Plasmid R394 is a Cointegrate

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Plasmids R394a and R394b which cointegrate to form R394 are described. They have molecular masses of 102 ± 4 and 11.0 ± 0.4 MDa, belong to the T and N incompatibility groups and confer resistance to kanamycin and ampicillin, respectively. R394a is self transmissible and mobilizes R394b, which is non-self transmissible. These findings clarify anomalies in the behaviour of R394 and support suggestions based on the properties of variant phages derived from R394.

INTRODUCTION

R factor R394 from *Proteus rettgeri* (Coetzee *et al.*, 1972) is a T group plasmid conferring kanamycin (Km) and ampicillin (Ap) resistance to host organisms. It has been used in various transduction studies involving *Proteus mirabilis* phage 5006M (Coetzee *et al.*, 1973; Coetzee, 1974; Coetzee, 1975*a*; Coetzee & Krizsanovich-Williams, 1976; Pretorius & Coetzee, 1980) and Providence phage PL25 (Coetzee, 1975*b*). It was also used in chromosome mobilization experiments involving *P. mirabilis* (Coetzee, 1979) and *Proteus morganii* (Beck & Coetzee, 1980). The β -lactamase determined by R394 has been studied (Hedges *et al.*, 1974; Matthew & Hedges, 1976).

Anomalous interactions of R394 with other plasmids have been observed, for example, in some experiments it appeared incompatible with N group plasmids R46 and R390, but in others was not incompatible (Coetzee *et al.*, 1972). This was interpreted in terms of 'dislodgement' and it was suspected that R394, although belonging to group T, contained N group DNA and that it was this segment that was excised under the influence of an N plasmid. The mechanism of dislodgement was not understood (Coetzee *et al.*, 1972).

Molecular studies (Pretorius & Coetzee, 1980) on the genome of *P. mirabilis* phages 5006M HFT *k* and 5006M HFT *ak*, containing the kanamycin and ampicillin resistance markers of R394 (Coetzee, 1974; 1975*a*) prompted characterization of R394. It was observed that R394-carrying strains contained two plasmids, and this report deals with this finding in relation to the anomalous properties of R394 and the structure of the above variant phages.

METHODS

Bacteria, plasmids and phages. *Escherichia coli* K12 strains: J53 (Clowes & Hayes, 1968); J53-1 (Coetzee *et al.*, 1972); J62 (Clowes & Hayes, 1968); J62-1 (Coetzee *et al.*, 1972); C600 r^-m^- (*E. coli* Genetic Stock Center); M259 r^-m^- (N. E. Murray, University of Edinburgh). *Staphylococcus aureus*: clinically isolated extracellular DNAase producing strain. Plasmids: R394, conferring resistance to ampicillin and kanamycin (Coetzee *et al.*, 1972); R401, an Inc group T plasmid, conferring resistance to ampicillin and streptomycin (Coetzee *et al.*, 1972); N3, an Inc group N plasmid, conferring resistance to streptomycin, spectinomycin, sulphonamides, tetracycline and mercury (Datta & Hedges, 1971); RP4, an Inc group P plasmid, conferring resistance to ampicillin, kanamycin and tetracycline (Datta *et al.*, 1971); R15, an Inc group N plasmid, conferring resistance to streptomycin,

sulphonamides and mercury (Datta & Hedges, 1971). Phages: phage τ , an Inc group T plasmid-dependent bacteriophage (Bradley *et al.*, 1981); phage PR4, specific for pili coded for by plasmids of Inc groups P, N and W (Stanisich, 1974).

Isolation of plasmids. This was done from *E. coli* strains (see above) using the method of Prakash *et al.* (1981).

Separation of plasmids and transformation. A plasmid R394 preparation (1 ml containing 300 μg DNA) was layered on to a 5–20% (w/v) sucrose gradient in TE buffer (10 mM-Tris/HCl pH 8, 1 mM-EDTA) and centrifuged for 1 h in a Spinco SW41 rotor at 200000 *g* at 4 °C. Eleven fractions of approximately 1.2 ml each were taken and 70 μl of the first and last fractions, containing approximately 1 μg DNA each, were adjusted to 30 mM- CaCl_2 in TEN buffer (20 mM-Tris/HCl pH 8, 1 mM-EDTA, 20 mM-NaCl). These were introduced independently into calcium-treated *E. coli* C600 r^-m^- cells as described by Cohen *et al.* (1972). Transformants were selected on MacConkey agar supplemented with 30 μg kanamycin or ampicillin ml^{-1} . Colonies were subsequently replicated to reciprocal plates.

Electron microscopy of DNA. The methods of Coetzee & Pretorius (1979) involving benzyldimethylalkylammonium chloride (BAC) were used. Plasmid RP4 DNA (molecular mass 36 MDal; Jacob *et al.*, 1977) was used as internal length standard for measurements of electron micrograph negatives which were rear-view projected. An Electronic Graphics calculator (Numonics Corp., Lansdale, Pa, U.S.A.) was used for measurements.

