Phage pilH α : a Phage Which Adsorbs to IncHI and IncHII Plasmid-coded Pili

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Phage pilH α was specific for bacterial strains, of various genera, harbouring plasmids of the HI and HII incompatibility groups. Plaque formation was temperature sensitive in that plaques formed at 26 °C but not at 37 °C. Plaques were fairly clear, irregular in outline and varied from pin point to about 2 mm in diameter on all hosts where plaques were detected. The phage had an isometric hexagonal outline with a diameter of 25 nm. It contained RNA but differed from all but one other plasmid-dependent RNA phage by being sensitive to chloroform. It adsorbed along the length of the shafts of IncHI and HII plasmid-coded pili.

INTRODUCTION

The IncH group of plasmids (Grindley *et al.*, 1972; Anderson & Smith, 1972) was subdivided into H₁ and H₂ because, although incompatible, the groups share minimal DNA homology. These groups are now designated H1 and H2 (Taylor & Grant, 1977). Plasmid MIP233 which, despite being incompatible with both the latter groups (Le Minor *et al.*, 1976), also has little DNA homology with them and was allocated to Inc group H3 (Roussel & Chabbert, 1978).

Plasmids of these H subgroups have molecular masses which range from 110 MDal to 170 MDal (Anderson *et al.*, 1975; Bradley *et al.*, 1982a) and transfer by conjugation occurs at low frequencies (Grindley *et al.*, 1972; Bradley *et al.*, 1980) (but see below). The transfer process is thermosensitive (Williams Smith, 1974; Taylor & Levine, 1980). Some IncH1 plasmids displace the F plasmid from *Escherichia coli* K12 F⁺ despite the fact that they share minimal DNA homology with the latter plasmid (Smith *et al.*, 1973) and H2 plasmids inhibit the growth of a number of bacteriophages (Taylor & Grant, 1976).

Pili specified by H1 and H2 plasmids are of the thick flexible variety, belong to morphological group 1 and are serologically related (Bradley, 1980b). Pili of H3 plasmids have not yet been identified (Bradley *et al.*, 1980, 1982a).

Plasmids pHH1457 and pHH1508a are incompatible, have masses of 110 and 100 MDal respectively and code for the constitutive production of thick flexible pili which are serologically related to those produced by the H1 and H2 plasmid groups (Bradley *et al.*, 1982a). Conjugative transfer of plasmids pHH1457 or pHH1508a to IncH1, H2 or H3 plasmid-containing strains is not thermosensitive and the plasmids are compatible with plasmids of all three IncH subgroups (Bradley *et al.*, 1982a). Because of the morphological and serological similarity of pili coded for

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by plasmids of the IncH groups and by pHH1457 and pHH1508a it was decided to make the latter plasmids prototypes of a new group, IncHII and, by analogy with IncF, to name the original IncH plasmids IncHI with subgroups HI1, HI2 and HI3 (Bradley *et al.*, 1982a). Two more members of the IncHII group, pHH1532b-1 and pMG110, have recently been identified (Bradley *et al.*, 1982a; Wolfson *et al.*, 1982).

IncH plasmids harboured by human pathogenic bacteria are an important cause of antibiotic resistance exhibited by these strains and create therapeutic problems (Anderson, 1975; Bradley *et al.*, 1982a). The mechanisms involved with the temperature sensitivity of conjugative transfer of HI plasmids (Taylor, 1983), the high-temperature elimination of some IncHI plasmids (Taylor & Levine, 1980; Williams Smith *et al.*, 1978) and processes concerned in the displacement of IncF plasmids by plasmids of the HI1 group (Smith *et al.*, 1973) are other important areas requiring further work.

A phage adsorbing specifically to IncH plasmid-coded pili has not yet been described. Such a phage could be of use in the above experiments by monitoring production of these pili by bacteria and could possibly even be considered for use as a therapeutic agent (Williams Smith & Huggins, 1983; Editorial, 1983). Here we describe the properties of phage pilH α which was isolated from sewage as lysing *E. coli* strain JE2571(pHH1508a) but not the isogenic strain lacking the plasmid.

