Further Characterization of a Bacteriophage Recovered from an Avian Strain of *Chlamydia psittaci*

By **CHRISTOPHER C. STOREY, MERYL LUSHER, SHIRLEY J. RICHMOND** and **JANE BACON**

Department of Medical Microbiology, Medical School, University of Manchester, Oxford Road, Manchester M13 9PL, U.K.

(Accepted 14 February 1989)

**SUMMARY**

The genome of a 22 nm icosahedral phage which infects some avian *Chlamydia psittaci* strains recovered from domestic ducks has been characterized as a ss circular DNA molecule of about 4850 bases. The replicative form of this genome was isolated from purified chlamydial organisms. A restriction endonuclease cleavage site map of the genome was constructed from dsDNA synthesized in vitro from ss phage DNA and EcoRI fragments were then cloned into pUC9. The phage genome was detected only by Southern blot hybridization in *C. psittaci* which was productively infected with phage; no evidence was found for the integration of phage DNA into the chlamydial chromosome. Three viral polypeptides, of approximate Mr values 75K, 30K and 16.5K were identified when phage was analysed by SDS-PAGE. This virus, which we have designated Chpl, is either an aberrant member of the *Microviridae* or the first member of a new bacteriophage family.

**INTRODUCTION**

In 1982, phage infection was described in two *Chlamydia psittaci* isolates (N352 and N232) (Richmond *et al.*, 1982). These isolates were recovered from domestic ducks that had been implicated in an outbreak of ornithosis in poultry workers (Andrews *et al.*, 1981). Crystalline arrays of virus within distended reticulate bodies and free virus within mature chlamydial inclusions were seen by transmission electron microscopy when these isolates were grown in McCoy cell monolayers. A 22 nm icosahedral virus, with a buoyant density of 1.37 g/ml in CsCl, was recovered from the supernatants of these infected cell cultures (Richmond *et al.*, 1982). We now further characterize this virus, the only phage known to infect a well documented *Chlamydia* species.

**METHODS**

*Chlamydial strains.* Strains N352, N232 (Richmond *et al.*, 1982) and 360 (Bacon *et al.*, 1986) are all wild avian *C. psittaci* isolates recovered from domestic ducks. Strain N352 is infected with the bacteriophage we have designated Chpl. Phage was also initially detected in N232 (Richmond *et al.*, 1982); on subsequent passage in cell culture however, phage expression ceased and the stocks of N232 used in this work did not contain phage detectable either serologically (Bacon *et al.*, 1986) or by electron microscopy. Phage has never been detected in strain 360. The laboratory *C. psittaci* strain 6BC (Morgan & Wiseman, 1946), supplied by Dr T. Hatch, University of Tennessee, Memphis, Tenn., U.S.A., is also of avian origin. *Chlamydia trachomatis* serotype L2 (strain 434) was provided by Professor J. Schachter, University of California, San Francisco, Ca., U.S.A. All chlamydiae were grown in cycloheximide-treated McCoy cell monolayers under standard conditions (Richmond *et al.*, 1985).

*Phage DNA preparation.* Phage was harvested from McCoy cell monolayers, which had been heavily infected with N352 after a 48 to 72 h incubation period, by three cycles of freezing and thawing, followed by low speed centrifugation at 2500 g for 10 min to remove cellular debris. The supernatant was then filtered (0.2 μm Acrodisc...
which contained proteinase K (200 µg/ml) and 0.5% (w/v) SDS. After incubation at 60 °C for 30 min, DNA was synthesized and digested with restriction enzymes under conditions recommended by the manufacturer (Boehringer). pH 7.0), then washed in 0.5 x SSC which contained 0.1% (w/v) SDS at 65 °C. Filters were autoradiographed using filters at 65 °C. Filters were washed twice in 2 x SSC (SSC consisted of 0.15 M-NaCl and 0.15 M-trisodium citrate, pH 7.0), then washed in 0.5 x SSC which contained 0.1% (w/v) SDS at 65 °C. Filters were autoradiographed using Fuji RX-G film. Hybridization probes were produced from DNA purified by electrophoresis through low melting temperature agarose by the random-primed oligolabelling method of Feinberg & Vogelstein (1984).