For demonstration of hairpin loop structures, plasmid (150 μg ml^{-1}) was denatured in 1 mM-Tris/HCl, pH 8, 13 mM-EDTA and 90% formamide at 60 °C for 30 min and left on the bench for 30 min. Formaldehyde was added to 2.5% and the mixture was kept on ice for 1 h. A 10^{-2} dilution was made into droplet solution (100 mM-sodium acetate, 50 mM-triethanolamine pH 8, 30% (v/v) formamide and 0.01% (w/v) BAC). Droplets of 50 μl were formed and processed for electron microscopy according to Coetzee & Pretorius (1979).

Temperature sensitivity. Plasmid-bearing strains (10^{-5} dilution of stationary phase stock) were grown overnight with aeration in nutrient broth (Oxoid, no. 2, CM67) at 25, 37 and 43 °C, plated on non-selective MacConkey agar and grown at 37 °C. Colony counts were made and plates replicated to MacConkey agar containing either kanamycin or ampicillin at 30 μg ml^{-1} . Replica colonies were scored.

Phage τ sensitivity. Phage τ was spot tested (Bradley *et al.*, 1981) at various concentrations (10^8 – 10^3 p.f.u. ml^{-1}) on lawns of bacteria carrying different plasmids.

DNAase production. Plasmid-bearing strains in exponential growth and a *Staphylococcus aureus* control strain were inoculated into DNAase test agar plates (Gibco Bio-cult Diagnostics), grown at 37 °C and flooded with 1 M-HCl. Clear zones revealed extracellular DNAase activity.

Transfer of R factors. Methods were as described by Datta *et al.* (1971) and Datta & Hedges (1971).

RESULTS

Identification of plasmids R394a and R394b. Plasmid isolates from R394 carrying *E. coli* strains J53, J53-1 and J62-1 revealed the presence of two plasmids. Those from strain J62-1 were separated by sucrose gradient centrifugation and transformation as described above. Two colonies that were resistant only to kanamycin contained a single plasmid, R394a. Transformants that were resistant only to ampicillin (2000 colonies) contained another plasmid, named R394b. Neither plasmid coded for resistance to any other antibiotic. No heavy metal resistance was observed.

No extracellular DNAase was detected in cultures of C600 r^-m^- (R394a), whereas Matsumoto *et al.* (1978) observed DNAase activity in strains carrying the T group plasmid Rts1.

Temperature sensitivity. R394a and R394b were retained in *E. coli* C600 r^-m^- at growth temperatures of 25, 37 and 43 °C, in agreement with Coetzee *et al.* (1972) with respect to R394 in *E. coli* J53. This is in contrast to the temperature sensitivity displayed by other plasmids of the IncT group (Coetzee *et al.*, 1972).

Electron microscopy. Purified plasmid DNA of R394a and R394b, co-mounted with plasmid RP4 DNA, facilitated molecular mass determinations. These were 102 ± 4 MDal (9 molecules) for R394a and 11.0 ± 0.4 MDal (15 molecules) for R394b.

Complete denaturation and limited renaturation of plasmid DNA revealed one hairpin loop structure in R394a, with dimensions of 800 and 900 base pairs for the stem and loop respectively. These are in agreement with those found by Pretorius & Coetzee (1980). No such structure was observed in R394b.

Conjugational transfer and incompatibility. R394a was transferred by conjugation between strains of *E. coli* K12, with an efficiency of about 10^{-4} per donor h^{-1} . R394b was not transmissible from strains carrying this plasmid alone, but ampicillin resistance was efficiently mobilized by R394b from donors carrying both plasmids.

Conjugational transfer of N3 into C600 $r^{-}m^{-}$ (R394b) led to elimination of ampicillin resistance, and transformation of M259 $r^{-}m^{-}$ (N3) by R394b DNA led to elimination of the resident plasmid. Thus R394b is an N plasmid. Phage PR4 did not lyse C600 $r^{-}m^{-}$ (R394b), thus this plasmid is probably unable to produce N pili, consistent with the observation that it is transfer defective.

Conjugation of R401 into strains carrying R394a led to loss of kanamycin resistance and the reciprocal transfer induced loss of the markers of R401. Thus, R394a is a plasmid of group T. This finding was confirmed by the observation that phage ϕ plated upon C600 $r^{-}m^{-}$ (R394a) but not upon C600 $r^{-}m^{-}$ (R394b).

Cointegrate formation and transposition. Strain J53, carrying R394a and R394b, was mated with J62(N3) (Table 1). Transfer of N3 led to the elimination of ampicillin resistance, but kanamycin resistance was retained. Hence, the two plasmids were maintained separately in this culture. Transfer of kanamycin resistance from J53(R394a)(R394b) to J62(N3) did not lead to co-transfer of ampicillin resistance or the loss of tetracycline resistance. Selection for transfer of ampicillin resistance to J62(N3) led to the elimination of tetracycline resistance from more than half of the recipients, but about 40% retained the markers of N3 (Table 1, experiment 5) and these were stable during growth in drug-free medium.