METHODS

Bacteria and plasmids. The *Escherichia coli* K12 strains were J53, J53-2, J62-1 (Datta *et al.*, 1980) and JE2571 (Bradley, 1980a). *Klebsiella pneumoniae* strain G162 and *K. oxytoca* strains G147 and G147-1 were provided by Dr I. Simpson, Glaxo Group Research, Greenford, Middlesex, UK. 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. Sr41-2 is a spontaneous *rif* mutant of the latter strain (J. N. Coetzee, unpublished). *Proteus mirabilis* strain PM5006nal (Coetzee, 1974) and *P. morganii* 2815nal-r (Coetzee *et al.*, 1979) were also used. Plasmids originated from the collection of Naomi Datta (Datta, 1977, 1979) with the exceptions of pDT933, which was kindly supplied by D. E. Taylor (Taylor, 1983) and pMG110 (Wolfson *et al.*, 1982), which was sent by D. C. Hooper, J. S. Wolfson and M. N. Swartz. Apart from plasmids belonging to the IncHI incompatibility group (see Table 1), only those which encoded constitutive pilus synthesis (as judged by a transfer frequency of $>1 \times 10^{-3}$ per donor h 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), F ϕ lac (Bradley *et al.*, 1981b), HI1 (Bradley *et al.*, 1982a), I $_1$, I $_2$ (Coetzee *et al.*, 1982), J (Bradley *et al.*, 1982b), N, P (Coetzee *et al.*, 1979), t (Bradley *et al.*, 1981c), W (Coetzee *et al.*, 1979) and X (Bradley *et al.*, 1981a) were used. J62-1 (PlacR1drd19) (Sirgel *et al.*, 1981) and J53(R6K) (Bradley *et al.*, 1981a) were the indicator strains for phages C-1 and X (see below) respectively. Plasmid-carrying strains were maintained on selective antibacterial drug-containing media to minimize plasmid segregation (see Bradley *et al.*, 1982a).

Bacteriophages. Lysis of bacterial strains by plasmid-specific phages MS2 (Davis *et al.*, 1961), C-1 (Sirgel *et al.*, 1981), F ϕ lac (Bradley *et al.*, 1981b), PR64FS (Coetzee *et al.*, 1980), J (Bradley *et al.*, 1982b), M (Coetzee *et al.*, 1983), PR772 (Coetzee *et al.*, 1979), t (Bradley *et al.*, 1981c) or X (Bradley *et al.*, 1981a) was used to demonstrate constitutive pilus production by strains carrying appropriate plasmids. Phages C-1 and X were also used as controls in RNAase-chloroform and 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, antibacterial agents, general phage techniques, transfer of plasmids by conjugation, organic solvent and ribonuclease sensitivity of phage and production of phage lysates by a soft agar layer technique. These were done as described and referred to by Coetzee *et al.* (1979), Bradley *et al.* (1980, 1981a), Ackermann *et al.* (1978) and Hershey *et al.* (1943). Broth and the overlay agar contained the particular plasmid-selective antibacterial agents (Bradley *et al.*, 1982a). Conjugative transfer of IncHI plasmids was done at 26 °C (Taylor & Levine, 1980). Phage isolation and initial experiments with phage pilH α (see below) were performed at an incubation temperature of 37 °C. When it became apparent that plaque formation by this phage was temperature sensitive, all further experiments were conducted at 26 °C unless otherwise stated.

Phage isolation. Attempts were made to isolate an IncHII plasmid-specific phage from sewage specimens obtained from various sewage works in the Pretoria area according to the method of Bradley *et al.* (1981a). One pair of partner strains used for sewage enrichment was JE2571 and M827-2 carrying plasmid pHH1457, the second set comprised the same hosts carrying plasmid pHH1508a and plasmid pHH1532b-1 in the two hosts made the third set. After the incubation period, enriched sewage filtrates were spotted separately on soft agar lawns of all the above strains individually as well as on isogenic organisms lacking plasmids. Plating the filtrates on the same

strain used for enrichment ruled out selection (see Meynell & Lawn, 1968) but was adopted in view of the host specificity of certain plasmid-specific phages (see Coetzee *et al.*, 1979; Sirgel *et al.*, 1981; Bradley *et al.*, 1981c, 1982b; J. N. Coetzee, unpublished results).

Bacteriophage propagation. Propagation of phage pilH α (see below) was done as described by Bradley *et al.* (1981a; see also Coetzee *et al.*, 1982). Briefly, an exponentially growing culture of the donor plasmid-bearing organisms under test (0.1 ml) was mixed with an equal volume of stationary phase plasmid-free isogenic recipient 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 (see Bradley & Fleming, 1983), were added 0.1 ml phage suspension (1×10^{10} p.f.u. ml $^{-1}$) and 4 ml of melted soft top-layer agar 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 JE2571(pHH1508a) determined. This titre was compared to that obtained in a similar experiment using the same recipient but no donor. A phage titre increase of at least 10-fold that of the negative control, in two separate experiments, was taken as a positive result. Experiments which yielded negative or doubtful results were repeated on at least a further two occasions.

