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An Explanation for the Apparent Host Specificity of *Pseudomonas* Plasmid R91 Expression

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Pseudomonas aeruginosa strain 9169 has been reported to contain a plasmid that expresses resistance to carbenicillin (Cb), kanamycin (Km), and tetracycline (Tc) in *Escherichia coli* but resistance only to Cb in certain *Pseudomonas* recipients. The triply resistant plasmid in *E. coli* belonged to incompatibility (Inc) group P or P-1, whereas the singly resistant plasmid in *P. aeruginosa* was compatible with IncP-1 plasmids and other plasmids of established Inc specificity but incompatible with plasmid pSR1 that is here used to define a new *Pseudomonas* Inc group P-10. Additional physical and genetic studies showed that strain 9169 contained not one but two plasmids: IncP-1 plasmid R91a, determining the Cb Km Tc phenotype, and IncP-10 plasmid R91, determining Cb that differed in molecular weight and in *Eco*RI and *Bam*HI restriction endonuclease recognition sites. Plasmid multiplicity rather than host effects on plasmid gene expression can account for differences in the phenotype of strain 9169 transconjugants to *E. coli* and *P. aeruginosa*.

With few exceptions expression of resistance genes on plasmids is strain independent. The level of antibiotic resistance produced by a plasmid may vary, but some degree of resistance is generally detectable in every host. A deviation from this rule was reported by Chandler and Krishnapillai (6), who found that *Pseudomonas aeruginosa* strain 9169, which is resistant to carbenicillin (Cb), kanamycin (Km), and tetracycline (Tc), could transfer the Cb Km Tc phenotype to *Escherichia coli* or *Shigella flexneri* but transferred only Cb to strain PAO or PAT *P. aeruginosa* recipients. In one instance Cb was transmitted from a Cb PAO transconjugant to *E. coli* C where triple resistance was expressed. Accordingly, they suggested that a plasmid termed R91 was present in strain 9169 that expressed Cb Km Tc in *E. coli*, but, that in the *P. aeruginosa* recipients, Km Tc was not expressed.

Expression of the gene or genes determining plasmid incompatibility is also generally the same in hosts as different as *E. coli* and *P. aeruginosa* (16). Consequently we were surprised to find that while the plasmid from strain 9169 that determines triple antibiotic resistance in *E. coli* belongs to incompatibility (Inc) group P (or P-1 in the *Pseudomonas* system of nomenclature), the plasmid determining Cb transmitted from strain 9169 to *P. aeruginosa* does not. Despite this difference, R91 in *Pseudomonas* shares a number of properties with IncP-1 plasmids from strains isolated in the same clinical outbreak as strain 9169.

Strain 9169 was isolated in 1969 by Lowbury et al. (26) at the Birmingham Accident Hospital where intensive use of carbenicillin led to the emergence of a variety of Cb *P. aeruginosa* strains. Strain 1822 is probably the best known of these since it contains the widely used, broad host range IncP-1 plasmid RP1 that determines Cb Km Tc, inhibits the propagation of phage G101, and allows propagation of phages PRR1 and Pf3 that attach specifically to P-1 pili, as well as propagation of phages PRD1, PR3, and PR4 that bind to pili made by other Inc groups (3, 19). Phages PRD1, PR3, and PR4 will propagate in liquid medium on a *P. aeruginosa* recipient carrying plasmid R91 (31). In addition, conjugally derepressed derivatives of R91, such as R91-5, readily allow plating of phages PRD1, PR3, and PR4 and also inhibit the propagation of phage G101 (7). However, despite these similarities in phage responses, RP1, in contrast to R91, expresses Cb Km Tc and IncP-1 specificity in both *P. aeruginosa* and *E. coli*.

We have investigated the genetic and physical

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properties of R91 and related plasmids in greater detail in an effort to understand its exceptional behavior.

MATERIALS AND METHODS

Bacterial strains and plasmids. Table 1 lists the bacterial strains and plasmids used. For incompatibility testing, a derivative of plasmid R91-5 carrying Tn*7* (1) was constructed by selecting for transfer of Sm from a donor strain carrying R91-5 and a Tra$^-$ (transfer deficient) derivative of RP4::Tn*7* selected as resistant to donor-specific phage PR4 (31). R91-5::Tn*7* is Cb Sm Tp (trimethoprim resistant) and by agarose gel electrophoresis (21, 27) has an estimated molecular mass of 43 megadaltons (Mdal).

