Isolation and Characterization of a Bacteriophage Si1 for *Spirillum itersonii*

By G. D. CLARK-WALKER and S. B. PRIMROSE*

The Research School of Biological Sciences and The John Curtin School of Medical Research, The Australian National University, Canberra, A.C.T., Australia

(Accepted 18 January 1971)

**SUMMARY**

A large icosahedral bacteriophage, Si1, infectious for *Spirillum itersonii* has been isolated and characterized. It contains double-stranded DNA which has 53 % G+C, no unusual bases and a molecular weight $2.6 \times 10^7$.

**INTRODUCTION**

The bacterium *Spirillum itersonii* has been used in studies of the regulation of cytochrome synthesis (Clark-Walker, Rittenberg & Lascelles, 1967), but we felt that these studies would be aided if a transducing bacteriophage was available for genetic analysis. Attempts to isolate such a phage by induction with mitomycin C and ultraviolet light were unsuccessful, but examination of induced cultures in the electron microscope revealed the presence of phage tails (Clark-Walker, 1969). Subsequently, natural habitats of the bacterium were investigated for the presence of viruses. We report here the isolation of a phage, designated Si1, for *S. itersonii*. Although we have not established that this virus is capable of transduction, we are prompted to report our results because of some unique characteristics of the phage and also because it is, to our knowledge, the first virus isolated for any species of the genus *Spirillum*.

**METHODS**

*Bacterial cultures.* The strain of *Spirillum itersonii* used as host for the isolation of the phage was the same as in previous studies (Clark-Walker, 1969) and was originally obtained from Dr R. Martinez, Bacteriology Department, University of California, Los Angeles. *S. itersonii* var. *vulgatum* ATCC 11331 and *S. lunatum* ATCC 11337 were obtained from Dr V. B. D. Skerman, University of Queensland, and *S. serpens* from Dr R. Rittenberg, University of California, Los Angeles. We have also isolated from a local pond a *Spirillum* resembling *S. itersonii*.

*Media.* The YEMG medium has been described previously (Clark-Walker, 1969). PGY medium contains per l. K$_2$HPO$_4$, 0.2 g.; MgSO$_4$·7H$_2$O, 0.04 g.; CaCl$_2$·2H$_2$O, 0.04 g.; monosodium glutamate 1.86 g.; Difco yeast extract, 1.0 g. and Difco proteose peptone 1.0 g. The pH of this medium was adjusted to 7.2 with NaOH. This medium diluted 1:1 with water and supplemented with 1.5 % (w/v) agar was used for plates. Agar overlays of PGY/2 contained PGY/2 medium supplemented with 0.6 % agar for phage assay and 0.25 % agar for the preparation of phage stocks.

*Isolation of bacteriophage Si1.* After numerous unsuccessful attempts to isolate a virus

* Present address: Department of General Microbiology, University of Edinburgh, Edinburgh, Scotland.
from ponds and streams around Canberra, raw sewage was obtained from the treatment works at Weston Creek. This was centrifuged at 5000g for 10 min. to remove major debris and the supernatant fluid centrifuged at 30,000g for 1 hr. The pellet was allowed to resuspend overnight in the drainings of the centrifuge tube and then filtered through a 0.8 μm filter. The filtrate was plated with actively growing cells using YEMG and YEMG/2 plates and overlays. An average of 3 plaques/plate was obtained, the plaques being larger on the diluted medium. One plaque was picked and purified by several successive single plaque isolations on YEMG/2 plates. A working stock of phage was prepared by taking a plate showing confluent lysis and flooding it with YEMG/2 broth to elute the phage.

**Plaque assay of S. i.** YEMG/2 medium did not give reproducible titres, but reproducibility was improved by reducing the concentrations of phosphate, calcium and magnesium in the medium. Consequently, PGY/2 plates and overlays were used. For plaque assays, 0.2 ml. of a PGY broth culture of *S. iersonii* (*E*540 = 1.0) were added to 0.1 ml. of phage suspension. After standing for 5 min., 4 ml of PGY/2 agar at 47° was added and the contents poured on to approximately 15 ml. of PGY/2 agar in an 8-5 cm. diameter Petri dish. Clear plaques measuring about 1 mm. diameter could be counted after 16 hr at 30°.

**Host range.** A preparation containing 8 × 10⁸ p.f.u./ml. was plated on the strains of *Spirillum* listed above, using both YEMG/2 and PGY/2 media. The virus was only active against the particular strain of *S. iersonii* on which it had been isolated.