Concentration of phage. The optimal final concentrations of polyethylene glycol (mol. wt 6000) and NaCl (Yamamoto *et al.*, 1970) for concentrating phage pilH α (see Results) were 11% (w/v) and 0.5 M respectively. Pellets were suspended in small volumes of distilled water.

Electron microscopy. Negative staining for electron microscopy was done as described by Bradley *et al.* (1981a, b, c).

RESULTS

Transfer of plasmids

IncHII plasmids pHH1457, pHH1508a and pHH1532b-1, and IncHI plasmids pDT933 and R478 (Table 1) transferred between various *E. coli* K12 strains and these strains to *Klebsiella* strains G162 and G147, *S. typhimurium* M827-2, *Sr. marcescens* Sr71-2 or *P.morganii* 2815nal-r at frequencies of $> 3 \times 10^{-3}$ transconjugants per donor h $^{-1}$. Plasmids R27, R726, pW23 and MIP233 transferred from *E. coli* K12 strains to the above hosts at frequencies between 5×10^{-7} and 1×10^{-5} transconjugants per donor h $^{-1}$. None of the above plasmids transferred from the *E. coli* hosts to *P. mirabilis* 5006nal (frequency $< 1 \times 10^{-7}$ transconjugants per donor h $^{-1}$). The host range of plasmids may be limited to particular hosts (Datta & Hedges, 1972; Hedges & Jacoby, 1980) and the present case may be another example.

Whereas the transfer of the IncHI plasmids is temperature sensitive (Williams Smith, 1974; Williams Smith *et al.*, 1978; Anderson, 1975; Taylor & Levine, 1980; Bradley *et al.*, 1980; Taylor, 1983), temperature sensitivity has not been reported for the conjugative transfer of the IncHII plasmids pHH1532b-1 and pMG110. Plate matings with plasmid-bearing derivatives of *K. oxytoca* strain G147 and strains of *E. coli* J62-1 as recipients yielded transfer frequencies of $> 5 \times 10^{-2}$ transconjugants per donor h $^{-1}$ for both plasmids in experiments conducted at 26 °C and 37 °C.

Isolation of a pHH1508a-dependent bacteriophage

From sample no. 361 of sewage examined over a period of 3 years and enriched with strain M827-2(pHH1508a), a phage was isolated on JE2571(pHH1508a) which formed plaques on these two strains but not on isogenic organisms lacking the plasmid. Because of the adsorption site and host range (see below) and to allow for different, as yet undiscovered, phages also active on IncH plasmid-carrying strains, it was named phage pilH α .

Properties of phage pilH α

Plaque formation and morphology. A problem present from the start was the inconsistency with which strains supported plaque formation by the phage. The fact that the phage also plated (intermittently) on bacteria carrying IncHI plasmids (see below) which are temperature sensitive for transfer, prompted an investigation of the possibility that plaque formation was a temperature sensitive process. Phage spot tests were accordingly done with all strains previously used carrying IncHI or IncHII plasmids and with seeded pre-warmed plates at 26 °C and 37 °C. Immediately after spotting the phage the uncovered plates were returned to empty incubators

Fig. 1. Phage pilH α virions adsorbed to an IncHII pilus synthesized by *K. oxytoca* strain G147-1(pMG110). Many phage plus pilus complexes were found in phage suspensions made by extracting confluent lysed plates. Detached virions found near the pili were probably reversibly adsorbed and separated during drying. Phage pilH α appears similar to other RNA phages. Bar, 100 nm.

maintained at the above temperatures. The incubators never contained more than eight plates singly placed and these were then incubated overnight after quickly replacing the Petri dish lids once the drops of phage had dried. The phage only lysed bacteria maintained at 26 °C. There was no visible action at 37 °C. Further experiments determined the critical temperature to be about 31 °C (not shown). Adopting a temperature of 26 °C for all further experiments removed all inconsistencies from the plating procedure. Plaques on bacterial lawns which had been incubated overnight at 26 °C were fairly clear and varied from about 2 mm in diameter to pin point. The larger plaques were irregular in outline.

