Lipid-containing Anthrax Phage AP 50: Structural Proteins and Life Cycle

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SUMMARY

The structural proteins of the purified lipid-containing anthrax phage AP 50 were studied by SDS–polyacrylamide gel electrophoresis. Nine major structural proteins were found. The pattern of phage DNA was determined by agarose gel electrophoresis after its treatment with AsuI restriction endonuclease. The mol. wt. of phage DNA was calculated as $9.0 \pm 0.5 \times 10^6$. The infection process was followed by thin sectioning and electron microscopy. During infection phage were attached to the cell wall of the host, and the adsorbed phage apparently injected their DNA through an electron microscopically visible channel formed across the cell wall. Maturation of the phage capsid appears to take place within the nuclear region of the cell. Before lysis, intact phage with DNA could be seen first in vesicles at the cell periphery and afterwards between the cell wall and the cytoplasmic membrane. Mature phage were finally released by the rupture of the cell wall.

INTRODUCTION

Bacteriophage AP 50, which is specific for Bacillus anthracis, was isolated from soil (Nagy, 1974) and characterized as one of the few known lipid-containing phages specific for the genus Bacillus (Nagy, 1974; Nagy et al., 1976). Its extremely narrow host specificity, infecting only B. anthracis strains inducible by mitomycin C and harbouring defective prophage structures (Nagy & Ivánovics, 1977), and the fact that phages very similar to AP 50 could be isolated from natural isolates of Bacillus cereus lysogens (Nagy & Ivánovics, 1982) makes further analysis of this phage worthwhile. The present communication describes the protein composition of a clear mutant of phage AP 50 (Nagy, 1974), the estimation of the molecular weight of its DNA and the restriction enzyme patterns obtained by treatment with AsuI. The different stages of the infection and maturation process of phage AP 50c were examined by electron microscopy.

METHODS

Growth and purification of the phage. The clear mutant of phage AP 50 (AP 50c) (Nagy, 1974) was propagated in yeast extract–peptone (YP) medium (Csiszár & Ivánovics, 1965) using B. anthracis strain Sterne (CN 18-74 cur) as host (Nagy & Ivánovics, 1972). The concentration and purification of the phage lysate was carried out as described previously (Nagy, 1974; Nagy et al., 1976). The 20 to 60% (w/v) sucrose gradient–centrifuged peak material with a titre of $10^{12}$/ml was used for the chemical studies.

DNA characterization. Nucleic acid was isolated from the concentrated and purified phage by phenol extraction in $1 \times$ SSC (0.15 M-NaCl, 0.015 M-sodium citrate) after digestion with Pronase K (Merck) in the presence of SDS. Nucleic acid samples were dialysed against 0-01


m-Tris, 0·01 m-NaCl and 0·001 m-EDTA, pH 8. Restriction enzymes were purchased from Boehringer-Mannheim (BamHI) and from Sigma (EcoRI). AsuI was isolated from Anabaena subcyclindrica CCAP 1403/4b in the Biological Research Centre, Szeged, Hungary. Molecular weights were calculated from the mobility of the fragments of BamHI- and EcoRI-treated λ DNA standards (Szybalski & Szybalski, 1979). Agarose gel electrophoresis was performed in a 1 % agarose gel (Sigma) at 4 mA/tube using a buffer containing 0·05 m-Tris, 0·02 m-NaOH, 2 mm-EDTA and 0·02 m-NaCl, pH 8.

Protein analysis. The protein pattern of the purified phage was studied using a discontinuous SDS-polyacrylamide gel system (Laemmli, 1970). The stacking gel contained 5 % (w/v) acrylamide, the separating gel contained 10% (w/v) acrylamide, and the electrode buffer contained 25 mm-Tris–HCl, 0·1 % SDS, 192 mm-glycine pH 8·6. Proteins were solubilized by incubating for 5 min in a boiling water bath and bromophenol blue was added as the tracking dye. The mol. wt. of each polypeptide was determined from the electrophoretic mobility using bovine serum albumin (mol. wt. 68 000), ovalbumin (mol. wt. 45 000), trypsin (mol. wt. 23 000), lysozyme (mol. wt. 17 500), ribonuclease (mol. wt. 15 000) and cytochrome c (mol. wt. 11 700) as standard proteins. Electrophoresis was carried out at 50 V until the samples reached the separating gel and after that at 100 V until the dye was in the lower end of the gel. The gels were stained with Coomassie Brilliant Blue according to Weber & Osborn (1969). The relative amount of each protein was determined from the scanning profiles of the stained gels, assuming that the amount of the protein is proportional to that of the dye bound to the protein.