The seven transcipts which stably carried resistance markers of all three plasmids were examined. One (Table 1, experiment 6) appeared to carry R394a and a plasmid coding for resistance to ampicillin and tetracycline. The latter was incompatible with R15 but not with R394a (data not shown) and was therefore a plasmid of group N. It was presumably produced by recombination (perhaps transposition) between R394b and N3.

Table 1. *Conjugations to test compatibility properties*

Experiment	Donor	Recipient	Selective antibiotic*	Number of transcipts selected	Non-selected markers of transcipts†
1‡	J53(N3)	C600 $r^{-}m^{-}$ (R394b)	Tc	16	Ap ^s (16)
2	J53(R401)	C600 $r^{-}m^{-}$ (R394a)	Sm	8	Km ^s (8)
3	C600 $r^{-}m^{-}$ (R394a)	J53(R401)	Km	8	Sm ^s (8)
4	J62(N3)	J53(R394a)(R394b)	Tc	15	Km ^r Ap ^s (15)
5	J53(R394a)(R394b)	J62(N3)	{ Km Ap	8 31	{ Tc ^r Ap ^s (8) Tc ^s Km ^s (3); Tc ^s Km ^r (15) Tc ^r Km ^s (6); Tc ^r Km ^r (7)
6	One TcKmAp transcipt of conjugation 5	J53	{ Km Ap Tc	12 14 10	Ap ^s Tc ^s (11); Ap ^r Tc ^r (1) Km ^s Tc ^r (14) Ap ^r Km ^s (10)
7	A second TcKmAp transcipt of conjugation 5	J53	{ Km Ap Tc	16 16 10	Ap ^r Tc ^s (15); Ap ^s Tc ^s (1) Km ^r Tc ^s (14); Km ^s Tc ^s (2) Km ^s Ap ^s (10)
8	ApKmTc ^s transcipt of conjugation 7	J62(N3)	{ Ap Km	16 16	Km ^r Tc ^s (16) Ap ^r Tc ^s (14); Ap ^r Tc ^r (2)
9	J62(N3)	Donor strain used in conjugation 8	Tc	16	Ap ^r Km ^r (16)

* Antibiotics were incorporated into minimal agar containing supplements permitting growth of the recipient but not the donor strain. Antibiotics: Tc, tetracycline; Sm, streptomycin; Km, kanamycin; Ap, ampicillin.

† Figures in parentheses show the number of strains of each resistance pattern.

‡ The reciprocal to this transfer, that of R394b to a host carrying N3, was effected by transformation and led to elimination of the resident plasmid in all of ten cases studied.

The other six transciipients all behaved similarly to each other. On conjugation, two sorts of transciipients, Tc only or ApKm predominated (Table 1, experiment 7 gives typical results). The plasmid conferring resistance to tetracycline seems to be N3 unaltered, but the other two resistances appear to be conferred by a single plasmid. We interpret this as a cointegrate structure, since (Table 1, experiments 8 and 9) it is able to eliminate N3 but is not itself eliminable by entry of this plasmid.

DISCUSSION

Results indicate that plasmid R394 (Coetzee *et al.*, 1972) consists of two plasmids, R394a and R394b. The original interpretation of R394 was that this was a single plasmid with anomalous properties ascribed to a phenomenon termed 'dislodgement' (Coetzee *et al.*, 1972). We have shown that R394 consists of two separate plasmids each with a single resistance determinant, that are capable of forming cointegrate structures corresponding perhaps to transposition intermediates (Gill *et al.*, 1978). Pairs of plasmids which can exist either as separately replicating structures or as cointegrates may explain many of the anomalous results described as dislodgement. In its independent state a plasmid may be eliminated by an incompatible R factor that would be unable to effect the elimination of the cointegrate. Hence, the results of incompatibility testing will depend upon the state of the plasmids in the clone selected for testing and this may vary on different occasions.

The hairpin loop structure observed on R394a was first detected (Pretorius & Coetzee, 1980) on the genome of *P. mirabilis* phage 5006M HFT *k* (Coetzee, 1974). This phage is a derivative of high frequency transducing phage 5006M and originated from transduction of R394 to *P. mirabilis* strain PM5006 (Coetzee, 1974). The kanamycin resistance marker on the phage 5006M HFT *k* genome may be located on the hairpin loop structure (Pretorius & Coetzee, 1980) and the present observation is consistent with this. A similar structure was not evident on R394b, supporting the observation that the ampicillin resistance marker on the genome of phage 5006M HFT *ak* is not located in a hairpin loop structure (Pretorius & Coetzee, 1980). The latter phage, a derivative of phage 5006M HFT *k*, was obtained by transduction of the ampicillin resistance marker of R394 (Coetzee, 1975).

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