Electron microscopy. Nucleic acid (1 ng/µl) was prepared in 20 µl of hyperphase (30% v/v formamide, 25 µg/ml cytochrome c, 0.1 M-Tris–HCl pH 8.5 and 0.02 M-EDTA) and spread on a hypophase which consisted of 10% (v/v) formamide, 10 mM-Tris–HCl pH 8.5 and 1 mM-EDTA. Nucleic acid was picked up on collodion-coated grids, stained in 0.05 M-uranyl acetate in 90% ethanol, then rotary-shadowed with platinum (Burkardt & Puhler, 1984); 18 MΩ water was used throughout these procedures. Grids were examined on an AEI Corinth electron microscope. Measurements were made directly from electron micrographs using ‘Measuremouse’ (Analytical Measuring Systems Ltd.).

Nuclease digestion. ChpI nucleic acid (200 ng) was incubated at 37 °C for 30 min with either DNase I (20 µg/ml, Sigma), RNase A (20 µg/ml, Sigma), DNase S1 (1 unit, Sigma) or exonuclease VII (1 unit, BRL) under the reaction conditions recommended by the manufacturers. Each digest was analysed by electrophoresis on a 0.8% agarose gel stained with ethidium bromide. Linear dsDNA (800 base fragment of cloned DNA), ssDNA (M13mp18) and RNA (RNA, Sigma) were included as controls.

Second strand synthesis. Single-stranded ChpI DNA was primed with random hexanucleotides and the second strand was synthesized by the Klenow fragment of DNA polymerase I (15 units/µg of DNA) in 50 mM-Tris–HCl pH 8.0, 200 mM-HEPES pH 6.6, 5 mM-MgCl2 and 1 mM-2-mercaptoethanol after the addition of dATP, dCTP, dGTP and TTP, each at a concentration of 0.5 mM. The mixture was incubated at 15 °C for 15 h. The dsDNA was phenol–chloroform-extracted and precipitated with ethanol.

Restriction endonuclease analysis. Double-stranded DNA of ChpI was obtained from ssDNA by second-strand synthesis and digested with restriction enzymes under conditions recommended by the manufacturer (Boehringer Mannheim, Pharmacia or BRL). Digests were analysed by electrophoresis on agarose gels and a restriction endonuclease cleavage site map was constructed from the analysis of single and double digests (Maniatis et al., 1982).

Cloning of phage DNA. Double-stranded DNA constructed from ss ChpI was digested with EcoRI and ligated into the EcoRI site of the pUC9 plasmid vector. Ligated DNA was used to transform Escherichia coli JM83 by the method of Kushner (1978). Colonies were screened by restriction analysis of plasmid DNA extracted by the method of Birnboim & Doly (1979). Double-stranded covalently closed circular (CCC) DNA preparation. This was prepared from purified chlamydial DNA by the method of Birnboim & Doly (1979), except that the lysozyme step was omitted.

Southern blotting. Southern blotting was carried out by standard methods (Maniatis et al., 1982). DNA was transferred to Hybond-N nylon filters (Amersham) and then hybridized with 32P-labelled probe DNA overnight at 65 °C. Filters were washed twice in 2 x SSC which contained 0.1% (w/v) SDS at 65 °C. Filters were autoradiographed using Fuji RX-G film. Hybridization probes were produced from DNA purified by electrophoresis through low melting temperature agarose by the random-primed oligolabelling method of Feinberg & Vogelstein (1984).

Restriction endonuclease cleavage. Double-stranded DNA of ChpI was obtained from ssDNA by second-strand synthesis and digested with restriction enzymes under conditions recommended by the manufacturer (Boehringer Mannheim, Pharmacia or BRL). Digests were analysed by electrophoresis on agarose gels and a restriction endonuclease cleavage site map was constructed from the analysis of single and double digests (Maniatis et al., 1982).

Cloning of phage DNA. Double-stranded DNA constructed from ss ChpI was digested with EcoRI and ligated into the EcoRI site of the pUC9 plasmid vector. Ligated DNA was used to transform Escherichia coli JM83 by the method of Kushner (1978). Colonies were screened by restriction analysis of plasmid DNA extracted by the method of Birnboim & Doly (1979). Native ds phage DNA extracted from chlamydial organisms was also cloned into the EcoRI site of pUC9 as described above, except that the colonies were screened by colony-blot hybridization (Grunstein & Hogness, 1975) using 32P-labelled ss phage DNA as the probe.

