Function of Pili in Bacteriophage φ6 Penetration

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SUMMARY

The genome of bacteriophage φ6, which has a lipid protein envelope, consists of three pieces of dsRNA. Virus infection is initiated by attachment to a φ6-specific host pilus followed by fusion of the phage membrane and the bacterial outer membrane. In this study we analysed several different φ6 hosts as well as more than 200 independently isolated φ6-resistant variants derived from Pseudomonas syringae pv. phaseolicola. It is shown that φ6-specific pili are coded by genes located in the host chromosome. It appears that pilus retraction is needed to pull the pilus-associated virus through the extracellular polysaccharide of the host and thus to bring it into contact with the outer membrane where membrane fusion can take place.

INTRODUCTION

Bacteriophage φ6 has two unique features; first, its genome consists of three pieces of dsRNA (Semancik et al., 1973) and secondly, it possesses a lipid-protein envelope surrounding the viral nucleocapsid (Vidaver et al., 1973; Mindich et al., 1976a, b; Bamford & Palva, 1980). The phage attaches to the pilus of its usual laboratory host Pseudomonas syringae pv. phaseolicola HB10Y (Vidaver et al., 1973; Bamford et al., 1976; Bamford & Lounatmaa, 1982), and in a second step fusion of the phage membrane with the host outer membrane occurs (Bamford et al., 1976; Bamford & Lounatmaa, 1978, 1982; Kakitani et al., 1980). The protein involved in adsorption is the virion-specific protein P3 (Mindich et al., 1976b), located on the surface of the particle (van Etten et al., 1976). The lytic enzyme, protein P5, associated with the virion (Kakitani et al., 1978; Mindich & Lehman, 1979; Iba et al., 1979), is located between the membrane and the nucleocapsid (Bamford & Palva, 1980). It has been suggested that after the fusion of the phage membrane with the bacterial outer membrane this protein digests the host peptidoglycan and thus allows the nucleocapsid to penetrate the cell wall (Kakitani et al., 1980). A study of P. syringae pv. phaseolicola mutants resistant to φ6 showed that resistance arose by random mutation and not by one common event, such as loss of a plasmid and/or attachment sites (Cuppels et al., 1979). These authors also isolated φ6-resistant phage-producing strains. This type of strain was later shown to have an altered cell wall structure which was more resistant to phage-coded lysozyme (P5), hindering virus entry and release of mature virions (Romantschuk & Bamford, 1981).

In this study, we used phage-resistant derivatives of P. syringae pv. phaseolicola and other Pseudomonas species as host strains to investigate the adsorption of φ6 to bacterial pilus and to address the question of whether the φ6-specific pilus or other attachment sites are coded by independently replicating plasmids.

METHODS

Phage and bacterial strains. Bacteriophage φ6 and its original host P. syringae pv. phaseolicola HB10Y (HB) (Vidaver et al., 1973) were received from Dr A. K. Vidaver, University of Nebraska, Lincoln, Ne., U.S.A. A double host range mutant, φ6h1s, and its host, P. pseudoalcaligenes ERA S4 (S4) (Mindich et al., 1976a), were obtained from Dr I. Mindich, The Public Health Research Institute of the City of New York, N.Y., U.S.A. An
Table 1. Bacterial strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Abbreviation</th>
<th>Auxotrophy</th>
<th>$\phi$65/$\phi$68</th>
<th>Piliation*</th>
<th>Source/reference</th>
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<tr>
<td>ERA (pLM2) S4</td>
<td>S4</td>
<td></td>
<td>$\phi$6h1s$^b$</td>
<td>Super</td>
<td>Mindich et al. (1976a)</td>
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<tr>
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<td></td>
</tr>
<tr>
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<td>AO</td>
<td></td>
<td>$\phi$6h1sb$^b$</td>
<td>Super</td>
<td>A. K. Vidaver (University of Nebraska)</td>
</tr>
<tr>
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<tr>
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<tr>
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<td>MP11</td>
<td>leu his</td>
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<td>Super</td>
<td>Deriv. of MP22; this study</td>
</tr>
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* The degree of piliation in HB was defined as normal; other strains were compared to this.

additional host range mutant, $\phi$6h1sb, was isolated in our laboratory. This phage was able to infect *P. syringae* pv. *syringae* HS191 (HS), harbouring a 34 MDal plasmid, and a derivative of it. *P. syringae* pv. *syringae* AO111 (AO), both obtained from Dr A. K. Vidaver. The bacterial strains used in this study are listed in Table 1.

Phage-resistant ($\phi$6$^a$) bacterial strains were isolated as described previously (Romantschuk & Bamford, 1981) and are listed in Table 1. These are representatives of different mutant classes and were selected from a total of 223 independently isolated $\phi$6$^a$ bacterial strains.

