Bacteriophage M: an Incompatibility Group M Plasmid-specific Phage

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Phage M was specific for bacterial strains, of various genera, harbouring plasmids of the M incompatibility group. It formed turbid plaques which varied from pin point to more than 2 mm in diameter on all hosts where plaques were detected. The phage had an hexagonal outline with a diameter of 27 nm. It contained RNA but differed from other plasmid-dependent RNA phages in being sensitive to chloroform. It adsorbed along the length of shafts of M pili.

INTRODUCTION

The IncM plasmid incompatibility group (for Morgan, 1906) was established to accommodate plasmid R446b present in a strain of *Proteus morganii*, which was compatible with representative plasmids of all incompatibility groups tested (Hedges *et al.*, 1973). Datta (1975) and Novick *et al.* (1976) listed IncM and Com7, an incompatibility group named by Chabbert *et al.* (1972), as synonymous, but the tendency is to abandon the latter designation in favour of the former (Datta, 1979; Richards & Datta, 1979). Some plasmids from *Serratia marcescens* were described as being members of incompatibility group L (Hedges *et al.*, 1975), but these were shown to be typical members of group M with molecular masses of 40–65 MDal (Richards & Datta, 1979) and plasmids ascribed to the former group are now included in the latter.

IncM plasmids code for pili which are rigid and belong to morphological group 3 (Bradley, 1980*b*). These plasmids render bacterial hosts sensitive to the wide host-range filamentous phage X (Bradley *et al.*, 1981*a*). Here is reported the isolation and some properties of a new phage specific for bacteria carrying these plasmids.

METHODS

Bacteria and plasmids. *Escherichia coli* strains were J53, J53-2, J62-1 (Datta *et al.*, 1980) and JE2571 (Bradley, 1980*a*). *Klebsiella pneumoniae* strain G162 and *K. oxytoca* strain G147 were provided by Dr I. Simpson, Glaxo Research, Greenford, Middlesex, U.K. The *Salmonella typhimurium* strain was M827-2, a spontaneous *rif* mutant of LT2 strain M827 (Spratt *et al.*, 1973); *Serratia marcescens* Sr41 (Matsumoto *et al.*, 1973) was provided by Dr H. Matsumoto. *Proteus mirabilis* strain PM5006 (Coetzee & Smit, 1970) and *P. morganii* 2815 (Coetzee, 1972) were also used. Plasmids originated from the collection of Naomi Datta (Datta, 1977, 1979). Apart from plasmids belonging to the IncM incompatibility group (see Table 1), only those which encoded constitutive pilus synthesis (as judged by a transfer frequency of greater than 10^{-3} per bacterium h^{-1} or by plaque formation by appropriate plasmid-specific phages) were used. Thus one or more plasmids belonging to Inc groups B (Coetzee *et al.*, 1982), C (Sirgel *et al.*, 1981), FI to FIV (Jacob *et al.*, 1977), F₀lac (Bradley *et al.*, 1981*c*), HII (Bradley *et al.*, 1982*b*), I₁, I₂ (Coetzee *et al.*, 1982), J (Bradley *et al.*, 1982*a*), N, P (Coetzee *et al.*, 1979), T (Bradley *et al.*, 1981*b*), W (Coetzee *et al.*, 1979) and X (Bradley *et al.*, 1981*a*) were employed.

Bacteriophages. Lysis of bacterial strains by plasmid-specific phages MS2 (Davis *et al.*, 1961), C-1 (Sirgel *et al.*, 1981), F₀lac (Bradley *et al.*, 1981*c*), PR64FS (Coetzee *et al.*, 1980), J (Bradley *et al.*, 1982*a*), t (Bradley *et al.*, 1981*b*) or X (Bradley *et al.*, 1981*a*) were used to demonstrate constitutive pilus production by strains carrying appropriate

Table 1. *Host range of phage M*

Drops of phage suspension (titre 5×10^9 p.f.u. ml⁻¹) were spotted on lawns of organisms contained in top-layer agar. Phage propagation was determined as described in Methods.