Bacteriophages. The phages used were donor-specific phages PRR1 (29), PRD1 (28), Pf3, PR3, and PR4 (31) and female-specific phages B39 (22) and G101 (13). They were propagated as described (15).

Plasmid transmission by conjugation. Procedures for plasmid transmission by conjugation have been described (12, 15). When prototrophic clinical isolates were used as donors, plates contained rifampin (100 μg/ml) for counterselection. Counterselection of other donors was by omission of their nutritional requirements.

Plasmid transmission by transformation. *E. coli* or *P. aeruginosa* recipients were transformed as described by Lederberg and Cohen (24) with plasmid DNA purified from cesium chloride-ethidium bromide gradients.

Incompatibility testing. Procedures for determining plasmid incompatibility have been described (9, 15). To overcome marker overlap, R91-5::Tn*7* was used in tests with plasmids RIP64, Rms149, R2, and RP1-1. Retention of RP1-1 was scored by resistance to phage B39 (22).

Isolation of plasmid DNA. Plasmid DNA was isolated by a modification of the technique of Clewell and Helinski (8). Cells were harvested in log phase from 100 ml of L broth (25) and suspended in 7.0 ml of 25% sucrose in 50 mM tris(hydroxymethyl)-aminomethane (Tris), pH 8.0. One milliliter of 250 mM ethylenediaminetetraacetate (EDTA), pH 8.0, was added, and the mixture was incubated with gentle shaking at 37°C for 10 min. A solution of lysozyme was added to a final concentration of 1 mg/ml, and incubation was continued at 37°C for 10 min. A 0.1-ml amount of Pronase solution (20 mg/ml in TES buffer [50 mM Tris–5 mM EDTA–50 mM NaCl, pH 8.0]; predigested for 1 h at 37°C) was added, and incubation was continued for 15 min at 37°C. A 2.5-ml amount of 5 M NaCl was added, and the cells were lysed by gentle inversion after adding 1.0 ml of 20% (vol/vol) Triton X-100 and 0.5 ml of 5% (wt/vol) sodium deoxycholate. The mixture was kept in an ice bath for 30 min and then centrifuged at 25,000 × *g* for 60 min. A 8.0-ml sample of supernatant was decanted; 0.5 ml of 25% Sarkosyl was added; and the cleared lysate was prepared for ethidium bromide-cesium chloride equilibrium density centrifugation. After 40 h at 33,000 rpm in a type 40 rotor on a Beckman model L ultracentrifuge, the plasmid band was visualized by UV light, collected, extracted twice with 1 volume of ice-cold CsCl-saturated 2-isopropanol, and dialyzed against TES buffer.

TABLE 1. *Bacterial strains and plasmids used*

Strain or plasmid	Relevant characteristics[a]	Reference
E. coli		
J53-2	F$^-$ *pro-22 metF63* Rif^r	18
C Ilv$^-$	F$^-$ *ilv* Res$^-$ Mod$^-$ Rif^r	20
P. aeruginosa		
Ros38	Clinical isolate[b]	
9169	Clinical isolate[c]	26
PU21	FP$^-$ *ilvB112 leu-1 str^r-1* Rif^r	15
PAO303	FP$^-$ *argB18*	15
Plasmid		
pMG4	Km Sm Tc IncP-1	19
RP4::Tn*7*	Cb Km Sm Tc Tp IncP-1	2
R702	Km Sm Su Tc Hg Pma IncP-1	19
R751	Tp IncP-1	19
R931	Sm Tc Hg Ter Uv IncP-2	19
RIP64	Cb Cm Gm Su Tm Hg IncP-3	19
R1162	Sm Su IncP-4	19
Rms163	Cm Su Tc IncP-5	19
Rms149	Cb Gm Sm Su IncP-6	19
Rms148	Sm IncP-7	19
FP2	Hg Pma IncP-8	19
R2	Cb Sm Su Uv IncP-9	19, 21
pSR1	Gm Km Su Tm Hg Pma IncP-10	This paper
R91-5	Cb IncP-10	6, 7
RP1-1	Cb (unclassified)	19
R716	Sm Hg (unclassified)	19

[a] Plasmid markers indicate resistance to carbenicillin (Cb), chloramphenicol (Cm), mercuric ion (Hg), gentamicin (Gm), kanamycin (Km), phenylmercuric acetate (Pma), streptomycin (Sm), sulfonamides (Su), tetracycline (Tc), tellurite (Ter), tobramycin (Tm), and trimethoprim (Tp). Inc, Incompatibility group.