Growth and purification of phage. Purified phage was obtained as described previously (Bamford & Palva, 1980), using L broth (LB) (Maniatis et al., 1982) as a rich growth medium.

Radioactive labelling of the phage. Exponentially growing HB cells in M9 medium (Maniatis et al., 1982) supplemented with 0.4% glucose, 2 mM-MgSO$_4$, 0.5 mM-CaCl$_2$, 10 $\mu$M-FeSO$_4$, and 0.1% casamino acids were pelleted and resuspended to a density of 10$^8$ c.f.u./ml in the same medium but containing only 0.01% casamino acids and 10 $\mu$g/ml each of tryptophan, methionine, cysteine and histidine. A 1 ml sample of the culture was shaken for 20 min at 24 °C and then infected with phage at a multiplicity of 10. Sixty min post-infection, 5 $\mu$Ci of a $^{14}$C-amino acid mixture (Amersham, CFB. 152) was added. At 2.5 h post-infection cell debris was removed and the phage was purified as above.

Adsorption assays. Exponentially growing bacteria in LB medium were pelleted (5000 r.p.m., 5 min), resuspended in LB medium (3 × 10$^8$ c.f.u./ml) and incubated (20 min at 24 °C). Radioactively labelled purified phage was added (m.o.i. 0.1) and 1 ml samples were withdrawn at different times. Cells were immediately pelleted, and the supernatant and pellet were assayed for radioactivity.

The estimation of adsorption rates using unlabelled phage was done as previously described (Romantschuk & Bamford, 1981).

Phage-producing cells were assayed by infecting exponentially growing bacteria with phage at a multiplicity of 10. Samples were taken at various times, centrifuged and washed twice with LB, and plated on a lawn of HB cells.

Electron microscopy. Negative staining electron microscopy was done as described previously (Romantschuk & Bamford, 1981). For thin sections, bacteria were infected with phage at a m.o.i. of about 100, and samples were taken 6 min after infection. Samples were prepared and micrographs taken as previously described (Bamford & Mindich, 1980).

For demonstrating the extracellular polysaccharide, exponentially growing HB and *Escherichia coli* MC4100 (Casadaban, 1976) cells were added to purified virus preparations (approx. 1 mg/ml). Polyethylene glycol 6000 and NaCl were added to a final concentration of 9% and 0.5 M respectively. The material was precipitated by centrifugation, incubated for 80 min at room temperature and fixed with glutaraldehyde.

Isolation of plasmids. Plasmids were isolated using a modification of the method of Hansen & Olsen (1978). A 5 ml overnight LB medium culture was pelleted (Sorvall SS-34 rotor, 6000 r.p.m., 10 min), resuspended in 1 ml ice-cold lysozyme solution (5 mg/ml in 50 mM-glucose, 2 mM-EDTA, 25 mM-Tris–HCl pH 8.0), and incubated at room temperature for 15 min. Two ml of 1% SDS, 50 mM-EDTA was added and the tubes were gently inverted several times. One and a half ml of 3 M-potassium acetate pH 6.0 was added dropwise to precipitate chromosomal DNA and cell debris. Tubes were left on ice for 1 h, after which the white precipitates were removed with a hook. Pancreatic R Nase was added to a final concentration of 50 $\mu$g/ml and incubation continued for 30 min at room temperature. Plasmid DNA was ethanol-precipitated, then dissolved in 0.5 ml of DNA buffer (10 mM-Tris–HCl, 0.1 mM-EDTA, pH 8.0).
Penetration of phage Jp6

_Gel electrophoresis of DNA._ Isolated plasmids were analysed in a horizontal 0.7% agarose slab gel, as described by Maniatis et al. (1982). Alternatively, the method of Eckhardt (1978) was used to analyse plasmids in extracts of bacterial cells.

_Isolation of outer membranes._ Outer membranes of bacterial cells were routinely isolated by the rapid method of Palva (1978) or by the Triton X-100 method, in the presence of magnesium ions (Schnaitman, 1970).

_Gel electrophoresis of proteins._ Proteins were characterized by SDS-PAGE, as described by Bamford et al. (1981), except that the separation gel buffer had a pH of 8.6.

**RESULTS**

**Resistance frequency**

The frequency of phage-resistant cells in overnight cultures inoculated from single colonies varied considerably for different bacterial strains [HB, 4 (± 3) × 10⁻⁶; HS, 2 (± 2) × 10⁻⁵; AO, > 10⁻²; S4, > 10⁻²]. In the particular cases of AO and S4, the frequencies of phage-resistant variants were far too high to be caused by spontaneous mutations.