Host	Plasmid Inc group	Plaque formation by phage:*		Plasmid reference
		M	X	
<i>E. coli</i> J53(RIP69)	M	+	+	Witchitz & Gerbaud (1972)
<i>K. pneumoniae</i> G162(RIP69)	M	+	+	Witchitz & Gerbaud (1972)
<i>K. oxytoca</i> G147(RIP69)	M	+	+	Witchitz & Gerbaud (1972)
<i>S. typhimurium</i> M827-2(RIP69)	M	+	+	Witchitz & Gerbaud (1972)
<i>Sr. marcescens</i> Sr41(RIP69)	M	+	+	Witchitz & Gerbaud (1972)
<i>P. morgani</i> 2815(RIP69)	M	+	+	Witchitz & Gerbaud (1972)
<i>P. mirabilis</i> PM5006(RIP69)	M	—(—)	—(+)	Witchitz & Gerbaud (1972)
<i>E. coli</i> J53(R446b)	M	+†	—(+)	Bradley <i>et al.</i> (1980)
<i>K. pneumoniae</i> G162(R446b)	M	+	+	Bradley <i>et al.</i> (1980)
<i>E. coli</i> J53(R831b)	M	+	+	Bradley <i>et al.</i> (1980)
<i>P. mirabilis</i> PM5006(R831b)	M	—(—)	—(+)	Bradley <i>et al.</i> (1980)
<i>Sr. marcescens</i> Sr41(R831b)	M	+	+	Bradley <i>et al.</i> (1980)
<i>E. coli</i> J53(R471)	M	—(—)	—(+)	Richards & Datta (1979); Hedges (1980)
<i>K. oxytoca</i> G147(R471)	M	+	+	Richards & Datta (1979); Hedges (1980)
<i>K. pneumoniae</i> G162(N3)	N	—	+	Hedges (1972)
<i>K. pneumoniae</i> G162(RP4)	P	—	+	Coetzee <i>et al.</i> (1979)
<i>K. pneumoniae</i> G162(RSa)	W	—	+	Coetzee <i>et al.</i> (1979)

* +, Plaque formation to titre; —, no visible action. Signs in parentheses indicate results of bacteriophage propagation experiments.

† Approximately 30-fold lower efficiency of plating.

plasmids. Phages C-1 and X were also used as controls in RNAase-chloroform and RNAase-diethyl ether sensitivity tests. The former phage is sensitive to RNAase but resistant to the organic solvents (Sirgel *et al.*, 1981), while the latter is resistant to RNAase and diethyl ether but sensitive to chloroform (Bradley *et al.*, 1981a).

Media, antibiotics, transfer of plasmids by conjugation, general phage techniques, production of phage lysates and organic solvent sensitivity of phage. These were done as described and referred to by Coetzee *et al.* (1979) and Ackermann *et al.* (1978). The indicator organism for phage M (see below) was G162(RIP69); J62-1(P-lacR1drd19) (Sirgel *et al.*, 1981) and J53(R6K) (Bradley *et al.*, 1981a) were the indicator strains for phages C-1 and X, respectively. The incubation temperature was 37 °C.

Phage isolation. Sewage specimens were obtained from various sewage works in the Pretoria area. Attempts were made to isolate a RIP69 plasmid-specific phage from sewage according to the method of Bradley *et al.* (1981a). The pairs of organisms used for enrichment of sewage with subsequent reciprocal testing for phage activity of the supernatants were J53-2(RIP69), M827-2(RIP69) and G162(RIP69), G147(RIP69). All enriched samples were then treated as before.

Ribonuclease sensitivity. This was done according to Bradley *et al.* (1981a) using the same phages and corresponding indicator strains mentioned above.

Bacteriophage propagation. Propagation of phages M (see below) and X was done as described by Coetzee *et al.* (1982). Briefly, an exponentially growing culture of the donor plasmid-bearing organism under test (0.1 ml) was mixed with an equal volume of stationary phase recipient (strains J53 or PM5006) on a Millipore membrane resting on nutrient agar. After overnight incubation, the growth was gently washed off by suspending the membrane in 5 ml warm broth. To 1 ml of this broth suspension, possibly containing transconjugants temporarily derepressed for pilus synthesis, were added 0.1 ml phage suspension (1×10^{10} p.f.u. ml⁻¹) and 4 ml soft top-layer and the mixture was poured over the surface of a nutrient agar plate and allowed to set. After overnight incubation, the phage was harvested and the titre on strain G162(RIP69) was determined. For the J53 system the recipient was J53 and donors were J53(R471) or J53(R446b). Donors and recipients for experiments with PM5006 were PM5006(RIP69) or PM5006(R831b) and PM5006, respectively. Negative donor controls were J53 and PM5006, while the positive donor control was J53(RIP69) for both systems.