[b] Strain Ros38 is a Gm Km Su Tm clinical isolate from Jacobi Hospital, New York, described by S. Rosenthal, J. Davies, L. Freundlich, and B. Hoffman (Abstr 205, 16th Intersci. Conf. Antimicrob. Agents Chemother., American Society for Microbiology, October, 1976). It was obtained from J. Davies, University of Wisconsin, Madison.

[c] Strain 9169 is a Cb Km Tc clinical isolate supplied by E. J. L. Lowbury (26).

Electron microscopy of DNA. Isolated plasmid DNA was nicked with 1,000 rads of gamma irradiation and spread for electron microscopy by the formamide technique of Davis et al. (10). Grids were stained with uranyl acetate, rotary shadowed (platinum, 80%; palladium, 20%), using a JEOLCO vacuum evaporator, and photographed with an RCA electron microscope. Phage PM2 DNA was used as the internal standard. Its molecular mass was taken to be 6.58 Mdal (30). Contour lengths were measured with a Hewlett-Packard digitalizer.

Restriction endonuclease digestion of DNA. *Eco*RI and *Bam*HI endonucleases were obtained from Miles Laboratory and New England BioLabs. Plasmid DNA was dialyzed against 10 mM Tris–1 mM EDTA, pH 7.45. Dialyzed DNA (40 μl) was mixed with 1 μl of restriction enzyme and 10 μl of 450 mM Tris–50 mM $MgCl_2$ (pH 7.4) and incubated for 2 h at 37°C. The digest was then mixed with 25 μl of 60% sucrose–1% bromophenol blue tracking dye and was subjected to

electrophoresis with Tris-acetate buffer (33) on a 0.7% agarose gel for 4 h at 50 mA, 100 V. An *Eco*RI digest of lambda DNA (λcI857*S7*) served as a standard.

RESULTS

Transfer of Cb from strain 9169. After a 2-h mating with a *P. aeruginosa* PAO recipient such as PU21, strain 9169 transferred Cb at a frequency of 1.5×10^{-3} per donor. Of 30 such Cb transconjugants, none also acquired Km or Tc. This singly resistant plasmid from strain 9169 was termed R91.

As reported by Chandler and Krishnapillai (6), transfer of Cb from strain 9169 to *E. coli* occurred at a low rate. After an 18-h mating with *E. coli* J53-2 or *E. coli* C Ilv$^-$, Cb transconjugants appeared at a frequency of 1×10^{-10} to 10×10^{-10} per donor. With both recipients, Cb transconjugants were also Km and Tc. This triply resistant plasmid from strain 9169 was termed R91a.

Neither PU21(R91) nor J53-2(R91a) was visibly lysed by phages PRR1, PRD1, Pf3, or PR4. PU21(R91) propagated phage G101 normally. Plasmid R91 was readily transmissible between *Pseudomonas* strains at a frequency of 1×10^{-3} to 3×10^{-3} per donor in a 2-h mating. Transfer of R91a between *E. coli* strains was occasionally detectable at a frequency on the order of 10^{-9} per donor after mating for 18 h. Transfer of R91 from *P. aeruginosa* to *E. coli* or of R91a from *E. coli* to *P. aeruginosa* by conjugation was not observed (frequency less than 10^{-9} per donor).

Incompatibility behavior of plasmids R91 and R91a. Although R91a did not confer susceptibility to phages active on P-group plasmids, it proved to have P-group incompatibility specificity. As shown in Table 2, IncP plasmid R751 transferred into an *E. coli* recipient carrying R91a at a reduced frequency and 0 of 20 Tp transconjugants retained R91a-determined Cb Km or Tc.

In *Pseudomonas*, however, plasmid R91 was compatible with R751 and other IncP-1 plasmids (Table 2). Twenty of 20 Cb transconjugants retained Tp determined by R751. Similarly R91 was compatible with IncP-1 plasmids pMG4 (15) or R702 and, furthermore, could be transferred independently from a donor carrying both R91 and R702 (data not shown).