**Stability.** The phage was dialysed for 72 hr at 4° against the indicated buffers and then assayed.

**Preparation of S11.** Since we have been unable to find conditions permitting growth in liquid culture, preparations of the phage have been made from plate lysates. One hundred and forty PGY/2 plates were overlaid with 4 ml. of PGY/2 0.25% agar containing 2.5 × 10⁵ p.f.u. and 2 × 10⁷ bacteria (corresponding to 0.2 ml. PGY broth culture at *E*540 = 1.0). After overnight incubation at 30° the semi-solid agar overlays were scraped off into a beaker and each plate was washed with approximately 2 ml. of 0.02 m-sodium phosphate buffer pH 7.2 and the washings added to the combined overlays. The agar and major debris were removed by centrifugation at 8000g for 10 min. and the agar pellet washed by resuspension in 0.02 m-sodium phosphate buffer pH 7.2 and followed by recentrifugation as above. The combined supernatant fluids were centrifuged at 30,000 rev./min. for 90 min. in a Spinco no. 42 Rotor at 2°. Each pellet was allowed to resuspend overnight at 0° in a small volume of sodium phosphate buffer pH 7.2 and the washings added to the combined overlays. The agar and major debris were removed by centrifugation at 8000g for 10 min. and the agar pellet washed by resuspension in 0.02 m-sodium phosphate buffer pH 7.2 and subsequently assayed. The combined supernatant fluids were centrifuged at 30,000g for 10 min. and the agar pellet washed by resuspension in 0.02 m-sodium phosphate buffer pH 7.2 and subsequently assayed.

**Preparation of S11 DNA.** A phage preparation in 2 ml. of 0.05 m-NaCl, 0.005 m-EDTA and 0.05 m-tris + HCl pH 8.0 was mixed with an equal volume of water-saturated redistilled phenol and gently agitated for 30 min. at room temperature. Aqueous and phenolic layers were separated by centrifugation and the aqueous layer subjected to a second phenol extraction. The aqueous layer was dialysed against three changes of 2 l. of 1/10 diluted 0.15 m-NaCl, 0.015 m-trisodium citrate pH 7.0 (SSC) for 1 hr and then incubated for 45 min. at 37° with 1 ml. of self-digested pronase (1 mg./ml.). The phenol extraction and dialysis procedures were repeated and the concentration of DNA in the final extract was estimated from the extinction at 260 nm.

**Preparation of Spirillum iersonii DNA.** *S. iersonii* was grown to stationary phase in 200 ml. of YEMG medium. The cells were harvested by centrifugation at 3000g for 5 min.,
resuspended in 50 ml of tris + HCl and resedimented. The bacterial pellet was resuspended in 5 ml tris + HCl containing 100 mg/ml sucrose, 1 mg/ml lysozyme and 0.5 mg/ml of heat-treated ribonuclease and incubated at 30°C for 10 min. The cells were lysed by the addition of 1 ml of 2% Sarkosyl and the DNA extracted from the resulting viscous solution by the method of Marmur (1963).

**Determination of DNA buoyant density.** Buoyant density was measured in a Spinco model E ultracentrifuge using an AN-D Rotor and a cell with a 12 mm, 4°C Kel-F centrepiece. Approximately 2 µg of DNA, together with an equal quantity of *Micrococcus lysodeikticus* DNA, was dissolved in CsCl and the density adjusted to 1.710 g/cm³. The centrifuge was run at 44,770 rev./min. for 22 to 24 hr with the temperature controlled at 25°C. At the end of this time photographs were taken and buoyant densities and corresponding percentage of guanine + cytosine were determined as described by Mandel, Schildkraut & Marmur (1968). The buoyant density of the virus DNA was also determined in Cs₂SO₄ by the method of Grunwedel & Davidson (1967) and the corresponding G + C content taken from the tables provided by Szybalski (1968).

**Electron microscopy.** Samples were examined on thin carbon films supported on 400-mesh copper grids and negatively stained with 2% (w/v) ammonium molybdate after allowing 1 min. for adhesion to the carbon film. For estimation of the virus particle size, samples were mixed with catalase crystals and the preparation negatively stained as above. Measurements of virus particles were made from photographs showing both virus particles and catalase crystals and the size calculated from the lattice spacing of the catalase crystal which was taken as 86 Å (Wrigley, 1968). Electron microscopy was performed on a Hitachi HU11E.

**Electron microscopy of Si I DNA.** Virus DNA was examined by the procedure of Klein-schmidt et al. (1962). A small quantity of phage (0.1 ml. in 0.02 M-sodium phosphate pH 7-2) was mixed with 1 ml of distilled water containing 0.01% cytochrome c. The solution (0.2 ml.) was spread on a clean water surface in a Teflon-coated aluminium trough and allowed to stand at room temperature for 2 hr without disturbance. Areas of the surface were picked up on carbon films supported on 400-mesh copper grids, dehydrated in ethanol, dried and rotary shadowed with platinum at an angle of 6°. DNA was measured on photographs with a map-measuring device calibrated against photographs of negatively-stained catalase crystals taken at the same microscope settings.

