Bacteriophage X-2: a Filamentous Phage Lysing IncX-Plasmid-harbouring Bacterial Strains

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Phage X-2, a filamentous rod about 950 nm in length, was isolated from sewage as plating on strains of *Escherichia coli*, *Salmonella typhimurium* or *Serratia marcescens* carrying either the IncX plasmid R6K, or the unique plasmid R775. Phage X-2 differs morphologically from a previously described very broad host range filamentous phage X which also lyses plasmid R6K-carrying strains and the phages differ in their resistance to inactivation by diethyl ether. Phage X-2 is serologically unrelated to phage X and the X-like phages IKe and I₂-2. The adsorption site of the phage on the plasmid-bearing strains could not be determined but evidence implicating conjugative pili is presented.

INTRODUCTION

The IncX group of plasmids (Hedges *et al.*, 1973) was established with plasmid R6K (Kontamichalou *et al.*, 1970) and the apparently identical pair of plasmids, R485 and R487 (Hedges *et al.*, 1973), as members. Plasmid R6K, the prototype, has a mass of 26 MDa (Kontamichalou *et al.*, 1970) and codes for thick flexible pili belonging to morphological group 2 of Bradley (1980). The group now comprises eight members and conjugative pilus synthesis is repressed in all members apart from plasmid R6K, which forms these pili constitutively (Bradley *et al.*, 1981a).

The filamentous phage X was isolated from sewage as plating on an *Escherichia coli* host carrying plasmid R6K (Bradley *et al.*, 1981a). Apart from its specificity for IncX-harbouring bacteria, it differed from other filamentous phages in other respects: phage X has a frankly curly appearance (Bradley *et al.*, 1981a; Bradley *et al.*, 1983) compared to filamentous phages like f1 (Caro & Schnös, 1966), IKe (Khatoun *et al.*, 1972; Bradley *et al.*, 1983), PR6FS (Bradley, 1983), C-2 (Bradley *et al.*, 1982a), SF (Coetzee *et al.*, 1986) and tf-1 (Coetzee *et al.*, 1987). Secondly the phage had a very broad host range in that it plated on bacterial strains harbouring plasmids of Inc groups I₂, M, N, P-1, U and W. This host range partially overlaps that of phages IKe and I₂-2 (Coetzee *et al.*, 1982) and these phages are here named X-like for that reason (see Bradley *et al.*, 1983).

During a search for an RNA-containing phage specific for IncX plasmid-containing bacteria, another filamentous phage adsorbing to IncX plasmid-bearing bacterial strains was isolated. Here properties of this phage are described.

METHODS

Bacteria. *E. coli* strains used were JE2571 and the corresponding nalidixic-acid- and rifampicin-resistant (rif^R) mutants JE2571-1 and -2, respectively (Bradley, 1980, 1985). The *Salmonella typhimurium* strain was M827-2, a

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spontaneous rif^R mutant of the LT2 strain M827 (Spratt *et al.*, 1973). Sr41-2 is a spontaneous rif^R mutant (J. N. Coetzee, unpublished) of *Serratia marcescens* strain 41 (Matsumoto *et al.*, 1973). *Klebsiella pneumoniae* G162 and *K. oxytoca* G147 were provided by Dr I. Simpson (Glaxo, Greenford, Middlesex, UK). Members of the tribe *Proteae* used were *Proteus mirabilis* PM5006 (Coetzee *et al.*, 1979), *P. rettgeri* NCTC8893 (Coetzee, 1983), *P. morganii* 2815-1 (Coetzee *et al.*, 1985b) and *Providencia alcalifaciens* P29-2 (Coetzee *et al.*, 1985a).