Phage morphology and adsorption site. Phage pilH α had a semi-transparent appearance, an hexagonal outline and a diameter of about 25 nm. It thus resembled other RNA-containing phages such as MS2 (Davis *et al.*, 1961), F $_{\phi}$ lac (Bradley *et al.*, 1981*b*), C-1 (Sirgel *et al.*, 1981) or M (Coetzee *et al.*, 1983). Phage preparations made by extracting confluent lysed double agar layer plates (see Methods) contained many examples of phage virions adsorbed uniformly along the lengths of IncHI or IncHII pili (Fig. 1).

Sensitivity to organic solvents or RNAase. The plaque-forming titre of a phage pilH α suspension was reduced by about 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 pilH α and the latter two phages were not reduced by diethyl ether treatment. Plaque formation by phages pilH α and C-1 was totally inhibited by RNAase, whereas that of phage X was unaffected.

Host range. Although the control phages mentioned above plated on strains carrying their respective plasmids belonging to the Inc groups listed above, phage pilH α only formed plaques or propagated on bacterial strains carrying plasmids of the IncHI or HII groups (not shown and Table 1). It plated with equal efficiency (or propagated) on *Klebsiella*, *E. coli*, *S. typhimurium*, *P. morganii* and *Sr. marcescens* strains carrying any one of the H-complex plasmids used (Table 1 and not shown). Plasmids of the IncHI subgroup have been reported as being repressed for pilus synthesis (Bradley *et al.*, 1980, 1982*a*; Bradley, 1980*b*; Taylor & Levine, 1980; Taylor & Grant, 1977) but the finding that phage pilH α plated on strains carrying R478 was not unexpected: plasmid R478 transferred at high frequencies and Taylor & Levine (1980) reported an overnight transfer frequency of 9×10^{-3} per organism. It may thus be assumed that the plasmid determines enough pili to support plaque formation (Meynell, 1978). Further correlation between plaque formation and transfer frequency was found with plasmid R27. This plasmid, which transferred at low frequencies (see above), did not allow the phage to plate, but a Tn7 insertional mutant (pDT933) of the plasmid, which transferred at much higher frequencies (see above; Taylor, 1983), permitted the phage to plate on strains harbouring it (Table 1).

Table 1. *Host range of phage pilHα*

Drops of phage pilHα suspension (titre 1×10^{10} p.f.u. ml⁻¹) were spotted on lawns of organisms contained in top-layer agar. Incubation temperature was 26 °C. Phage propagation was done as described in Methods.

Host	Plasmid Inc group*	Phage action		Plasmid reference
		Plaque formation†	Propagation‡	
<i>E. coli</i> JE2571(pHH1457)	HII	+	ND	Bradley <i>et al.</i> (1982a)
<i>S. typhimurium</i> M827-2(pHH1457)	HII	+	ND	Bradley <i>et al.</i> (1982a)
<i>Sr. marcescens</i> Sr41(pHH1457)	HII	+	ND	Bradley <i>et al.</i> (1982a)
<i>K. oxytoca</i> G147(pHH1457)	HII	+	ND	Bradley <i>et al.</i> (1982a)
<i>E. coli</i> JE2571(pHH1508a)	HII	+	ND	Bradley <i>et al.</i> (1982a)
<i>Sr. marcescens</i> Sr41-2(pHH1508a)	HII	+	ND	Bradley <i>et al.</i> (1982a)
<i>K. pneumoniae</i> G162(pHH1508a)	HII	+	ND	Bradley <i>et al.</i> (1982a)
<i>E. coli</i> J62-1(pHH1532b-1)	HII	+	ND	Bradley <i>et al.</i> (1982a)
<i>S. typhimurium</i> M827-2(pHH1532b-1)	HII	+	ND	Bradley <i>et al.</i> (1982a)
<i>K. pneumoniae</i> G162(pHH1532b-1)	HII	+	ND	Bradley <i>et al.</i> (1982a)
<i>K. oxytoca</i> G147(pMG110)	HII	+	ND	Wolfson <i>et al.</i> (1982)
<i>E. coli</i> J53-2(R27)	HII (H1)	—	+	Bradley <i>et al.</i> (1982a)
<i>S. typhimurium</i> M827-2(R27)	HII (H1)	—	+	Bradley <i>et al.</i> (1982a)
<i>E. coli</i> J53-2(pDT933)	HII (H1)	+	ND	Taylor (1983)
<i>S. typhimurium</i> M827-2(pDT933)	HII (H1)	+	ND	Taylor (1983)
<i>Sr. marcescens</i> Sr41(pDT933)	HII (H1)	+	ND	Taylor (1983)
<i>P.morganii</i> 2815nal-r(pDT933)	HII (H1)	+	ND	Taylor (1983)
<i>K. oxytoca</i> G147(pDT933)	HII (H1)	+	ND	Taylor (1983)
<i>E. coli</i> J53(R726)	HII (H1)	—	+	Bradley <i>et al.</i> (1982a)
<i>S. typhimurium</i> M827-2(R726)	HII (H1)	—	+	Bradley <i>et al.</i> (1982a)
<i>E. coli</i> J53(R478)	H12 (H2)	+	ND	Taylor & Levine (1980)
<i>Sr. marcescens</i> Sr41-2(R478)	H12 (H2)	+	ND	Taylor & Levine (1980)
<i>K. pneumoniae</i> G162(R478)	H12 (H2)	+	ND	Taylor & Levine (1980)
<i>P.morganii</i> 2815nal-r(R478)	H12 (H2)	+	ND	Taylor & Levine (1980)
<i>E. coli</i> J53-2(pWR23)	H12 (H2)	—	+	Taylor & Levine (1980)
<i>S. typhimurium</i> M827-2(pWR23)	H12 (H2)	—	+	Taylor & Levine (1980)
<i>Sr. marcescens</i> Sr41-2(pWR23)	H12 (H2)	—	+	Taylor & Levine (1980)
<i>E. coli</i> J53(MIP233)	H13 (H3)	—	+	Taylor & Levine (1980)
<i>S. typhimurium</i> M827-2(MIP233)	H13 (H3)	—	+	Taylor & Levine (1980)
<i>Sr. marcescens</i> Sr41-2(MIP233)	H13 (H3)	—	+	Taylor & Levine (1980)

