Characterization of *Bdellovibrio bacteriovorus* Bacteriophage MAC-1

By RICHARD C. ROBERTS, MEGAN A. KEEFER AND RAJINDER S. RANU*

Department of Microbiology, Colorado State University, Fort Collins, CO 80523, USA

(Received 11 March 1987; revised 7 July 1987)

The bacteriophage MAC-1, which specifically infects *Bdellovibrio bacteriovorus*, was plaque purified and raised to high titre. The phage was purified by NaCl/polyethylene glycol precipitation, followed by two cycles of isopycnic density gradient centrifugation in CsCl. The purified phage exhibited a density of 1.363 g cm\(^{-3}\) and a sedimentation coefficient of 94S. Nucleic acid isolated from purified phage was resistant to hydrolysis under alkaline conditions and to digestion with RNAase, but it was hydrolysed by DNAase, providing evidence that the phage genome is made up of DNA. The lack of hyperchromic effect upon denaturation, hydrolysis of phage DNA by S1 nuclease, characteristic fluorescent staining with acridine orange, and resistance to digestion with a variety of restriction endonucleases are consistent with the DNA being single-stranded. A buoyant density of 1.722 g cm\(^{-3}\) and a sedimentation coefficient of 17-9S were obtained for the phage DNA. The molecular mass of phage DNA was determined as 1.58 MDa by agarose gel electrophoresis with single-stranded DNA as standards. Electron microscopy of the DNA showed that the genome is circular in nature. In addition, using Southern blots, the two replicative forms, RF1 (supercoiled) and RF2 (circular) have been identified and isolated from infected cell extracts.

**INTRODUCTION**

*Bdellovibrio bacteriovorus* species are extremely small, highly motile, Gram-negative, soil-inhabiting bacteria that attack and replicate in other Gram-negative bacteria (Burnham *et al.*, 1968; Rittenburg, 1983; Shilo & Bruff, 1970; Starr & Seidler, 1971; Stolp & Starr, 1963). Host-independent mutants devoid of parasitic activity and capable of growing on an artificial medium have been described (Diedrich *et al.*, 1970; Seidler & Starr, 1969; Shilo & Bruff, 1970). A group of bacteriophages have been isolated by Althauser *et al.* (1972), Hashimoto *et al.* (1970) and Varon & Levisohn (1972) that specifically infect *B. bacteriovorus* species. Heretofore, none of these phages have been well characterized. In-depth knowledge of the phages of bdellovibrios could serve as a tool in the classification of these unique bacteria. Moreover, a study of the molecular biology of these phages could lead to the development of phage cloning vectors for soil micro-organisms. Eventually these findings could provide the molecular basis for barriers that exist in the expression of genes in different hosts. With these factors in mind, we have initiated studies on the molecular characterization of one of the bdellovibrio phages. We selected MAC-1 for its small size and lack of head or tail structure. Thus, it may lend itself to detailed molecular dissection. In this communication we describe some characteristics of the phage and its genome.

**METHODS**

Materials. Pancreatic RNAase was obtained from Sigma, DNAase I from Worthington Biochemicals, proteinase K from Miles, restriction endonucleases and T4 polynucleotide kinase from International Biotechnologies, and S1 nuclease, λ DNA, M13mp19 DNA and φX174 DNA (+ strand) from Bethesda Research Laboratories. Sources of other materials have been described (Ranu, 1983; Ranu & Kaji, 1972).

Abbreviations: RF, replicative form; ssDNA, single-stranded DNA.
Bacteria, bacteriophage and culture medium. A host-independent strain of *B. bacteriovorus* (ATCC 25631) was obtained from the American Type Culture Collection; the bacteriophage MAC-1 stock was kindly provided by M. Althausser (formerly of the Department of Microbiology, Colorado State University, Fort Collins). The bacteria were propagated in peptone/yeast extract (PYE) as described by Althausser et al. (1972). All incubations were at 30 °C. Liquid cultures were grown in a shaker. The phage titre was determined according to Adams (1959) with some modifications. The high-titre phage stock was prepared essentially according to Hershey that large Petri plates (150 mm) were used.