Plasmids and bacteriophages. Plasmids were from the collection of N. Datta (Datta, 1979). Some of the plasmids (Bradley *et al.*, 1981a) are listed in Table 2. Strain JE2571, carrying the IncF1 plasmid R386 (Dennison, 1972) was the indicator for phage fd (see below). Other plasmids belonging to Inc groups C (Sirgel *et al.*, 1981), D (Coetzee *et al.*, 1985a), HI and HII (Coetzee *et al.*, 1985b), J (Bradley *et al.*, 1982a), S (Coetzee *et al.*, 1986) and T (Bradley *et al.*, 1981b; Coetzee *et al.*, 1987) were also used in phage host-range experiments. Constitutive production of conjugative pili by the above plasmids was assessed by the ability of corresponding plasmid-specific phages, described in the same references, to form plaques on lawns of the plasmid-bearing organisms. Filamentous phage X (Bradley *et al.*, 1981a) the X-like phages IKe (Khatoon *et al.*, 1972) and I₂-2 (Coetzee *et al.*, 1982; Bradley *et al.*, 1983), phages PR64FS (Coetzee *et al.*, 1980), fd (Marvin & Hoffmann-Berling, 1963), SF (Coetzee *et al.*, 1986) and tf-1 (Coetzee *et al.*, 1987) were compared serologically with phage X-2 (see Results). The RNA-containing phage C-1 (Sirgel *et al.*, 1981) and filamentous phages X, IKe, PR64FS and fd were used as controls in organic solvent and RNAase susceptibility tests. Phage PR64FS was also used to assess constitutive production of conjugative pili by plasmids of IncI incompatibility complex (see Table 2) while phages IKe and I₂-2 were also tested for possible lytic action on bacterial strains harbouring the IncX plasmid R6K.

Media and antibacterial agents. These were according to Coetzee *et al.* (1979).

General phage techniques and production of phage lysates. The methods of Adams (1950) were used. The incubation temperature was 37 °C, except for phages D (Coetzee *et al.*, 1985a) or pilHα (Coetzee *et al.*, 1985b) when the temperature was 26 °C. Plates were incubated overnight.

Selection of phage-resistant clones of strain JE2571(R6K). Selective top-layer nutrient agar (5 ml) containing ampicillin (40 µg ml⁻¹) and streptomycin sulphate (30 µg ml⁻¹) were seeded with about 4 × 10⁶ organisms of the plasmid-carrying strain in the presence of 1 × 10¹⁰ p.f.u. phage X-2 before pouring over the surface of a nutrient agar plate. After setting, the plate was incubated. Overgrowing colonies on the otherwise relatively clear surface were then picked off and purified by repeated single colony isolation on selective nutrient agar.

Ribonuclease and organic solvent sensitivity of phage suspensions. The former was assessed as described by Bradley *et al.* (1981a). The latter tests involved the mixing of solvent and broth lysate of phage 1:3 (v/v) in flat stoppered vessels and shaking in a shaker at two oscillations s⁻¹ at 25 °C for 1 h as recommended by Ackermann *et al.* (1978).

Phage isolation. Sewage samples (obtained from various sewage works in the Pretoria-Johannesburg area) were enriched with broth cultures of JE2571-2(R6K) or M827-2(R6K) according to the method of Bradley *et al.* (1981a). Dilutions of the supernatants of the lysates were then spotted on lawns of the above two plasmid-carrying strains growing on nutrient agar and on the same medium containing 25 µg RNAase ml⁻¹ (BDH). As an RNA-containing phage was being sought (see Introduction) this approach enabled such prospective phages to be detected immediately by noting the absence of plaques on the RNAase-containing member of a pair of plates (see Coetzee *et al.*, 1985a).

Transfer of plasmids by plate mating. This was done by the method of Bradley *et al.* (1980).

Phage antiserum. Ten injections of 1 ml of broth suspensions of phages X and X-2 (see Results) (titres > 1 × 10¹⁰ p.f.u. ml⁻¹) were given intravenously at 2 d intervals to groups of three rabbits respectively. After resting the animals for 15 d, a booster injection of 5 ml of the corresponding phage suspension was administered subcutaneously; 10 d later, blood was collected from the animals and the serum warmed at 56 °C for 30 min to inactivate complement.

Phage growth on bacterial strains harbouring plasmids repressed for pilus synthesis. The methods of Coetzee *et al.* (1986) were used employing newly formed transconjugants, produced by the temporary derepression methods of Bradley *et al.* (1980) and Bradley & Fleming (1983), of the plasmids under study.

Electron microscopy. Techniques for visualizing phage-pilus and bacterium-phage mixtures were as described by Bradley (1980) and Bradley *et al.* (1981a, 1982a). For immune electron microscopy, grid-labelling with antiserum to phages X, X-2 (see Results) or IKe_h (anti-IKe_h kindly supplied by R. B. Grant) was done by the method of Lawn (1967).