**Chemicals.** Caesium chloride was obtained from Rare Earth Division of the American Potash and Chemical Corporation, West Chicago, Illinois, and purified by recrystallization three times. Caesium sulphate was obtained from British Drug Houses, Poole, Dorset, U.K., and used without further purification. Phenol was obtained from E. Merck, Darmstadt, W. Germany, and redistilled before use. Sarkosyl L was obtained from Geigy Industrial Chemicals, Ardsley, New York, ribonuclease A and lysozyme were obtained from Worthington Biochemical Corporation, Freehold, New Jersey, pronase from Calbiochem, Los Angeles, California, cytochrome c from Sigma Chemical Company, St Louis, Missouri, and catalase crystals from C. E. Boehringer, Mannheim, W. Germany. *Micrococcus lysodeikticus* DNA was a generous gift from Dr A. J. D. Bellett.

### RESULTS

**Purification of bacteriophage Si I**

The stages in the purification of bacteriophage Si I are given in Table 1. Only 11% of the original infectivity was recovered in the high-speed pellet. There was a further marked loss in titre after additional purification in a CsCl gradient.
The virus could be stored in YEMG/2 broth at 2 °C in the presence of chloroform for 6 months with only a threefold decrease in titre. However, storage of the virus at acid pH or in the presence of tris buffer lead to a marked loss in titre (Table 2).

Characterization of bacteriophage Si1

Morphology of Si1. The particles of purified virus hexagonal in outline and icosahedral in shape, were 63 nm. in diameter (Fig. 1). The particles did not appear to have any tail, although occasional particles were seen which suggested that one vertex of the virus may be different in structure from the rest. We considered the possibility that a tail or tail fibres had been lost during the course of preparation and that this could have accounted for the loss in titre observed during purification. However, when the washings from a plate showing confluent lysis were examined, no detectable change was observed in the appearance of the phage.

Table 1. Preparation of bacteriophage Si1

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Combined agar overlays</td>
<td>1000</td>
<td>1.4 x 10^8</td>
<td>1.4 x 10^10</td>
<td>100</td>
</tr>
<tr>
<td>Combined supernatants from agar washing</td>
<td>780</td>
<td>3.1 x 10^6</td>
<td>2.4 x 10^10</td>
<td>17</td>
</tr>
<tr>
<td>Supernatant from high-speed centrifugation</td>
<td>780</td>
<td>0.074 x 10^8</td>
<td>0.58 x 10^10</td>
<td>0.4</td>
</tr>
<tr>
<td>Resuspended pellet</td>
<td>5</td>
<td>3.0 x 10^8</td>
<td>1.5 x 10^10</td>
<td>11</td>
</tr>
<tr>
<td>Particle-containing band from CsCl gradient after removal of CsCl</td>
<td>2</td>
<td>1.2 x 10^8</td>
<td>0.024 x 10^10</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Table 2. Stability of Si1 in different buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>p.f.u.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M-sodium phosphate</td>
<td>6.9</td>
<td>4.0 x 10^8</td>
</tr>
<tr>
<td>0.05 M-sodium borate</td>
<td>7.3</td>
<td>2.7 x 10^8</td>
</tr>
<tr>
<td>YEMG/2</td>
<td>7.3</td>
<td>3.1 x 10^8</td>
</tr>
<tr>
<td>0.1 M-tris+HCl</td>
<td>7.0</td>
<td>3.0 x 10^8</td>
</tr>
<tr>
<td>0.1 M-tris+HCl</td>
<td>7.0</td>
<td>3.0 x 10^8</td>
</tr>
<tr>
<td>0.1 M-tris+HCl</td>
<td>7.0</td>
<td>3.0 x 10^8</td>
</tr>
<tr>
<td>0.1 M-sodium acetate</td>
<td>5.8</td>
<td>1.7 x 10^8</td>
</tr>
</tbody>
</table>

Properties of Si1 nucleic acid. The buoyant density of the phage nucleic acid was determined in caesium chloride and caesium sulphate, since Szybalski (1968) has pointed out that if both determinations do not lead to the same percentage guanine + cytosine values, some unusual bases or abnormal structural features of DNA can be expected. In CsCl, the buoyant density was estimated as 1.712 g./cm.³ (Fig. 4), a value consistent with double-stranded DNA containing 53 % guanine + cytosine (Mandel et al. 1968). Si1 DNA differed significantly from the host DNA which had a buoyant density of 1.721 g./cm.³, corresponding to a guanine + cytosine content of 62 %. In caesium sulphate the buoyant density of Si1 DNA was 1.428 g./cm.³, corresponding to a guanine + cytosine content of 55 % (Szybalski, 1968). Since this value was in reasonable agreement with the value found in caesium chloride, it is unlikely that any unusual bases were present.