**Purification of bacteriophage.** All manipulations were performed at 4 °C. To a litre of clear phage suspension, 25 g NaCl was added and stirred gently. Then, 10 g polyethylene glycol (PEG) (average M, 6000) per 100 ml of phage preparation was added slowly with constant stirring. The suspension was kept in an ice bath for 1 h, then centrifuged at 10000 r.p.m. for 1 h in a GSA Sorvall rotor to sediment the granular precipitate that formed. The NaCl/PEG precipitation method quantitatively precipitates the phage from the high-titre phage stock. The precipitate was homogenized in 40 ml phosphate-buffered saline (20 mM-potassium phosphate pH 7.4, 0.15 M-NaCl and 5 mM-magnesium acetate; PBS), and then stirred overnight in the cold in the presence of 50 µg pancreatic RNAase and 100 µg DNAase I. The suspension was centrifuged at 30000 g for 45 min. Essentially all of the phage stock was present in the clear supernate, which was then centrifuged at 40000 r.p.m. in a Spinco Ti 50.2 rotor for 20–22 h. The supernate was discarded and the pellet from each centrifuge tube was suspended in 2 ml PBS. The phage suspension was brought to a density of 1.360 g cm⁻³ by the addition of solid CsCl. The sample was clarified by centrifugation of 20000 r.p.m. in a Spinco Ti 50.2 rotor for 30 min. The clear supernate was then centrifuged at 48000 r.p.m. in a Spinco 50.1 rotor for 24 h. Under those conditions, the phage formed a sharp, light milky band in the CsCl density gradient. The gradients were fractionated and the phage titre in each fraction was determined. The fractions containing the majority of phage were pooled and the CsCl density gradient centrifugation was repeated as described above. The peak fraction containing the phage was dialysed against either PBS or TE buffer [Tris/HCl (pH 8.0) 10 mM and EDTA 1 mM]; in either buffer the phage is stable when maintained at 0 °C. A titre of 5 × 10⁴ to 1 × 10⁵ p.f.u. of purified phage was obtained from 40–60 large Petri plates.

*Isolation of MAC-1 nucleic acid and DNA manipulations.* A purified phage preparation (1 ml containing 6 × 10¹⁴ p.f.u.) was used to isolate phage nucleic acid according to Maniatis et al. (1982). Similarly, standard procedures were used for restriction endonuclease digestions, agarose gel electrophoresis [1-2-3% (w/v) in the TAE buffer, containing 40 mM-Tris/acetate (pH 8.0) and 2 mM-EDTA], digestion with SI nuclease or DNAase or RNAase, molecular mass determinations, and buoyant density and sedimentation coefficient determinations (Maniatis et al., 1982). Electron microscopy of MAC-1 DNA was performed according to Davis et al. (1971). Fluorescent staining of phage nucleic acid with acridine orange and subsequently with molybdic acid and tartaric acid was done according to Bradley (1966).

*Identification of MAC-1 replicative forms (RFs).* Infected cells (multiplicity of infection 1:5) were lysed after 6 h and the non-membrane-bound DNA was isolated (Pagano & Hutchison, 1971). The RFs of MAC-1 in the extracted DNA were identified and then isolated from agarose gels by interception with DEAE-impregnated membranes. The DEAE-bound DNA was eluted with high salt and precipitated with ethanol (Winberg & Hammerskjold, 1980). Aliquots of these samples were electrophoresed in a 1-2% agarose gel in the presence of ethidium bromide. The DNA was denatured at high pH (alkali) and blotted onto a nitrocellulose filter according to Southern as described by Maniatis et al. (1982). The filter was probed using ³²P-labelled MAC-1 single-stranded (ss) DNA, either linearized by shearing or digested with *Hae*III to produce two DNA fragments. These ssDNA probes were end-labelled with ³²P using T4 polynucleotide kinase and [γ³²P]ATP, following either the exchange or forward reaction conditions (after treatment with alkaline phosphatase) as described by Maniatis et al. (1982). Specific activity of the labelled MAC-1 ssDNA probes ranged from 1-0 × 10⁶ to 7-0 × 10⁶ c.p.m. µg⁻¹.

**RESULTS**

*Characterization of MAC-1 phage and its genome.*

The growth of MAC-1 in *B. bacteriovorus* under optimal conditions (at OD₅₄₀ 0-5–0-6 and a multiplicity of infection 0-05–0-1) yielded a titre of 1 × 10¹¹ p.f.u. ml⁻¹ after 48 h incubation. The purified phage preparation displayed a UV absorption spectrum characteristic of a nucleic acid–protein complex with a 260/280 nm ratio of 1-79. The purified phage and its isolated nucleic acid on CsCl density gradient centrifugation exhibited buoyant densities of 1.363 ± 0.003 and 1.722 ± 0.003 g cm⁻³, respectively (results not shown). The sedimentation coefficients of the phage and its nucleic acid, determined by sucrose density gradient centrifugation, were 94S and 17.9S, respectively (results not shown).