Additional tests indicated that R91 or R91 derivatives were compatible with plasmids belonging to *Pseudomonas* Inc groups P-2 (R931), P-3 (RIP64), P-4 (R1162), P-5 (Rms163), P-6 (Rms149), P-7 (Rms148), P-8 (FP2), P-9 (R2), and unclassified plasmids RP1-1 and R716 (data not shown). However, R91 proved to be incompatible with plasmid pSR1 that determines Gm (gentamicin resistance) Km Su (sulfonamide resistance) Tm (tobramycin resistance) Hg (mercuric ion resistance) Pma (phenylmercuric acetate resistance) and that was found in a clinical *P. aeruginosa* isolate from New York. As shown in Table 2, R91 showed a reduced transfer frequency into a recipient carrying plasmid pSR1, and only 2 of 20 Cb transconjugants retained markers determined by pSR1. In a reciprocal cross, with plasmid R91-5 in the recipient, 0 of 20 Gm transconjugants retained Cb determined by R91-5 (Table 2). Like plasmid R91 plasmid pSR1 is compatible with plasmids belonging to Inc groups P-1 to P-9 (unpublished observations). We propose a new *Pseudomonas* Inc group P-10 for R91, R91-5, and pSR1.

Physical properties of plasmids R91 and R91a. By electron microscopy plasmid R91 from *P. aeruginosa* had a molecular mass of 35.0 ± 1.1 Mdal, whereas plasmid R91a from *E. coli* had a molecular mass of 38.7 ± 0.9 Mdal. These values differ significantly ($P < 0.02$), but the molecular mass of R91a is similar to that determined for IncP-1 plasmid RP1 (39 Mdal) by electron microscopy (4).

A more striking difference between the plasmids was evident after digestion with restriction endonucleases. The prototype IncP-1 plasmids RP1 and RP4 have a single *Eco*RI recognition site (18). Figure 1 shows that because of its size

TABLE 2. *Incompatibility studies with plasmids R91a, R91, and R91-5*

Donor	Recipient	Selection	Transfer frequency	Properties of transconjugants[a]
J53(R751)	C Ilv$^-$	Tp	7×10^{-3}	
J53(R751)	C Ilv$^-$ (R91a)	Tp	1×10^{-5}	0 of 20 Cb Km Tc
PAO303(R91)	PU21	Cb	3×10^{-3}	
PAO303(R91)	PU21(R751)	Cb	1×10^{-3}	20 of 20 Tp
PAO303(R91)	PU21(pSR1)	Cb	2×10^{-4}	2 of 20 Gm Su Hg
PU21(pSR1)	PAO303	Gm	3×10^{-4}	
PU21(pSR1)	PAO303(R91-5)	Gm	2×10^{-6}	0 of 20 Cb

[a] Transconjugants were selected after a 2-h mating at 37°C, purified, and tested for retention of markers carried by the plasmid present in the recipient (see Table 1, footnote *a* and text). In recipient controls the resident plasmid was uniformly stable.

the linear DNA fragment produced from RP4 by *Eco*RI digestion is not well separated from the largest *Eco*RI lambda fragment. Plasmid R91a gave a similar pattern to RP4, but plasmid R91 was cleaved by *Eco*RI into at least nine fragments (Fig. 1). Plasmid P91-5 gave an *Eco*RI digestion pattern identical to plasmid R91, and plasmid pSR1 was also cleaved at multiple sites. Endonuclease *Bam*HI also differentiates the plasmids. *Bam*HI cleaves R91a (or RP4) at a single site, whereas cleavage of plasmid R91 produced at least 10 fragments (data not shown).

Behavior of plasmid R91a in *P. aeruginosa*. Whereas the physical data strongly suggested that R91 and R91a were distinct plasmids, it was still desirable to show that the properties of the plasmids were host independent. Since they could not be transmitted intergenerically by conjugation, transformation with purified DNA was attempted. Transformation of an *E. coli* recipient with R91 DNA from *P. aeruginosa* was unsuccessful, but transformants were obtained using *P. aeruginosa* recipients and R91a DNA from *E. coli*. With either PAO303 or PU21 as recipient, transformants with Cb Km and Tc phenotypes were found. The antibiotic resistance phenotype of R91a is thus fully expressed in *P. aeruginosa*. Neither PAO303(R91a) or PU21(R91a) could transfer antibiotic resistance at a detectable frequency. As shown in Table 3, compatibility studies demonstrated that R91a in *Pseudomonas* is compatible with plasmid pSR1 but incompatible with plasmid R751, indicating that it retained IncP-1 specificity. Finally, Fig. 1 shows that PU21(R91a) has the same *Eco*RI digestion pattern as J53-2(R91a), and thus that the physical structure of the plasmid was also conserved after transfer from *E. coli* to *P. aeruginosa*.