In the electron microscope the phage DNA was a linear molecule (Fig. 2) 13.0 to 13.5 μm. long (Fig. 5). At least 2 hr were needed for the extrusion of the DNA molecule from the
Fig. 1. Bacteriophage SiI stained with ammonium molybdate after purification on a CsCl density gradient. The arrows point to possible modified vertices.
Fig. 2. A linear molecule of SiI DNA.
Fig. 3. DNA associated with particles of SiI 2 hr after spreading on a protein film.
virus, and DNA could still be seen emerging at this time (Fig. 3). The molecular weight of the phage DNA was $2.6 \times 10^7$, based on a length of $13.25 \mu m$ and a weight:length ratio of 196 daltons/Å for the sodium salt of DNA (Thomas, 1966).

![Fig. 4](image1)

**Fig. 4**

Fig. 4. Caesium chloride (left) and Cs$_2$SO$_4$ (right) density gradient ultraviolet absorbence patterns from the analytical ultracentrifuge. *Micrococcus lysodeikticus* DNA ($\rho = 1.731$ g./cm.$^3$ in CsCl and 1.435 g./cm.$^3$ in Cs$_2$SO$_4$) was used as a marker.

![Fig. 5](image2)

**Fig. 5**

Fig. 5. Histogram showing length distribution of Si I DNA measured from electron micrographs using catalase crystals for calibration.

**DISCUSSION**

To our knowledge the bacteriophage, Si I, infective for *Spirillum itersonii* is the first bacteriophage isolated for any species in the genus *Spirillum*. It is interesting to note that the bacterium used for the isolation of the virus was originally obtained from Lake Eire, U.S.A. (R. Martinez, personal communication), whereas the bacteriophage found in sewage from Canberra was not infective for similar locally isolated strains of *Spirillum*.

It was impossible to obtain good yields of virus by lysis of broth cultures, although we found that plaque formation could be improved by reducing the phosphate, calcium and magnesium concentrations in YEMG/2 agar medium. Even with this improved medium, increases in phage titre in broth cultures have been no larger than 10- to 20-fold. However, poor multiplication of bacteriophages in broth culture is not unusual (Eisenstark, 1966).

Electron microscopic evidence, together with buoyant density determinations, indicate that Si I contains a single linear molecule, 13.0 to 13.5 μm. long, of double-stranded DNA with a guanine+cytosine content of 53%. Although the base composition of the phage DNA is significantly different from that of the host DNA (62% G+C), it is not uncommon for double-stranded DNA phages to differ by this amount from their hosts (Szybalski, 1968).

Bacteriophage Si I is an icosahedral virus of diameter 63 nm., and is therefore similar in size to the head of bacteriophage λ which is 60 nm. in diameter (Eiserling & Boy de la Tour, 1965). However, Si I appears to have smaller head capsomeres than λ and to be significantly different from other double-stranded DNA bacteriophages in that it completely lacks a tail.
According to classifications of Bradley (1968) and Tikhonenko (1970) all viruses containing double-stranded DNA have tails, whereas those without tails are not only much smaller but possess RNA or single-stranded DNA.

Although the initial purpose of this study was to isolate a transducing phage for S. inter- sonii, it is unlikely that phage Si I is capable of mediating transduction since to our knowledge all phages of this type so far isolated have been found to possess tails.

The absence of a tail on bacteriophage Si I also raises the interesting question as to how this virus infects its host. The small icosahedral phage φX 174 has short spikes which are involved in adsorption and which probably play a role in the penetration of the cell wall and membrane by the phage DNA (Hutchison, Edgell & Sinsheimer, 1967), whereas the small spherical RNA phages such as R 17 are believed to inject their nucleic acid through the F pilus of Escherichia coli (Crawford & Gesteland, 1964). However, Spirillum strains are not known to possess pil, and phageSi I does not show any spikes. From its appearance it is possible that it has a modified vertex which could be involved in adsorption. This question remains to be clarified.

We thank Mr B. Younghusband for instruction in the use of the analytical ultracentrifuge and Elizabeth Gooch and Joan Hutchison for helpful technical assistance.

REFERENCES


(Received 2 November 1970)