The nature of the phage genome was determined to be ssDNA by several criteria. (1) It was
Bdellovibrio bacteriophage MAC-1

Fig. 1. Determination of molecular mass of MAC-1 DNA. ssDNA standards for a molecular mass ladder were generated with M13mp19 and φX174 DNA, and with pBR322 and λ DNA digested with various restriction endonucleases and subsequently heat denatured. Standards and MAC-1 were electrophoresed in a 1.35% agarose gel prepared in TAE buffer and ethidium bromide (a) (lane 1, pBR322 DNA; lane 2, pBR322 DNA digested with EcoRI; lane 3, pBR322 DNA digested with SalI and PstI; lane 4, pBR322 DNA digested with EcoRI and heat denatured; lane 5, pBR322 digested with EcoRI and PstI and heat denatured; lane 6, pBR322 digested with SalI and PstI and heat denatured; lane 7, φX174 DNA; lane 8, M13mp19 DNA; lane 9, MAC-1 DNA; lane 10, λ DNA; lane 11, λ DNA treated with HindIII; lane 12, λ DNA digested with HindIII and heat denatured). The migration distance for each DNA standard is plotted in (b): ○, pBR322 DNA fragments of 0-46, 0-977, 1-19 and 1-44 MDa; △, λ DNA fragments of 0-67, 0-766, 1-43 and 3-11 MDa; ●, φX174 DNA of 1-78 MDa; □, M13mp19 DNA of 2-39 MDa. MAC-1 ssDNA migrated slightly faster than φX174 ssDNA and does not fall exactly at the same point on the molecular mass curve as the φX174 DNA.

Replicative forms of MAC-1

To recognize the RFs of MAC-1, DNA from an infected cell lysate was separated by agarose gel electrophoresis, Southern blotted and probed with MAC-1 ssDNA as the specific hybridization probe (Fig. 3). Three DNA bands contained sequences complementary to MAC-1 and appeared to nearly co-migrate in the gel with the circular, linear, and supercoiled forms of resistant to hydrolysis under alkaline conditions. (2) It was resistant to digestion by pancreatic RNAase (results not shown). (3) It was digested completely by DNAase I (results not shown). (4) The acridine orange fluorescent staining procedure of Bradley (1966) showed a characteristic red fluorescence under UV light at 254 nm. The change of the red colour to pale green upon exposure to either 1% (w/v) molybdic acid solution or 0-1 m-tartaric acid was consistent with the results obtained with M13 phage ssDNA. Bacteriophage λ DNA yielded a bright green fluorescence, which remained green in 1% molybdic acid but changed to orange in the presence of 0-1 m-tartaric acid. (5) The phage nucleic acid was completely resistant to digestion by a variety of restriction endonucleases (BamHI, EcoRI, HindIII, AluI, HaeII, SalI, HpaI, PstI, BglII, SstI, XbaI and XhoI; results not shown) (it was, however, cleaved by HaeIII, which is known to give site-specific cleavage of ssDNA). (6) Finally, the phage DNA was cleaved by S1 nuclease, which is specific for single-stranded nucleic acid (results not shown).

The molecular mass of MAC-1 DNA was determined by agarose gel electrophoresis (Fig. 1). For this purpose, a ssDNA ladder composed of denatured DNA from pBR322 and λ DNA cut with various restriction nucleases, and φX174 and M13mp19 DNA, was used. A molecular mass of 1-58 MDa was calculated, corresponding to 4-6 kbp for the double-stranded form (compared to 5-38 kbp for φX174 double-stranded DNA). The MAC-1 DNA moved a little faster than φX174 DNA (Fig. 1a). Electron microscopy of the phage DNA showed it to be circular in nature (Fig. 2).

Replicative forms of MAC-1

To recognize the RFs of MAC-1, DNA from an infected cell lysate was separated by agarose gel electrophoresis, Southern blotted and probed with MAC-1 ssDNA as the specific hybridization probe (Fig. 3). Three DNA bands contained sequences complementary to MAC-1 and appeared to nearly co-migrate in the gel with the circular, linear, and supercoiled forms of
Fig. 2. Visualization of MAC-1 DNA by electron microscopy. Purified MAC-1 DNA was decorated with cytochrome c as described by Davis et al. (1971) and spread on Formvar-coated copper grids (100 mesh). The DNA was stained with uranyl acetate and shadowed with platinum at a long angle (8 degrees) while being rotated at 60 r.p.m. to coat all sides of the molecules. The sample was viewed with a Philips 400T microscope. The bar represents 400 nm.