Poly peptide analysis. Phage was labelled with 35S by growing N352 in the presence of the Trans35S label (ICN Biochemicals) throughout the chlamydial developmental cycle. Trans35S is a hydrolysate of E. coli grown in the presence of 35SO4²⁻, which contains a maximum concentration of 70% [35S]methionine, 15% [35S]cysteine, 7% labelled methionine sulfoxide, 3% [14C]cysteic acid and 5% of other 35S-labelled compounds; 72 µCi was added in methionine-free medium to N352-infected McCoy cell monolayers grown in a 25 cm² flask. Phage was harvested as described previously except that nucleic treatment was omitted and the phage pellet was resuspended in an SDS sample buffer (1% w/v SDS, 0.1 M-Tris–HCl pH 8.6, 1% w/v dithiothreitol) at a concentration of 5000 c.p.m./µl. Radioimmunoprecipitation was carried out by the method of Kessler (1975). Unlabelled phage particles were prepared as above but the phage pellet was further purified on a glycerol–potassium tartrate gradient (Ashley & Cauf, 1982). SDS–PAGE was performed by standard methods (Laemmli, 1970) and 35S-labelled gels were dried and autoradiographed at -70 °C on Fuji RX-G film. Some gels were treated with En3Hance (DuPont) prior to drying. Unlabelled gels were stained with Coomassie Brilliant Blue.
Characterization of a chlamydiaphage

RESULTS

Phage nucleic acid

Nuclease digestion showed that Chpl nucleic acid was DNA, because it was sensitive to DNase I but resistant to RNase A (Fig. 1, lanes 3 and 4). Sensitivity to S1 nuclease but resistance to exonuclease VII suggested this DNA was ss and circular (Fig. 1, lanes 5 and 6). This was consistent with the observation that it could act as a template for the Klenow fragment of E. coli DNA-dependent DNA polymerase I. Electron microscopy confirmed that the phage genome was circular (Fig. 2). Its appearance was typical of ssDNA and it had a mean length of 4920 bases (n = 40, s.d. ± 779) when M13mp10 was used as an internal calibration standard.

![Figure 1](image1.png)

**Fig. 1.** Ethidium bromide-stained agarose gel of Chpl nucleic acid digested with nuclease. Lane 1, markers (kb; 1 kb ladder, BRL); lane 2, undigested Chpl nucleic acid; lane 3, DNase I; lane 4, RNase A; lane 5, DNase S1; lane 6, exonuclease VII.

![Figure 2](image2.png)

**Fig. 2.** Electron micrograph of Chpl nucleic acid. The bar marker represents 500 nm.
Double-stranded DNA for restriction endonuclease analysis and cloning experiments was produced from native Chp1 DNA by second-strand synthesis with the Klenow fragment after priming with random hexanucleotides. The resultant molecules were not uniform in size but they were cleaved to standard lengths with restriction enzymes. A restriction endonuclease map of the Chp1 genome is shown in Fig. 3. An estimated length of 4800 bases was obtained \((n = 36, \text{ S.D. } \pm 116)\) by summation of the sizes of the different restriction fragments, which was in good agreement with the size of the genome estimated by electron microscopy.

Urografin-purified chlamydiae were subjected to a standard SDS/alkaline lysis preparation that extracts preferentially CCC DNA, in order to see whether ds Chp1 DNA was present in N352. Although the total yield of DNA was extremely low, two DNA bands could be seen on ethidium bromide-stained agarose gels. These were thought to be the 7.5 kb plasmid (pCpA) that has been demonstrated in N352 (Lusher et al., 1989) and ds phage DNA.

Southern blot hybridization of an EcoRI digest of this material probed with \(^{32}P\)-labelled pCpA and a phage probe constructed from native ss phage DNA, confirmed that both ds phage DNA and plasmid DNA were present (Fig. 4). This ds phage DNA is probably the replicative form of Chp1.

**Cloning**

The native ds form of Chp1 was chosen initially to clone phage DNA. This was cut with EcoRI, then ligated into the EcoRI site of pUC9 and transformed into JM83. Chp1 DNA was present in only two clones designated 3.304 and 3.163, which both contained the same 800 base insert.

Since the above method of cloning proved very inefficient, an alternative technique was tried which utilized ds phage constructed from ssDNA. Chp1 DNA was then cleaved by EcoRI, ligated into pUC9 and the clones were identified by size and restriction mapping. Clones that contained inserts which corresponded to all five EcoRI fragments of sizes 800, 750, 450, 1350 and 1500 bases were obtained; these were designated Chp1 E1, E2, E3, E4 and E5 respectively. The total size of these five cloned fragments confirmed previous estimates of the length of the Chp1 genome obtained by electron microscopy and restriction analysis.
Characterization of a chlamydiaphage

Fig. 4. Southern blot hybridization of N352 extrachromosomal DNA probed with \(^{32}\)P-labelled Chpl (a) and *C. psittaci* plasmid pCpA (b). Lanes 1, markers (kb; \(^{32}\)P-labelled 1 kb ladder, BRL); lanes 2, N352 extrachromosomal DNA; lanes 3, Chpl DNA.

