-11*drd*)	J6-2	K	10^{-5}	S	>1
8	J6-2(RP4)(R64-11*drd*)	J5-3	K	2×10^{-5}	S	>1
	Recipient from mating no. 7		A	5×10^{-3}		
9	J5-3(RP4)(R64-11*drd*)	J6-2	K	2×10^{-5}	S	>1
	Recipient from mating no. 8, selected on K		A	6×10^{-4}		
10	J5-3 (recombinant)	J6-2	K	>1	S	>1
	Recipient from mating no. 8, selected on A					

[a] Ten colonies from selection plates above were purified by streaking and picking single colonies, first on MacConkey agar containing the same drug and then on drug-free MacConkey. (Since donors and recipients differed in lactose fermentation, recombinants were readily differentiated from donors on these media.) Resistance patterns were determined in Table 5).

[b] Abbreviations: K, kanamycin; A, ampicillin; C, chloramphenicol; S, sulfonamide.

TABLE 5. *Resistance patterns of recombinants*[a]

Mating	Selection	
	For RP4	For other
5	5/10 ATK (RP4 alone) 5/10 ATKSCSu (both factors)	10/10 SCSu (R538-1 alone)
6	10/10 ATK (RP4 alone)	10/10 ST (R64 alone)
7	3/3 ATKS (both factors)	10/10 ST (R64-11*drd* alone)
8	*Kanamycin selection* 10/10 ATKS (both factors) *Ampicillin selection* 10/10 AST, Ifl sens (recombinant)	10/10 ST (R64-11*drd* alone)
9	*Kanamycin selection* 10/10 ASTK (both factors) *Ampicillin selection* 10/10 ASTK (both factors)	10/10 ST (R64-11*drd* alone)
10	10/10 AST (recombinant plasmid)	10/10 AST (recombinant plasmid)

[a] See footnote to Table 1.

tained. Attempts to isolate derepressed mutants after treatment with various mutagens (U.V., nitrosoguanidine, ICR191, and ethyl nitrosourea) were uniformly unsuccessful.

No multiplication of Ifl phage was demonstrated with J5-3(RP4) either in established cultures or in cultures containing newly infected cells.

Mobilization of RP4 by I-like factors. When J5-3(RP4) was mated with J6-2(R64-11), the frequency of transfer of RP4 was much increased. The same was true with other derepressed I-like factors but not with F-like factors (Table 6). The kinetics of transfer suggested that RP4 was being transferred with high efficiency from cells newly infected with R64-11 (Table 7).

Host range. RP4 was transferred from J5-3 to *P. aeruginosa* PAO2 with a frequency of 2.3×10^{-3}, but we were unable to transfer the recombinant R factor described above into this strain. Nor were we able to transfer any other R factor, F-like or I-like, repressed or derepressed, to PAO2.

RP4 was transmissible to *Chromobacterium violaceum* D254, *Rhizobium trifolii* 24G, and *Rhizobium meliloti* 1.5 at an efficiency of 10^{-6} or greater. Transfer from J5-3 of derepressed mutant R factors, with F or I pili, was never observed to these genera (efficiency $< 10^{-10}$), although they transferred with 100% efficiency between K-12 strains. Transfer of RP4 to *Agrobacterium tumefaciens* B6 occurred with low efficiency (10^{-8} to 10^{-9}); no other R factor could be introduced into this organism.

DISCUSSION

The *fi*$^-$ R factors do not constitute a homogeneous group. Those known to produce I pili show mutual superinfection immunity, manifested by both exclusion and lack of stable coexistence. RP4 does not show superinfection immunity to known I plasmids. Coexistence of *fi*$^-$ plasmids has been reported several times, e.g., R factor S-a showed no superinfection immunity with any of several *fi*$^+$ and *fi*$^-$ factors (24) and Bouanchaud and Chabbert (4) identified three different *fi*$^-$ factors in a single, naturally occurring strain of *Salmonella panama*, which could be transferred to and coexisted stably in K-12.

The compatibility groups defined by our experiments are exemplified by R64 and RP4. RP4 is unrelated to *fi*$^+$ R factors by any of the tests used. There is no proof that it produces I pili. The negative results with phage Ifl provide strong evidence against such production, but without derepressed mutants, or satisfactory HFT preparations, their absence cannot be proved. Until now, the only R factors in which mutants derepressed for pilus synthesis have been isolated are ones in which derepression in newly infected cells is marked so that HFT preparations are readily obtained.