Concentration of phage. Phage M lysates were concentrated using polyethylene glycol (mol. wt 6000) and NaCl at final concentration of 11% (w/v) and 0.5 M, respectively (Yamamoto *et al.*, 1970). Pellets were suspended in small volumes of distilled water.

Electron microscopy. We found that when a culture of *E. coli* strain JE2571(R446) infected with phage M was shaken in broth, the culture fluids contained many free M pili with phage M virions attached. Accordingly, to demonstrate adsorption, 40 ml Brain Heart Infusion Broth was inoculated with an overnight broth culture of JE2571(R446b) to a concentration of 5×10^7 organisms ml⁻¹ and grown with shaking to a concentration of 1×10^8 organisms ml⁻¹. Phage M (5 ml) at a titre of 1×10^9 p.f.u. ml⁻¹ was then added and growth continued to a cell concentration of 2×10^9 organisms ml⁻¹. No visible lysis occurred. After removing bacteria by centrifugation, a carbon-coated electron microscope specimen support grid was touched on the surface. The grid was washed by floating on successive baths of 0.1 M-ammonium acetate solution. Negative staining was done with a mixture of equal volumes of 2% (w/v) sodium phosphotungstate and 0.1 M-ammonium acetate solutions. To investigate the effect of chloroform on the structure of the phage M virions, 5 ml phage suspension was shaken with 5 ml chloroform in a conical flask at room temperature for 2 h at 300 oscillations min⁻¹. After standing for 30 min to allow the layers to separate, specimens were prepared for electron microscopy as described above. Magnification calibration was done with catalase.

RESULTS

Isolation of a RIP69-dependent bacteriophage

From the fifth sample of sewage [enriched with G162(RIP69)] a phage was isolated on G147(RIP69) which formed plaques on the former strain but not on isogenic strains lacking the plasmid. The phage was named M and was routinely propagated on G162(RIP69).

Properties of phage M

Plaque morphology. The phage formed turbid plaques on all hosts where plaque formation was evident. Plaques varied from about 2 mm in diameter to less than 1 mm and the large plaques were usually irregular in outline.

Phage morphology and adsorption site. Phage M was morphologically similar to other RNA-containing bacteriophages (Fig. 1*a, b*), having a semitransparent appearance in negatively stained preparations and a diameter of 27 nm. The phage adsorbed more or less evenly along the whole of the M pilus shaft (Fig. 1*a, b*). Some virions had obviously become detached from pili during specimen preparation. A lysate of strain JE2571(R446b) contained many free M pili, 88% of which had one or more adsorbed virions (100 pilus sample). When 100 phage virions were scored, 81% were attached to pili. Phage virions less than 2 diameters from a pilus were considered attached (see Bradley *et al.*, 1981*b*; Coetzee *et al.*, 1982).

Sensitivity to organic solvents or RNAase. The plaque-forming titre of a phage M suspension was reduced by 80% as a result of chloroform treatment, whereas the titre of phage C-1 was unaffected. The titre of phage X was reduced by more than 99% following similar exposure. Titres of phage M and the latter two phages were not reduced by diethyl ether treatment. Plaque formation by phages M and C-1 was totally inhibited by RNAase, whereas that of phage X was unaffected.

Chloroform treatment of the phage M lysate described above (see Methods) detached all virions from the M pili. Phage inactivation appeared to be due to rupture of the capsid, this being the structural change observed; not all virions were completely disintegrated. A typical example (Fig. 1*c*) shows a virion with the break in the capsid arrowed. A count of 50 chloroform-treated virions revealed that 42 were ruptured, 8 remaining apparently intact. This agreed closely with the 80% reduction in titre that was obtained (see above). No ruptured particles were seen in control suspensions shaken in the absence of chloroform.