**Expression of pili**

The phage-sensitive bacterial strains HB, HS, AO and S4, as well as the phage-sensitive auxotrophic derivatives of HB (Table 1), were subjected to negative staining electron microscopy, together with an excess of phage. All the sensitive strains, except MP12 (one of the auxotrophic mutants of HB), showed pili with attached phages. Eighty-three of the independently isolated phage-resistant strains of HB were also examined and were classified by their pattern of piliation, as compared to the wild-type (Fig. 1). Sixty-two of the resistant strains showed no pili, 17 showed a reduced number, and three showed a clearly elevated number of pili.

**Adsorption**

Adsorption of phage was assayed by the ability of host bacteria to eliminate phage from suspension, as measured by plaque assay (data not shown), or by using radioactively labelled phage (Fig. 2). The adsorption efficiencies were correlated to the degree of piliation, as seen under the electron microscope.

HB and its φ6-resistant derivatives could be divided into three classes: (i) non-piliated strains that did not adsorb phage (e.g. MP0.17), (ii) strains showing a reduced number of pili (e.g. MP11.25), (iii) super-piliated strains with an elevated ability to adsorb phage (e.g. MP22.14). Hosts S4, HS and AO showed piliation similar to that of the super-piliated HB strains. The φ6-sensitive strain MP12 which showed no pili under the electron microscope behaved as the non-piliated strains in adsorption assays (Fig. 2). Only 7% of phage-exposed MP12 cells formed infective centres within 20 min, whereas the value for HB was about 70%. Phage-exposed cells of non-piliated phage-resistant mutants did not produce measurable numbers of infective centres.

Extracellular phages could be removed by washing the cells for 1 min with Triton X-100 (200 μg/ml in L broth). This treatment did not affect host cell viability; phage particles that had entered the cells, and phage production as assayed by infective centres and by one-step growth experiments, were not affected either (data not shown). From piliated resistant strains 95% of the adsorbed phages (m.o.i. 0.1) could be removed 20 min post-infection, but from the sensitive parental HB strain only 45% could be removed.

**Secondary attachment of φ6**

As shown earlier for HB (Bamford et al., 1976; Bamford & Lounatmaa, 1978), secondary attachment of the phage takes place by membrane fusion. All the phage-sensitive strains tested, including the non-piliated strain MP12, showed fusion of the phage membrane with the outer membrane of the bacterium when examined by thin sectioning. No fusions could be seen in any type of phage-resistant strain.

_P. syringae_ strains are known to produce extracellular polysaccharide (EPS) (Rudolph, 1978; El-Banoby & Rudolph, 1979) which is difficult to stain in sectioned material. HB and _E. coli_ MC4100 (Casadaban, 1976) cells were precipitated with polyethylene glycol together with an
excess of φ6 phages. It was observed that in the case of HB the phages stayed at least 150 nm distance from the outer membrane, leaving an apparently empty halo around the cells (Fig. 3). Only occasionally were phages in contact with the cell wall (arrows in Fig. 3a). The number of contact points equalled that of pili. As a control, non-EPS-producing *E. coli* cells showed no halo and the phages readily made contact with the bacterial cell wall (Fig. 3b).
Fig. 2. Phage adsorption as measured by the ability to eliminate radioactively labelled phage from suspension. The corresponding host range phage mutant was used for each bacterial strain. As a control all mutant phages were tested on HB. ○, HB; ■, MP12; ○, MP0.17; △, MP11.25; ▲, MP22.14; ▼, HS; □, AO; ▽, S4.

Fig. 3. Polyethylene glycol-precipitated cells in the presence of an excess of φ6. (a) Strain HB showing apparently empty halos around the cells. Only occasionally are phages in contact with the outer membrane (arrows). (b) E. coli MC4100 with φ6 particles in contact with the outer membrane. Bar marker represents 1 μm.
Plasmid analysis of HB

HB, the parental strain of the phage-resistant strains, harbours two plasmids (Cuppels et al., 1979). We tested 30 phage-resistant strains, representing all the different types of piliation, and found all of them to contain these two plasmids (Fig. 4).

On the other hand one of the phage-sensitive HB strains had only the larger of these two plasmids. The phage-sensitive strain HS contains one plasmid which is related to the pathogenicity of this strain (Gonzalez & Vidaver, 1979). The phage-sensitive non-pathogenic derivative of HS, AO, contains no plasmids. The φ6-sensitive strain S4 carries only plasmid pLM2 (Mindich et al., 1976a) and remains phage-sensitive when it loses the plasmid. The two plasmids in HB and the HS plasmid were all distinct as shown by preliminary restriction mapping (data not shown).

In order to test for the presence of possible megaplasmids the sensitive strains were also subjected to the plasmid analysis method of Eckhardt (1978). No additional plasmids could be observed when the 118 x 10^6 Dal plasmid Ti-B6S3 (Hernalsteens et al., 1978) was used as standard (data not shown).