pBR322 DNA (Fig. 3, lanes 1 and 5). Differential sensitivity to cleavage by several nucleases was used to identify the conformations of putative RFs. S1 nuclease converted the supercoiled form predominantly into the circular form; some linear form was also observed. The supercoiled and circular forms were resistant to digestion by exonuclease III while the linear form was completely degraded. Bal31 nuclease converted the supercoiled and circular forms to the linear form. The progressive nucleolytic activity of the enzyme decreased the apparent molecular mass of the linear form also. Identical results were obtained when the three forms of pBR322 DNA were subjected to digestion with these enzymes. Based on these results, the MAC-1 RF DNA bands were identified as supercoiled (RF1) (Fig. 3, lanes 5 and 7), relaxed circular (RF2) (Fig. 3, lanes 5 and 6), and linear (RF3) (Fig. 3, lane 5; DNA band between RF1 and RF2). The supercoiled and relaxed circular forms predominated in cell extracts. Also present, though in much smaller amounts, were the RF3 (perhaps formed during isolation) and a slower-migrating form which may represent a concatamer. It was necessary to expose the autoradiogram for a longer period to detect hybridization with RF3 and the concatamer (results not shown). These extracts also contained MAC-1 ssDNA (Fig. 3, lane 5; for comparison, see standard ssDNA in lane 3). It should be noted that uninfected cell extracts showed no hybridization with MAC-1 probes (Fig. 3, lane 4). The RFs of MAC-1 ssDNA are compatible with those expected for a circular ssDNA phage using a double-stranded intermediate for replication.

DISCUSSION

Electron microscopic visualization of the purified MAC-1 phage DNA showed that the genome is circular. Contour length measurements of the most extended molecules are consistent with the molecular mass determined by agarose gel electrophoresis. The circularity of the genome is also supported by its lack of susceptibility to digestion with spleen phosphodiesterase. Moreover, native MAC-1 DNA is a poor substrate for T4 polynucleotide kinase in either exchange or forward reactions (unpublished results). If the phage DNA is first linearized, however, it is readily phosphorylated by T4 polynucleotide kinase. Two major double-stranded
RFs have been identified in infected cell extracts, the supercoiled form (RF1) and the relaxed circular form (RF2). These two RFs of MAC-1 phage DNA (4.6 kbp) nearly comigrate with the supercoiled and relaxed circular form of pBR322 DNA (4.36 kbp). Consistent with a lower molecular mass of the DNA, the RFs of MAC-1 migrate faster than the two RFs of φX174 DNA, RFI and RFII (results not shown). The sedimentation coefficient of 17-9S of the MAC-1 ssDNA, compared with 24S of φX174 ssDNA, is also consistent with the smaller size of the MAC-1 phage genome. Southern blots of φX174 RFs show no detectable hybridization when probed with labelled MAC-1 genomic DNA (R. Gallegos, R. C. Roberts & R. S. Ranu, unpublished results). A direct co-relationship between purified phage DNA and the genome of the phage has been established by transfection of *B. bacteriovorus* (Roberts & Ranu, 1987). Previously a diverse family of isometric coliphages have been isolated (Godson, 1978). They are among the smallest DNA viruses. These phages share many different characteristics, in that they all contain a circular ssDNA genome and infect either different *Escherichia coli* strains or *Salmonella* (Godson, 1978). Such isometric phages with ssDNA as their genome have not been described for other bacteria (Godson, 1978). We believe this is the first report of a ssDNA isometric-shaped phage that infects bacteria other than *E. coli* and *Salmonella*. Further studies on the molecular biology of the phage are in progress.

Note added in proof. Using a single *Hind*III site in the replicative form of MAC-1 DNA, the entire phage genome has been cloned in pBR322.

We thank Richard Grant, Greg Hirschfield and Virginia Salas for participating in the preliminary stages of this work. We also thank Dr Martin Pato of the National Jewish Hospital and Research Center for his Bausch & Lomb ABBE-3L Refractometer and Ms Judith Pasternak for transporting the refractometer to us and for showing us how to use it. Finally, our thanks are due to Dr Mary Althauser for encouraging us to work with this system. This work is taken in part from a thesis submitted by R. C. R. in partial fulfilment of an MS degree requirement. This work is supported in part by USDA formula funds for animal health and disease and NIH BRSG grant 5S07-RR-05458-17 to R. S. R.
REFERENCES


