Isolation of Mycoplasmatales Virus-laidlawii 3,
a New Virus Infecting Acholeplasma laidlawii

(Accepted 30 January 1973)

Two fundamentally different viruses that infect Acholeplasma laidlawii have so far been isolated. They have been designated Mycoplasmatales virus-laidlawii 1 (MV-L1) and Mycoplasmatales virus-laidlawii 2 (MV-L2). MV-L1 is an unenveloped helical DNA virus, 16 nm in diameter and 90 nm in length (Bruce et al. 1972); while MV-L2 is a predominantly spherical enveloped virus with a mean diameter of 80 nm (range 52 to 125 nm). Its nucleic acid appears to be DNA (Gourlay et al. 1973).

This report describes the isolation of another virus which infects A. laidlawii and which differs from both MV-L1 and MV-L2.

Four serial tenfold dilutions of two clones of the M1305/68 strain of Acholeplasma laidlawii resistant to MV-L2 (Gourlay, 1972) were prepared in phosphate-buffered saline, pH 7.3, containing 5% foetal calf serum. Drops (0.02 ml) of each dilution were placed on glucose serum (GS) agar plates (Gourlay & Wyld, 1972) and the plates were examined after incubation at 37 °C for 48 h. The plate inoculated with acholeplasmas of clone 1 revealed confluent growth, within the areas of the drops, at all dilutions; the dilutions being distinguished one from the other only by variations in density of growth. The acholeplasmas of clone 2 had grown similarly but at the 10⁻¹ dilution, 20 to 30 very small plaques were visible within the area of the drop, and at the 10⁻² dilution, three slightly larger plaques were observed.

No plaques were visible at the neat and 10⁻³ dilutions. These plaques had apparently formed spontaneously and could be reproduced by the same method with clone 2.

The plaque-forming agent was propagated on a lawn of the M1305/68 strain of Acholeplasma laidlawii. Tenfold dilutions of clone 2 were prepared in buffered saline and drops were placed on the lawn. After incubation for 48 h confluent plaques were visible at the site of the neat, 10⁻¹ and 10⁻² dilution drops and an almost confluent plaque at the 10⁻³ dilution drop. All these plaques were cut out of the agar plate and placed in buffered saline for 5 h, and this fluid constituted the starting material for plaque-purification and characterization of the agent. The titre of the agent in this fluid was 6.25 log p.f.u./ml. The agent was cloned by picking a single plaque into buffered saline and subinoculating dilutions of the fluid onto a lawn of the M1305/68 strain of A. laidlawii on four successive occasions. A stock of the cloned material was then prepared (titre 10⁻² log p.f.u./ml) and stored at −70 °C. Three separate clones were obtained in this way and all behaved similarly in subsequent characterization studies.

At high dilutions stock material produced single discrete plaques, about 0.5 to 2 mm in diameter (Fig. 1). Plaques were formed during incubation at 22 and 37 °C.

When the agent, diluted 1/1000 in buffered saline, was exposed on two separate occasions for 3 min at a distance of 12 cm from a u.v. light source (Universal U.V. lamp, 254 nm, Gelman–Camag, model 54102) the titre was reduced by an average of 5.0 log p.f.u./ml. Exposure of the agent diluted 1/10 to a temperature of 60 °C for 30 min on three occasions reduced the titre by an average of 0.48 log p.f.u./ml. The agent, diluted 1/10 in buffered saline, was treated on two occasions with the detergent Nonidet-P40 (British Drug Houses,
Fig. 1. Plaques formed by serial tenfold dilutions of virus on a lawn of *Acholeplasma laidlawii* strain M1305/68.

Poole, Dorset), 0.4% (v/v) for 15 min at 37 °C and in both instances the titre was increased very slightly by an average of 0.09 log p.f.u./ml, compared with a buffered saline control. When one part of diethyl ether was added to 4 parts of the agent diluted 1/10 in buffered saline and placed at 4 °C for 18 h no reduction in activity was observed.

A sample of stock material diluted in serum saline was serially filtered under a positive pressure of 5 to 10 lb/in² through Millipore filters of decreasing pore size. In three separate attempts the agent (titre 8.2 to 9.2 log p.f.u./ml.) passed readily through a filter of 220 nm pore size without loss of titre. There was, however, an average loss in titre of 0.8 log p.f.u./ml when passed through a 100 nm filter, and a further average loss in titre of 1.6 log p.f.u./ml when passed through a 50 nm filter. No plaque-forming activity was detected in the 25 nm filtrates.

Antisera prepared in rabbits to MV-L1 and MV-L2 inhibited the formation of plaques by MV-L1 and MV-L2 respectively on lawns of the M1305/68 strain of *Acholeplasma laidlawii*, but both antisera failed to inhibit the formation of plaques by the new agent on lawns of the same acholeplasma.