Host range. Unlike the filamentous phage X (Bradley *et al.*, 1981*a*) which also plated on *Klebsiella* strain G162 carrying plasmids belonging to Inc groups N, P and W, phage M plated only on strains harbouring plasmids of the M group (Table 1 and not shown) and not on any other strains carrying plasmids belonging to other incompatibility groups tested. The M plasmid-bearing strains were derived from a number of bacterial genera. Neither phage X nor M formed plaques on *P. mirabilis* PM5006 harbouring various IncM plasmids (Table 1 and not shown). The latter strains also failed to propagate phage M (Table 1 and not shown) although

Fig. 1. (a) RNA phage M virions adsorbed along the shafts of M pili, from a lysate of *E. coli* strain JE2571(R446b) grown in nutrient broth. (b) High magnification electron micrograph of phage M virions showing similarity to other RNA phages. The virions near the bottom are presumed to have become detached from the pilus during specimen preparation. (c) Phage M virion with a break (arrow) in its capsid after chloroform treatment (see Methods). The bar markers represent 100 nm.

phage X registered a titre increase (Table 1 and not shown). The phages did not plate on *E. coli* strain J53 carrying the IncM plasmid R471 although this strain propagated phage X. Plasmid R446b conferred only partial phage sensitivity on this strain of *E. coli* (Table 1 and see Bradley *et al.*, 1981a). Plaque formation was evident on *Klebsiella* strains carrying either of these plasmids (Table 1).

DISCUSSION

Although M plasmids have been identified in a wide range of bacterial species including members of the *Escherichieae* and *Proteeae* (Hedges *et al.*, 1973; Chabbert & Roussel, 1977), they seem to be most closely associated with the *Klebsielleae* and form a high proportion of the R plasmids of this tribe (Witchitz & Gerbaud, 1972; Severn, 1977; Davey & Pittard, 1977; Romero *et al.*, 1978; Richards & Datta, 1979; Datta *et al.*, 1979; O'Brien *et al.*, 1980; Hedges, 1980). In all the above cases, M plasmids conferring significant resistance to antibiotics in hospital outbreaks were detected in members of the *Klebsielleae* but transfer to bacteria of other tribes was noted in most instances. Since a phage dependent for adsorption upon the pili determined by M plasmids must be expected to be adapted for growth in those cells in which such plasmids occur, we chose a *K. pneumoniae* strain carrying M plasmid RIP69 as host for selection of the new phage. This precaution proved unnecessary since phage M could grow on M⁺ strains of at least three other tribes of *Enterobacteriaceae* (Table 1). Thus, phage M is adapted to exploit the host range of M plasmids like phage PRR1 which lyses many hosts of the wide

host-range IncP-1 plasmids (Olsen & Thomas, 1973). The capacity of *Pseudomonas* strains to serve as hosts for the phage could not be tested as IncM plasmids do not transfer to these strains (J. N. Coetzee, unpublished; Jacoby, 1975).

Plasmids RIP69 and R831b clearly encoded constitutive production of phage receptors in all strains tested apart from *P. mirabilis* PM5006 (Table 1 and not shown; Bradley *et al.*, 1980; 1981*a*). The phenomenon of plasmid-specific phages not forming plaques or not propagating on species of all bacterial genera harbouring a plasmid which confers sensitivity, has been encountered repeatedly (Olsen & Thomas, 1973; Bradley, 1977; Bradley *et al.*, 1981*a*; Coetzee *et al.*, 1979; Sirgel *et al.*, 1981; Hua *et al.*, 1981). The behaviour of plasmids RIP69 and R831b in *P. mirabilis* PM5006 and plasmids R471 and R446b in *E. coli* strain J53 may be further examples (Table 1).

With the discovery of phage M, IncM plasmid-coded pili join those of Inc groups C (Sirgel *et al.*, 1981), FI to FIV (Jacob *et al.*, 1977; Datta, 1979; Dennison & Hedges, 1972), *F₀lac* (Bradley *et al.*, 1981*c*), I, (Coetzee *et al.*, 1982), P-1 (Olsen & Thomas, 1973) and T (Bradley *et al.*, 1981*b*) in possessing adsorption sites for specific RNA-containing phages.

M pili are the only rigid pili (Bradley, 1980*b*) described to date which possess RNA phage adsorption sites, except for P-1 pili, which are thinner than M pili (Bradley, 1980*b*) and which adsorb the RNA-containing phage PRR1 (Olsen & Thomas, 1973). Structural differences between phage M and the RNA phages adsorbing to the flexible pili mentioned above would thus possibly not be unexpected. The only such difference encountered was the sensitivity of the phage to chloroform (Fig. 1*c*).

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