RESULTS

Transfer of plasmids

The IncX plasmid R6K transferred on agar surfaces from *E. coli* strain JE2571-1 to *S. typhimurium* M827-2, *Serratia marcescens* Sr41-2 or *K. pneumoniae* G162 and *K. oxytoca* G147 at frequencies > 8 × 10⁻³ transconjugants per donor h⁻¹. Similar frequencies were recorded for transfer of the plasmid from these transconjugants back to *E. coli* strain JE2571-1. Transfer of

Fig. 1. Phage X-2 virions. Bar, 100 nm.

this plasmid between the *E. coli* or *S. typhimurium* strains and *P. mirabilis* PM5006, *P. morganii* 2815-2, *P. rettgeri* NCTC 8993 or *Pr. alcalifaciens* P29-2 occurred at much lower frequencies of about 5×10^{-5} transconjugants per donor h^{-1} . Similar low frequencies were recorded for the passage of plasmid R6K from the latter transconjugants back to *E. coli* JE2571-1 or *S. typhimurium* M827-2. The remainder of the IncX plasmids (Table 2) transferred between all the above strains also at frequencies of about 5×10^{-5} transconjugants per donor h^{-1} , in keeping with their repressed state of conjugative pilus synthesis (Bradley, 1980; Bradley *et al.*, 1981a).

Each of ten phage-X-2-resistant clones (see below) of JE2571(R6K) in matings with strain JE2571-1 failed to yield transconjugants: i.e. the transfer frequency was $< 4 \times 10^{-8}$ transconjugants per donor h^{-1} .

Isolation of a JE2571(R6K)-dependent bacteriophage

Many hundreds of enriched sewage samples were examined over a period of 7 years but none yielded a prospective RNA-containing phage which only plated on the non-RNAase containing member of a pair of plates. Occasionally, a lysate contained a phage which plated equally well on both the *E. coli* and *S. typhimurium* indicator strains. Some of these exceptional lysates were investigated. They usually yielded tailed somatic-adsorbing phages which infected members of the above two genera, or a phage similar to phage X (J. N. Coetzee, unpublished). One such lysate contained a phage which differed from phage X in many respects (see below). The phage was named phage X-2 and was routinely propagated on M827-2(R6K). Plaque-forming titres of $> 1 \times 10^{10} \text{ ml}^{-1}$ were regularly obtained.

Properties of phage X-2

Plaque morphology. The phage formed turbid plaques on susceptible organisms. The plaques were very uniform, about 1.1 mm in diameter, and did not vary on different hosts. The plaques were more turbid and slightly larger than those produced by phage X.

Morphology and adsorption site. The phage X-2 virion was filamentous, undulating, and had a thickness of about 10 nm. Measurement of 53 virions gave a mean length of $950 \pm 30 \text{ nm}$ and both ends were slightly pointed with no additional terminal structures evident (Fig. 1).

Table 1. *Immune electron microscopy of X-like phages*

For antisera to phages X-2 and X, see Results. Antiserum to phage *IKeh*, a broad host range mutant of phage *IKe* (Grant *et al.*, 1978), was kindly supplied by R. B. Grant. Grid-labelling of phages with antisera was done according to Lawn (1967).

Phage	Labelling with antiserum to phage:		
	X	X-2	<i>IKeh</i>
X	+	—	—
X-2	—	+	—
<i>IKe</i>	—	—	+
<i>I</i> ₂ -2	—*	ND	+*

ND, Not done.

+, Immunoglobulin labelling; —, no labelling.

*, Results from Bradley *et al.* (1983).

All attempts to demonstrate adsorption of phage X-2 to X pili or R775 pili (see below) in the electron microscope failed. This applies to studies of many pili which were attached to producer cells and large numbers of loose-lying pili. Even after 5 h incubation, mixtures of virions and X or R775 conjugative pili showed the objects separated and not forming complexes. A possible relationship between plasmid R6K conjugative pilus formation and phage X-2 susceptibility of the plasmid-harboring strain JE2571 was established by examining two phage-resistant colonies of JE2571(R6K) investigated for conjugal transfer of the plasmid (see above). Bacteria from the colonies were grown under optimum conditions for pilus production (3 h on Brain Heart nutrient agar) and then examined for the presence of pili by electron microscopy. No pili were detected in preparations of either of the two colonies (not shown).