In a serum neutralization test, 0.2 ml of antisera to MV-L1 and MV-L2, diluted from 1/4 to 1/64 in buffered saline, were added respectively to 0.2 ml saline suspensions of the agent (titre 5.1 log p.f.u./ml); the mixtures were incubated for 2 h at 37 °C and were then assayed for plaque-forming activity. Normal rabbit serum diluted 1/4 was used as control. Neither MV-L1 nor MV-L2 antisera, even at the 1/4 dilution, caused any reduction in titre of the agent. When MV-L1 and MV-L2 antisera, diluted 1/64, were tested against the homologous viruses, the titres were reduced; MV-L1 from 5.37 to < 1.25 log p.f.u./ml and MV-L2 from 5.2 to < 1.25 log p.f.u./ml.

The addition of 1 mg/ml of ribonuclease-A (Sigma, type 1-A) to the solid medium plate had no effect on the formation of plaques, as the titre of the agent grown in ribonuclease-containing plates and normal plates was similar.
Table 1. Titration of MV-L1, MV-L2 and the new agent on lawns prepared from clones of Acholeplasma laidlawii strain M1305/68 susceptible and resistant to each organism

<table>
<thead>
<tr>
<th>Host strain  </th>
<th>MV-L1</th>
<th>MV-L2</th>
<th>new agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible M1305/68</td>
<td>9.1</td>
<td>7.6</td>
<td>10.1</td>
</tr>
<tr>
<td>M1305/68 resistant to MV-L1</td>
<td>&lt;2.2</td>
<td>7.7</td>
<td>9.6</td>
</tr>
<tr>
<td>M1305/68 resistant to MV-L2</td>
<td>9.1</td>
<td>&lt;2.2</td>
<td>9.8</td>
</tr>
<tr>
<td>M1305/68 resistant to the new agent</td>
<td>9.2</td>
<td>8.1</td>
<td>&lt;2.2</td>
</tr>
</tbody>
</table>

Lawns were prepared from three clones of M1305/68, one clone resistant to MV-L1, one resistant to MV-L2 (not clone 2) and one resistant to the new agent. Control lawns were also prepared from fully susceptible M1305/68. MV-L1, MV-L2 and the new agent were each titrated and drops of each dilution placed on each lawn. After incubation at 37 °C for 48 h, MV-L1, MV-L2 and the new agent were all observed to produce plaques on the control M1305/68 lawns; MV-L1 and the new agent produced plaques on the MV-L2 resistant lawns, MV-L2 and the new agent produced plaques on the MV-L1 resistant lawns and both MV-L1 and MV-L2 produced plaques on lawns resistant to the new agent. Detailed results are given in Table 1.

In three out of three occasions the addition of 4.2 to 5.4 log p.f.u./ml of the agent to GS broth cultures of growing acholeplasmas resulted in a 3.8 to 5.2 log increase in titre of the agent after 22 h incubation at 37 °C. There was no evidence of replication in the absence of actively growing cells of the acholeplasma.

Stock material diluted 1/10 to 1/100 in serum saline was passed through a 220 nm Millipore filter and the filtrate was centrifuged at 150000 g for 1.5 h. The pellet was resuspended in a minimal amount of supernatant fluid, applied to carbon-collodion coated grids and negatively stained for a few seconds with 2 % potassium phosphotungstate at pH 7.2. Specimens were examined with a Philips EM300 electron microscope. Numerous polyhedral particles were observed (Fig. 2a–c). They appeared uniform in size (about 54 nm in diameter) and some particles were hexagonal in profile. No tails were visible, although the particles did sometimes appear to be attached to cellular material by a short process (Fig. 2c).

The characteristics of the new plaque-forming agent, described above, are consistent with those of a virus. This virus, however, differs in a number of important respects from both MV-L1 and MV-L2. First, electron micrographs reveal that the virus is morphologically unlike either MV-L1 or MV-L2. Secondly, it forms plaques on clones of M1305/68 immune to both MV-L1 and MV-L2 and conversely clones of M1305/68 immune to the virus are not immune to either MV-L1 or MV-L2. Finally, the virus is serologically dissimilar to both MV-L1 and MV-L2. In view of these differences it is obvious that this virus is new and intrinsically dissimilar to both MV-L1 and MV-L2 and represents a new group of mycoplasma viruses, for which the designation Mycoplasmatales virus-laidlawii 3 (MV-L3) is proposed.

The observations that the spontaneously produced plaques were seen only on lawns prepared from diluted acholeplasma cultures indicates that the density of lawn may be important for the recognition of small plaque-forming mycoplasma viruses.
Fig. 2. Electron micrograph of centrifuged deposit of filtered stock virus. Negatively stained with potassium phosphotungstate. Numerous polyhedral particles, presenting various views, can be observed in (a), (b) and (c). Empty particles are visible in (a) and (b). A short process apparently attaching a virus particle to cellular material is indicated by an arrow in (c).
Short communications

We wish to thank Mr I. Jebbett and Mr B. France for the photograph and electron micrograph respectively, and Miss E. Coleman for technical assistance.

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(Received 21 December 1972)