Preparation of samples for electron microscopy. A culture of CN 18-74 cur growing in YP medium was infected with phage AP 50c at a multiplicity of infection (m.o.i.) of approx. 20. At various times after infection 5 ml samples were removed, centrifuged for 5 min at 5000 g and pre-fixed with 2·5 % glutaraldehyde in 0·1 m-phosphate buffer pH 7·2 for 4 h at 4 °C. The samples were then washed three times with cold 0·1 m-phosphate buffer pH 7·2 and post-fixed with 1% osmium tetroxide for 2 h at room temperature in the same buffer. Osmium-fixed pellets were washed three times with buffer and dehydrated in a rising ethanol series. The samples were then embedded in Araldite (Fluka) and thin sections were prepared with an LKB ultramicrotome I and stained with uranyl acetate (Hayat, 1972) and lead citrate (Reynolds, 1963). Micrographs were taken with a JEOL 100B electron microscope operating at 80 kV.

RESULTS

Protein analysis

The pattern of the virion proteins obtained by electrophoresis is shown in Fig. 1. At least nine proteins with mol. wt. ranging from 63 000 to 12 400 were found. The major structural protein (band 2) which constitutes 50% of the particle protein had a mol. wt. of 48 000. Further purification of the phage on sucrose gradients did not significantly change the relative amounts of these nine proteins, indicating that all the bands are virus components.

Characteristics of AP 50c DNA

EcoRI and BamHI restriction endonucleases failed to cut the DNA of phage AP 50c, but it contained two sites for enzyme AsuI. The mol. wt. of the three fragments obtained were calculated as 6·5 × 10⁶, 1·8 × 10⁶ and 0·5 × 10⁶ using λ DNA fragments as markers (Fig. 2). The mol. wt. of the intact DNA of phage AP 50c was calculated as 9·0 ± 0·5 × 10⁶. This is approximately equal to 14 000 base pairs.
Structural proteins and life cycle of phage AP 50c

Fig. 1. SDS-polyacrylamide gel electrophoresis of phage AP 50c proteins. Lane 1, purified phage AP 50c; lane 2, phage AP 50c further purified on a sucrose gradient. Numbers indicate mol. wt. \((\times 10^3)\) of marker proteins.

Fig. 2. Agarose gel electrophoresis of phage \(\lambda\) DNA treated with \(BamHI\) (lane 1). AP 50c DNA treated with \(AsuI\) (lane 2) and phage \(\lambda\) DNA treated with \(EcoRI\) (lane 3). Numbers indicate mol. wt. \((\times 10^6)\) of \(\lambda\) DNA fragments used as markers (-----) and phage AP 50c DNA fragments (+); 9.0 \(\times\) 10^6 indicates intact phage AP 50c DNA.

Life cycle of phage AP 50c

Samples for thin sectioning were taken 5, 10, 30, 50, 60 and 90 min post-infection. \(B.\) \textit{anthracis} strain CN 18-74 cur was infected with phage obtained from a sucrose gradient-purified peak fraction of phage AP 50c at an m.o.i. of 20. At 5 min post-infection,
only a few phage could be seen attached to the cell wall, and those particles with intact capsids were mostly about 8 to 10 nm from the cell surface (Fig. 3a, arrow). In the preparation fixed 10 min post-infection most of the particles attached to the cell wall had a contracted inner part or were empty (Fig. 3b, arrow). At this stage of infection some complete phage or phage with contracted inner part (core) were observed connected to the inner membrane of the cell by an electron microscopically visible canal (Fig. 3c, arrow). The relative number of empty particles in contact with the cells increased during this period and by 30 min post-infection more than 80% of the adsorbed phage were empty. At this time no alterations to the cell structure had been observed. At 50 min post-infection phage-like structures (empty capsids) were observed in the nuclear region and near to the cell periphery (Fig. 4). The characteristic localization of the mature phage particles in the infected cells was also evident 50 min after infection. At this time, there appeared cells with dense cytoplasm and a vesicle at the periphery full of mature phage particles (Fig. 5a). In some cells the cytoplasmic membrane and the cell wall were distended; however, the cell wall was still intact. At 60 min post-infection intact mature phage particles were visible between the cell wall and the cytoplasmic membrane (Fig. 5b). The first lysed cells were also seen at this time; however, we did not observe bursting cells even in the sample taken at 90 min post-infection.