The effect of R64 in depressing the frequency of transfer of RP4, when present in the same cells, resembles the suppression of F transfer by *fi*$^+$ R factors. By this analogy, pilus production by RP4 is sensitive to the pilus repressor of R64, a typical I-like R factor. But RP4 does not repress con-

TABLE 6. *Transfer of RP4 to R^+ and R^- recipients*

Donor	Recipient	Frequency of transfer	Ratio of frequency of transfer to R^+ recipient/ R^- recipient
J5-3(RP4)	J6-2	7×10^{-4}	
J5-3(RP4)	J6-2(R64)	1.1×10^{-3}	1.6
J5-3(RP4)	J6-2(R64-11*drd*)	1.1×10^{-1}	160
J5-3(RP4)	J6-2(R144)	2.9×10^{-3}	4.1
J5-3(RP4)	J6-2(R144-3*drd*)	8.2×10^{-2}	20
J5-3(RP4)	J6-2(R163)	6×10^{-4}	0.8
J5-3(RP4)	J6-2(R163-7*drd*)	1.3×10^{-3}	1.9
J5-3(RP4)	J6-2(I-16*drd*)	3.8×10^{-2}	54
J5-3(RP4)	J6-2(F'*lac*)	4.5×10^{-3}	6.4
J5-3(RP4)	J6-2(R1)	6.2×10^{-4}	0.9
J5-3(RP4)	J6-2(R1-19*drd*)	5.6×10^{-4}	0.8
J5-3(RP4)	J6-2(R136)	7.2×10^{-4}	1.0
J5-3(RP4)	J6-2(R136-8*drd*)	4×10^{-3}	5.7

TABLE 7. *Mating of J5-3(RP4) with J6-2(R64-11drd)*

Time (min)	J5-3(RP4)	J5-3(RP4) (R64-11*drd*)	J6-2(R64-11*drd*)	J6-2(R64-11*drd*) (RP4)
0	2.6×10^7		1.5×10^8	
5		$>10^8$		2.4×10^5
10		8.5×10^8		3.5×10^5
20	3.3×10^7	2×10^7	2.1×10^8	1.3×10^6
40		2×10^7		1.6×10^6
60	3.3×10^7	2×10^7	2.1×10^8	2.4×10^6

stitutive I pilus synthesis by any of the mutant R factors tested, R64-11*drd*, R144-3*drd*, R163-7*drd*, and the sex factor I-16*drd* of ColI. This could mean that the mutants are operator constitutive (o^c), insensitive to repressor. No test to distinguish between o^c and repressor negative (i^-) mutants exists for I pilus production. When R64-11*drd* is newly introduced into J5-3(RP4), no inhibition of transfer of RP4 is found; on the contrary, RP4 is transferred at greater than normal frequency (Table 6). If R64-11*drd* is o^c, its repressor gene, i, will not function immediately upon entry into a new host, and this could account for the failure to depress RP4 transfer, but not for the markedly increased efficiency of transfer. If the mutation in R64-11*drd* is not o^c but i^-, the inhibition of transfer of RP4 in established double R^+ cultures may be due to another repressor, determined by R64 and R64-11*drd*, whose effect is apparently not expressed in newly infected cells. The very high efficiency of transfer of RP4 from cells newly infected with R64-11*drd* seems to be an example of mobilization of one plasmid by another, such as ColE1 by ColI (20) or nontransmissible R factors by Δ (1). This being so, there is no proof that RP4 determines its own transfer in the presence of R64-11*drd*, established or newly introduced.

RP4 is exceptional among R factors studied in being transmissible from K-12 to *P. aeruginosa* and members of the family *Rhizobiaceae*. Since DNA-mediated transformation has been frequently reported among members of the *Rhizobiaceae* (e.g., reference 26), it is possible that transfer of RP4 was by this mechanism. Because RP4 is transferred by conjugation to *P. aeruginosa* PAO which is not transformable (B. Holloway, *personal communication*), it evidently has a wider conjugation spectrum than other known R factors, and therefore we suppose that transfer to the *Rhizobiaceae* was by this mechanism.

The character by which RP4 was first identified, the high level of resistance to carbenicillin and other penicillins which it confers, depends on the production of a penicillinase which is apparently identical with that determined by at least one other R factor (RTEM) which is F-like (23). The penicillinase gene in one of our experiments was dissociated from RP4 and integrated into R64-11*drd*, which is I-like. Thus, the penicillinase gene is not specific to plasmid RP4; a plasmid of this type may act as a vector, bringing about genetic exchange between F-like and I-like plasmids.

RP4 is a transmissible plasmid; our results suggest that it has a novel type of conjugation mechanism although we have not proved that it does not specify I-type pili. It belongs to a compatibility group (P), different from other known plasmids and is transmissible to a different range of bacteria.

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