Fig. 5. Ethidium bromide-stained agarose gel electrophoresis (a) and Southern blot hybridization (b) of chlamydial chromosomal DNA from different strains. Lane 1, markers (kb; 1 kb ladder, BRL); lane 2, N352; lane 3, N232; lane 4, 360; lane 5, 6BC; lane 6, L2. Open arrowhead indicates chromosomal DNA, solid arrowheads, extrachromosomal DNA.

Chromosomal DNA from all five chlamydial strains studied (see Methods) was analysed by Southern blot hybridization to determine whether the Chpl genome was integrated into the chromosomal DNA of any of these strains. No hybridization with any chromosomal DNA occurred when \(^{32}\)P-labelled Chpl E4 was used as the hybridization probe (Fig. 5). This suggests that integration of the Chpl genome does not occur in chlamydial strains in which phage has been detected, irrespective of whether productive phage infection is currently occurring (as in
N352) or whether phage is no longer expressed (as in N232). It also suggests that chromosomal DNA of chlamydial strains in which phage has never been detected (C. psittaci strains 360 and 6BC and C. trachomatis serotype L2) do not contain phage nucleic acid sequences either. The Southern blot of N352 DNA revealed two extrachromosomal bands of DNA homologous to the Chp1 E4 probe (Fig. 5b, lane 2). The other four chlamydial strains, including strain N232 which previously expressed phage, did not contain any extrachromosomal DNA which reacted with the Chp1 probe.

**Polypeptides**

The number of structural polypeptides of Chp1 and their approximate Mr were determined by SDS-PAGE. Three major polypeptides designated VP1, VP2 and VP3 were detected when gels of 35S-labelled Chp1 were autoradiographed (Fig. 6). This result was confirmed when unlabelled Chp1 purified on gradients and Chp1 obtained by radioimmunoprecipitation both revealed the same three polypeptides on SDS-PAGE (data not shown). The mean Mr of VP1 was 75K (n = 4), VP2 was 30K (n = 4) and VP3 was 16.5K (n = 3).

**DISCUSSION**

Two well documented families of bacteriophage have ss circular DNA genomes comparable to the genome of the chlamydiophage Chp1 described in this work: the Inoviridae, of which M13 is a representative member, and the Microviridae, to which φX174 belongs (Matthews, 1982). The filamentous morphology of the Inoviridae clearly distinguishes them from Chp1, which like the Microviridae is an icosahedral virus. The Microviridae, however, all have knob-like spikes on their vertices which have not been observed in Chp1 (Richmond et al., 1982). The mean size (27 nm) and buoyant density (1.40 g/ml) of the Microviridae virion (Matthews, 1982) are both greater than those of Chp1 (size 22 nm, buoyant density 1.37 g/ml) (Richmond et al., 1982) as is the Microviridae genome, which is about 5400 bases in size or larger (Godson, 1978). On existing evidence, therefore, Chp1 does not conform exactly to any well recognized bacteriophage group. Further data on the organization of the Chp1 genome will determine whether this virus is an aberrant member of the Microviridae or the first member of a new family of bacteriophages.

Little is known at present about the nature and transmission of this phage infection of *Chlamydia*, prokaryotes which themselves parasitize the cytoplasm of eukaryotic cells. Early
Characterization of a chlamydiophage

observed based on serology and electron microscopy led us to postulate that phage infection may sometimes be cryptic because we were unable to detect phage in all *C. psittaci* isolates obtained from common source ducks and also because the proven phage-infected strain N352 did not always express phage (Bacon *et al.*, 1986). However the present work has produced no evidence to support the existence of a cryptic state. Phage DNA was detected only in avian *C. psittaci* stocks in which phage could also be detected serologically. N232, which ceased to express phage after passage in cell culture, appeared to be 'cured' of infection rather than cryptically infected.

We are currently developing *in situ* DNA hybridization techniques to study the presence of Chp1 DNA at the intra-inclusion level in order to elucidate further the distribution and mode of transmission of this phage.

This work was supported by a grant from the Wellcome Trust.

REFERENCES


(Received 14 December 1988)