Outer membrane proteins

The phage-sensitive strains and the resistant derivatives of HB were analysed with regard to their outer membrane protein composition. Outer membranes were isolated by a rapid method, as described in Methods. Although this did not result in pure outer membrane preparations, a marked enrichment of keto-deoxy-octonate (Dröge et al., 1970) and of outer membrane proteins could be observed (Fig. 5), taking as a reference outer membrane characterization experiments with strains HS and AO (Hurlbert & Gross, 1983). Outer membranes were also isolated with Triton X-100 by the method of Schnaitman (1970) with essentially the same results. The protein
Fig. 5. Comparison of the SDS-PAGE protein patterns of outer membranes obtained by rapid purification from various $\phi 6^s$ and $\phi 6^r$ bacterial strains. A 16% gel was used for separation. (a) HS; (b) AO; (c) HB; (d) MP12; (e) MP0.16; (f) MP22.14. (a) to (d) are $\phi 6^s$; (e) and (f) are $\phi 6^r$; (a), (b) and (f) are super-piliated; (c) is normally piliated; (d) and (e) are non-piliated. Protein standards (mol. wt.) shown with arrows were phosphorylase b (97000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase from bovine erythrocytes (30000), soybean trypsin inhibitor (20100) and $\alpha$-lactalbumin (14400), reading from top to bottom.

Compositions of the outer membranes of the different P. syringae strains were found to be very similar and no differences among phage-resistant derivatives and phage-sensitive parental strains could be detected, although 110 $\phi 6$-resistant variants of HB, representing all types of piliation, were tested (Fig. 5).
DISCUSSION

The high proportion of phage-resistant cells (in an overnight culture, approximately $10^{-5}$ for HB and HS, and $>10^{-2}$ for S4 and AO) could be explained by a phase variation phenomenon in pilus expression. Phase variation of both flagellar and fimbrial genes, caused by genomic rearrangements such as site-specific recombination, is a well-documented phenomenon (for review, see Silverman & Simon, 1983). Sensitivity to bacteriophage $\phi 6$ is associated with the presence of functional pili in the host bacterium; this was shown to be the case for all except one strain. Cells of this strain, MP12, showed no measurable phage adsorption and yielded only one-tenth of the infective centres, as compared to HB cells. Nonetheless, the plating efficiency was 0.1 to 0.3 of that on HB. No sign of phage-adsorbing pili could be observed with MP12, whereas normal membrane fusions were evident. We cannot rule out the possibility of short pilin oligomers being responsible for the sensitivity of this strain to $\phi 6$. These would not be visible by negative staining electron microscopy. Also, adsorption assays, which measure the bulk phage adsorption, are not sensitive enough to detect adsorption of only one or two phages on every tenth cell or so. It has also been shown that retracted F-pili lose their ability to adsorb small RNA phages but they remain sensitive to filamentous phages which use pilus tips as their receptors (Novotny & Fives-Taylor, 1974).

The resistant strains derived from HB could be divided into non-piliated and piliated ones. All of the piliated resistant strains had a mode of piliation that differed from the wild-type, and none of them showed phage-bacterial outer membrane fusions when assayed by thin sectioning. Almost all adsorbed phages could be rinsed off the piliated resistant strains with dilute Triton X-100, whereas only about half of the adsorbed phages could be removed from the sensitive strains by this method. These observations, together with the electron microscopically observed phage-free halo, suggest that a functional, retractable pilus is needed to pull the phage through the EPS and other possible steric hindrances, in order to make membrane fusion possible. Similar mechanisms have been proposed for other pilus-specific phages (Bradley, 1972; Novotny & Fives-Taylor, 1974; Schwartz, 1980). The function of at least some types of pili might involve polymerization and depolymerization of pilus subunits to the presumed benefit of the bacterium but at the same time offering a route for entrance of certain phages.

In our analysis of the outer membrane protein pattern we could not observe changes in any phage-resistant strain in comparison with the wild-type pattern. This, however, does not rule out the possibility that phage-out membrane fusion is dependent on outer membrane proteins, since functional changes in these proteins might not necessarily entail detectable changes in their molecular weights or their concentrations in membrane fractions.

Plasmid analysis showed that at least strains S4 and AO express $\phi 6$-specific pilus in the absence of independently replicating plasmids. The loss of an 80 kb plasmid in HB did not affect pilus expression either. This indicates that the $\phi 6$-specific pilus are most probably coded by chromosomal genes. Since it has been reported that $P. syringae$ pv. phaseolicola can integrate plasmids in the chromosome (Szabo & Mills, 1984), it can not be ruled out that piliation is due to expression of genes in integrated plasmids. The pilus of S4 and those of HB are not the products of the same genes since the molecular weights of the pilus subunits and pilus morphology are different in these strains (our unpublished results).

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