Phage serology. One of the rabbits yielded a phage X anti-serum with a *K* value of 110 min⁻¹ and an anti-phage X-2 serum with a *K* value of 225 min⁻¹ was obtained from another rabbit. Neither of the antisera had any neutralizing effect on the heterologous phage. The two phages thus had antigenically different adsorption sites. Neither of the antisera had a neutralizing effect on the other filamentous phages tested, namely the X-like phages *IKe* and *I*₂-2 and phages PR64FS, fd, SF and tf-1.

Immune electron microscopy (not illustrated; results listed in Table 1) proved that the remaining surfaces of phages X-2, X and *IKe* were different. By inference, phage *I*₂-2 which is serologically closely related to phage *IKe* (Bradley *et al.*, 1983) also differed from phages X-2 and X.

Sensitivity to RNAase and organic solvents. The plating titre of filamentous phages X-2, X, *IKe* and fd on their respective hosts was unaffected by the presence of 25 µg RNAase ml⁻¹ in media while that of the positive control, phage C-1, was reduced to zero.

Treatment with chloroform reduced the titres of phages X-2, X, *IKe* and fd to zero. Phage C-1 was unaffected. Phages X-2, *IKe*, fd and C-1 were refractory to diethyl ether but this solvent reduced the titre of phage X by about 95%.

These results are compatible with the filamentous morphology of phage X-2 and the reactions of the control phages, with the exception of phage X, were as expected (see Amako & Yasunaka, 1977). Results of the latter phage, with regard to diethyl ether treatment, are at variance with those previously reported (Bradley *et al.*, 1981*a*) and could be explained by the fact that, as was the case with chloroform (Coetzee *et al.*, 1979), exposure of phage X to diethyl ether was also only for 10 min.

Host ranges of phages. Phage X-2 only plated on *E. coli*, *Sr. marcescens* or *S. typhimurium* strains carrying the IncX plasmid R6K or the unique plasmid R775 (Table 2). In keeping with the repressed state of plasmid R6K pilus synthesis in strains of *Proteus*, *Morganella*, *Rettingerella*

Table 2. *Host range of phages X-2 and X*

Dilutions of phage suspensions (about 2×10^{10} p.f.u. ml⁻¹) were dropped on lawns of organisms contained in top-layer agar. Plates were incubated overnight at 37 °C. Phage propagation and temporary derepression spot tests were done as referred to in Methods. Phage action: +, plaque formation; -, no visible action; (+), positive phage propagation and temporary derepression spot tests obtained in at least two experiments; (-), negative phage propagation and temporary derepression spot tests obtained in three experiments.

Host	Plasmid Inc group	Phage action		Reference
		X-2	X	
JE2571(R6K)	X	+	+	Bradley (1980)
M827-2(R6K)	X	+	+	
SR41-2(R6K)	X	+	+	
G162(R6K)	X	-(-)	+	This investigation
G147(R6K)	X	-(-)	+	
PM5006(R6K)	X	-(+)	-(+)	
2815-2(R6K)	X	-(+)	-(+)	
NCTC8893(R6K)	X	-(+)	-(+)	
P29-2(R6K)	X	-(+)	-(+)	
JE2571-1(R485)	X	-(+)	-(+)	Bradley (1978)
M827-2(pHH1187)	X	-(+)	-(+)	
JE2571-2(TP231)	X	-(+)	-(+)	Bradley (1980)
M827-2(TP227)	X	-(+)	-(+)	
M827-2(TP228)	X	-(+)	-(+)	
JE2571(PVS112)	X	-(+)	-(+)	Caudrey & Stanisich (1979)
JE2571-2(PVS115)	X	-(+)	-(+)	
JE2571(R386)	F1	-(-)	-(-)	Dennison (1972)
JE2571(R64drd11)	I ₁	-(-)	-(-)	Datta <i>et al.</i> (1971)
M827-2(TP114drp1)	I ₂	-(-)	+	Bradley <i>et al.</i> (1983)
M827-2(Rip69)	M	-(-)	+	Coetzee (1979); Coetzee <i>et al.</i> (1983)
JE2571(R15)	N	-(-)	+	Jacob <i>et al.</i> (1977)
JE2571-2(RP4)	P	-(-)	+	Coetzee <i>et al.</i> (1979)
M827-2(RA3)	U	-(-)	+	Tschäpe <i>et al.</i> (1981)
JE2571(R1460)	U	-(-)	+	Bradley <i>et al.</i> (1982b)
JE2571-2(RSa)	W	-(-)	-(+)	Coetzee <i>et al.</i> (1979)
JE2571-1(R775)	Unique	+	+	Bradley <i>et al.</i> (1981a)
M827-2(R775)	Unique	+	+	
SR41-2(R775)	Unique	+	+	