* The former Inc subgroup (see Bradley *et al.*, 1982a) is given in parentheses.

† +, Plaque formation to titre; —, no visible action.

‡ +, At least a 10-fold phage titre increase above that of the negative control obtained on two separate occasions; ND, not done.

DISCUSSION

Phage pilHα is the first wild-type bacteriophage reported to be temperature sensitive for plaque formation. The mechanism, which is also expressed with IncHII plasmids which are not temperature sensitive for transfer (see above) remains to be ascertained. The reason why the phage was detected in the first instance with the use of 37 °C incubators was probably that bench incubators were usually filled to capacity with plates from the bench at the end of a day for overnight incubation. Under these conditions it takes hours for agar in the plates to reach 37 °C and plates will remain below 30 °C for long enough to allow a temperature sensitive system to develop significantly. Inconsistencies arose when the agar was temperature equilibrated in a much shorter period as a result of a 37 °C incubator containing a small number of plates.

Phage M (Coetzee *et al.*, 1983) was the first RNA-containing phage described which adsorbed to thick rigid pili. The fact that it differed from all other RNA phages in being sensitive to chloroform was thus not totally unexpected. This does not apply to the chloroform sensitivity of phage pilHα as all other RNA-containing phages which adsorb to specific thick flexible pili like C-1 (Sirel *et al.*, 1981), MS2 (Davis *et al.*, 1961), t (Bradley *et al.*, 1981c) and F₀lac (Bradley *et*

al., 1981*b*) are chloroform resistant. The degree of chloroform sensitivity of phage π lH α , like that of phage M, is less than that of suspensions of the lipid-containing phages belonging to the families *Tectiviridae*, *Corticoviridae* or *Cystoviridae* (Matthews, 1982), which are nearly totally inactivated under comparable conditions (Coetzee *et al.*, 1979; Wong & Bryan, 1978; Bradley & Rutherford, 1975; Vidaver *et al.*, 1973; Mindich, 1978). The RNA-containing phages also differ from phages of the above three families in that they are resistant to diethyl ether.

RNA-containing phages adsorb uniformly to the sides of shafts of sex pili or towards the distal tip only (Bradley *et al.*, 1981*b, c*). They are usually specific for plasmids of one particular incompatibility group (see Coetzee *et al.*, 1979, 1983). Exceptions occur with plasmids of IncF subgroups I–IV which constitute the F-complex. The pili coded for by these groups of plasmids are serologically related (Lawn & Meynell, 1970) and some RNA phages like MS2 or Q β plate on strains carrying plasmids belonging to any of these groups (Dennison & Hedges, 1972). Phage π lH α , by plating on bacterial strains carrying either HI or HII plasmids (Table 1), is thus in a rather analogous position to that of phages MS2 and Q β and lends support to the arrangement (see Bradley *et al.*, 1982*a*) of H plasmids into Inc groups HI and HII.

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