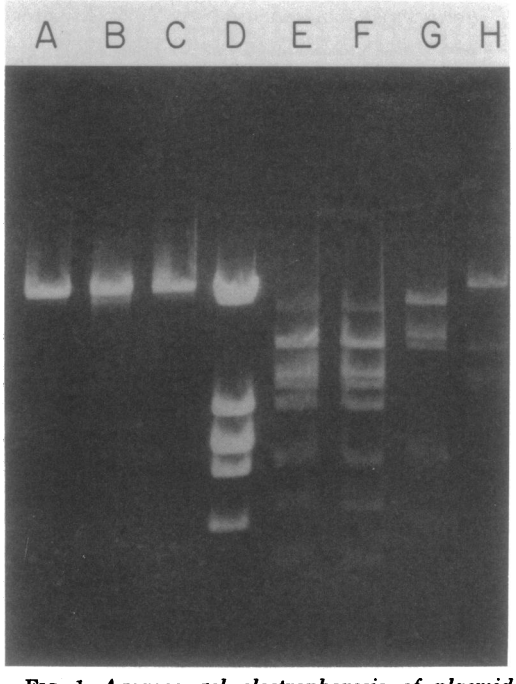

FIG. 1. *Agarose gel electrophoresis of plasmid DNA after EcoRI digestion. From left to right the plasmid sources were (A) J53(RP4), (B) J53(R91a), (C) PU21(R91a), (D) phage lambda standard, (E) PAO303(R91), (F) PAO303(R91-5), (G) PAO303-(pSR1), and (H) strain 9169.*

Properties of strain 9169. The simplest interpretation of these data is that strain 9169 contains two plasmids, the conjugative plasmid R91 determining Cb alone that belongs to IncP-10 and the virtually non-conjugative plasmid R91a determining Cb Km Tc that belongs to IncP-1. Physical and genetic studies confirmed this interpretation.

By agarose gel electrophoresis, strain 9169 contained a wide plasmid band in the 35- to 39-Mdal region. Figure 1 shows that when extrachromosomal DNA was purified from strain 9169 and subjected to *Eco*RI digestion, fragments characteristic of both plasmid R91 and R91a were present. Thus, strain 9169 must contain both R91 and R91a as separate plasmids of sufficiently similar molecular weights that the two plasmid bands are not resolved on electrophoresis of a cleared lysate.

Table 4 shows that when strain 9169 was used as a donor in an 18-h mating with a *Pseudomonas* recipient, Cb transconjugants appeared at a frequency of 3×10^{-1} per donor, whereas if tetracycline were used for selection, transconjugants were obtained at a frequency of 1×10^{-3} that were Cb Km Tc. Such triply resistant transconjugants transferred Cb alone at high fre-

TABLE 3. *Incompatibility studies with R91a in P. aeruginosa*

Donor	Recipient	Selection	Transfer frequency	Properties of transconjugants[a]
PAO303(pSR1)	PU21	Gm	7×10^{-4}	
PAO303(pSR1)	PU21(R91a)	Gm	3×10^{-4}	20 of 20 Cb Tc
PAO303(R751)	PU21	Tp	9×10^{-6}	
PAO303(R751)	PU21(R91a)	Tp	4×10^{-4}	0 of 20 Cb Tc

[a] See footnote *a* Table 2.

quency and Cb Km Tc at low frequency, like the parent 9169 strain, indicating that they contained both plasmid R91 as well as R91a. Thus, by genetic tests as well strain 9169 contained both plasmids.

The properties of plasmids RP1, R91a, R91, and R91-5 are summarized in Table 5.