and *Providencia* (see above), phage X-2 only propagated on them as efficiently as on *E. coli* or *S. typhimurium* strains carrying other repressed IncX plasmids. Phage X-2 did not even propagate on strains of *K. pneumoniae* or *K. oxytoca* carrying R6K, although the plasmid formed pili constitutively in these strains (Table 2). Neither did it propagate on strains of *E. coli* or *S. typhimurium* harbouring plasmids belonging to Inc groups I₂, M, N, P, U or W on which phage X plated or propagated. The reactions of phage X (Table 2) were as reported (Bradley *et al.*, 1981a, 1983). Both these phages failed to plate or propagate on strains harbouring IncI₁ or IncF1 plasmids (Table 2) or on *E. coli* and *S. typhimurium* strains carrying plasmids of the other Inc groups mentioned in Methods encoding thick flexible pili constitutively (not shown).

Phages IKE and I₂-2 had no visible action on strains of *E. coli*, *S. typhimurium* or *Sr. marcescens* carrying the IncX plasmid R6K (not shown).

DISCUSSION

Phage X-2 is serologically unrelated to phage X and the X-like phages IKE and I₂-2 (Table 1). Phage X-2 lacks the curly appearance of phage X (Bradley *et al.*, 1981a) and the phages differ in their reactions to diethyl ether. The host-range of phage X-2 also differs from those of phages IKE and I₂-2 (Table 2 and Bradley *et al.*, 1983), particularly in that the latter have no visible effect on JE2571(R6K), the host strain of the former phage.

While phages X and IKE adsorb to the tips of pili determined by plasmids of various Inc groups (Bradley, 1979; Bradley *et al.*, 1981*a*, 1983) the adsorption site of phage X-2 as well as that of phage I₂-2 (Coetzee *et al.*, 1982) has not been determined. For the latter phage it was concluded that either adsorption to IncI₂-plasmid-coded pili was very inefficient or that the preparative methods broke apart the phage-pilus complexes. For phage X-2 attention was also given to pili still attached to bacterial cells in case phage only adsorbed to such pili, but this approach also proved futile. However, indirect evidence implicating X pili in the adsorption process was obtained. Firstly, ten phage-X-2-resistant clones of strain JE2571(R6K), which still displayed the antibiotic-resistance markers of the plasmid, were sterile in matings with JE2571-1. Sex pili are implicated in the conjugative process (Ottow, 1975) and one explanation for this behaviour would be that these phage-resistant clones lacked sex pili. This reasoning was supported by the fact that no pili could be seen on the two of these phage-resistant clones examined. Thus loss of sensitivity to phage X-2 coincided with loss of X pili and adsorption of the phage to somatic sites coded by the plasmid may possibly also be excluded.

If adsorption of phage X-2 does occur to pili, the adsorption site must be different from that of phage X (see above) but could also be on the tips of these pili as tips of F pili can accommodate at least three f1 phages simultaneously (Caro & Schnös, 1966) and the tips of IncN pili adsorb phage IKE and also the lipid-containing phage PR4 (Bradley, 1979). Alternatively the adsorption site of phage X-2 could be on the pilus shafts. This would be in keeping with the restricted host range of phage X-2 in comparison with that of phage X (Table 2; see Coetzee *et al.*, 1979) and in that way resemble phage C2 (Bradley *et al.*, 1982*a*) or phage tf-1 (Coetzee *et al.*, 1987).

The host range of phage X-2 is limited to IncX plasmid- and the unique plasmid R775-harbouring strains of bacteria. Differences in the host range of phages X-2 and X with regard to the IncX plasmid R6K in the *K. pneumoniae* strain G162 and strain G147 of *K. oxytoca* (Table 2) could mean that the Klebsiella strains, while allowing adsorption, cannot support the intracellular growth of phage X-2 (see Bradley *et al.*, 1981*a, b*). Keeping the latter limitation in mind, the restricted host range of phage X-2 could make it more useful than phage X in identifying IncX and R775 plasmids.

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