**Discussion**

Two lipid-containing bacteriophages PM2 and φ6 (Franklin, 1978; Mindich, 1978) have been studied in detail, including analysis of their infectious cycle (Cota-Robles et al., 1968; Bamford et al., 1976). The life cycles of some plasmid-dependent, broad-host range
lipid-containing bacteriophages such as PR4 (Lundström et al., 1979) and PRD1 (L. Mindich & D. H. Bamford, personal communication) have also been reported. However, little is known of the mechanism of infection of the lipid-containing phages infecting Gram-positive bacteria.

Phage AP 50c, which is specific for B. anthracis, adsorbs to the cell surface somewhat more slowly than was observed in the case of phage PR4 (Lundström et al., 1979) which also has a receptor directly on the cell wall. By 30 min after infection about 80% of the adsorbed phage were empty.

AP 50c was reported earlier as a cubic phage in which the lipid layer lies inside the protein membrane, and with a tail-like structure which is usually detected in damaged particles that have lost their nucleic acid (Nagy, 1974; Nagy et al., 1976). This same phenomenon has been observed for other lipid-containing phages belonging to the same phage group (Tectiviridae),
e.g. PR3, PR4 (Bradley & Rutherford, 1975), PR5 (Wong & Bryan, 1978), Bam35 (Ackermann et al., 1978) and φNS11 (Sakaki & Oshima, 1976). It was therefore suggested that the tail-like structure in the case of all these phages is the nucleic acid ejection device which appears only when nucleic acid is to be released (Palva & Bamford, 1980). We presume that in the case of phage AP 50c this interesting mechanism of nucleic acid injection occurs with the aid of the lipoprotein membrane of the phage passing through the cell wall. This process is visible in our electron micrograph taken 10 min post-infection (Fig. 3c) as a canal connecting the phage particles with the cytoplasmic membrane. During further stages of the maturation process of phage AP 50c, which is similar to phage PR4 (Lundström et al., 1979), empty capsids were visible in the nuclear region and also at the periphery of the cells at 50 min post-infection. In samples taken at the same time and at later times mature phage particles appeared first in vesicles at the cell periphery and near to the cell wall. Before rupture of the cell wall and release of the mature phages, intact phage particles could be seen in the space formed between the cell wall and the cytoplasmic membrane.

SDS–polyacrylamide gel electrophoresis yielded nine protein components of phage AP 50c. The mol. wt. of the major protein component (48 000) is very similar to that observed for the Bacillus-specific lipid-containing phage φNS11 (mol. wt. 44 000; Sakaki et al., 1977) which has only five protein components.

Previously, phage AP 50c was reported to be an RNA-containing phage (Nagy et al., 1976) on the basis of a negative diphenylamine reaction. As the method of Burton (1956) is relatively insensitive, we have recently revised this finding (Nagy & Iványics, 1982), and on the basis of DNase sensitivity and RNase resistance of the nucleic acid of phage AP 50c it was concluded that it has a DNA genome. In the present study the mol. wt. of the DNA of phage AP 50c was calculated as $9.0 \pm 0.5 \times 10^6$. This value is very close to that of phage φNS11 DNA ($9.3 \times 10^6$) and that of the PRD1 group phages ($9.8 \times 10^6$ to $10.2 \times 10^6$; Sakaki et al., 1977; Bamford et al., 1981). As with the DNA of phages PRD1 and PR4, AP 50c DNA was also found to be resistant to the restriction enzymes BamHI and EcoRI (Nagy & Iványics, 1982). AsuI, a less specific restriction endonuclease, split the DNA of phage AP 50c into three fragments with a total mol. wt. of $8.8 \times 10^6$.

All these findings show that phage AP 50c belongs to the Tectiviridae subgroup of phages active on the genus Bacillus.

REFERENCES


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