DISCUSSION

Antibiotic-resistant isolates from Birmingham, England, have provided a wealth of plasmids useful for genetic studies in *P. aeruginosa*, including broad host range, IncP-1 plasmids such as RP1 (synonomous with R18 or R1822), RP2, R30, R68, and R88 that all determine Cb Km Tc but can be distinguished, in some cases, by other properties such as the ability to mobilize the *Pseudomonas* chromosome or their transfer frequency into a phage B3 lysogen (19, 23, 32). Not all of the original clinical isolates contained single plasmids. Strain 1822, in particular, contained both RP1 and a second plasmid, RP1-1, that determines only Cb and that differs from RP1 in host range, frequency of interstrain transfer, phage reactions, Inc specificity, and ability to be detected as extrachromosomal DNA (5, 14, 22, 31).

Our studies indicate that strain 9169 also contains two plasmids. One, R91a, determines Cb Km Tc, belongs to IncP-1, and has the size and *Eco*RI and *Bam*HI recognition sites characteristic of other IncP-1 Birmingham plasmids. R91a is unusual for an IncP-1 plasmid in that its transfer functions are expressed with barely detectable frequency. Like transfer-deficient mutants of RP1 (31), transfer of R91a alone by conjugation is virtually absent, and lysis of an R91a host by phages PRR1, PRD1, PR3, or PR4 is not observed. The second plasmid, R91, confers resistance only to Cb, belongs to an Inc group here defined as *Pseudomonas* group P-10, and has DNA sequences quite different from R91a, as indicated by its *Eco*RI and *Bam*HI endonuclease digestion patterns. Both R91 and R91a determine a TEM-2-type beta-lactamase (M. Matthew, personal communication), an enzyme known to be carried by a transposable sequence of DNA, Tn*1* (11), that is determined by *Pseudomonas* plasmids belonging to various Inc groups (G. A. Jacoby and M. Matthew, Plasmid, in press). Presumably R91 originated by transposition of Tn*1* to another plasmid present in the *P. aeruginosa* strains contributing to the Birmingham outbreak. Whether properties shared with IncP-1 plasmids, such as the ability of derepressed derivatives of R91 (for example, R91-5) to plate phages PRD1, PR3, and PR4 or to interfere with the propagation of phage G101 are fortuitously associated, remains to be determined.

The existence of R91a in strain 9169 initially escaped detection by genetic tests because the coexisting plasmid R91 predominates when Cb transconjugants are selected with a *Pseudomonas* recipient. The low frequency transfer of R91a appears to occur predominantly by mobilization, and since R91a, like other IncP-1 plasmids, has a broad host range, when strain 9169 is mated with an *E. coli* recipient, Cb Km Tc R91a transconjugants can be selected. These transconjugants carry a plasmid with resistance markers and Inc specificity different from Cb R91 transconjugants obtained in *Pseudomonas*, but the presence of two distinct plasmids in strain 9169 rather than an unusual host effect on gene expression by a single plasmid can account for the anomalous behavior of 9169 as a donor strain.

TABLE 4. *Selection of transconjugants from strain 9169 with carbenicillin or tetracycline*

Donor	Recipient	Selection	Transfer frequency[a]	Properties of transconjugants[b]
9169	PAO303 *rif*^{r}	Cb	3×10^{-1}	Cb
		Tc	1×10^{-3}	Cb Km Tc

[a] Mating was for 18 h at 37°C.
[b] See footnote *a*, Table 1.

TABLE 5. *Summary of properties of plasmids R91a, R91, and R91-5*

Plasmid	Resistance phenotype	Plate lysis with phages					Phi[a] G101	Tra[b]	Inc group	Mol wt ($\times 10^6$)	No. of *Eco*RI sites
		PRR1	PRD1	Pf3	PR3	PR4					
RP1	Cb Km Tc[c]	+	+	+	+	+	+	+	P-1	39[d]	1
R91a	Cb Km Tc	−	−	−	−	−	−	−	P-1	39	1
R91	Cb	−	−	−	−	−	−	+	P-10	35	At least 9
R91-5	Cb	−	+	−	+	+	+	++	P-10	ND[e]	At least 9

[a] Phi$^+$ (G101) plasmids inhibit the propagation of phage G101 (22).
[b] Tra$^-$ plasmids are transfer deficient, whereas Tra^{++} are derepressed for transfer.
[c] See footnote *a*, Table 1.
[d] As determined by electron microscopy (4).
[e] ND, Not determined by electron microscopy, but, by agarose gel electrophoresis (21), plasmids R91 and R91-5 have similar molecular weights.

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