Demonstration by Electron Microscopy of Intracellular Virus in Acholeplasma laidlawii Infected with either MV-L3 or a Similar but Serologically Distinct Virus (BN1 virus)

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SUMMARY
Cultures of Acholeplasma laidlawii strain M1305/68 were inoculated with Mycoplasmatales virus-laidlawii 3 (MV-L3) and examined by electron microscopy. Particles resembling MV-L3 were observed both intra- and extracellularly in thin sections prepared from MV-L3 infected cultures, but not from uninfected cultures. Similar particles were occasionally observed in uninoculated cultures of A. laidlawii strain BN1 cells, from which a virus (BN1 virus) was subsequently isolated. This virus was morphologically similar but not identical to MV-L3. It also differed serologically from, and in its resistance to, MV-L3 and the other mycoplasma viruses.

INTRODUCTION
Three morphologically distinct mycoplasma viruses have so far been isolated. These are Mycoplasmatales virus-laidlawii 1 (MV-L1), MV-L2 and MV-L3 (Gourlay, 1974). MV-L3 contains double-stranded DNA and has a polyhedral head (57 × 61 nm), a short tail (25 nm long) and collar; it infects Acholeplasma laidlawii (Garwes et al. 1975). Very little information is available about the replication of this virus, although recently Liss (1977) reported premature lysis experiments showing that mature virus particles accumulated within infected cells prior to their release. This is substantiated by our recent electron microscopic studies in which particles resembling MV-L3 were observed within cells of A. laidlawii infected with this virus, details of which are given in this paper. During a separate study, particles resembling intracellular MV-L3 were observed in cultures of another strain of A. laidlawii that had not been experimentally infected. Further investigation showed this virus to be not MV-L3 but a virus morphologically similar but serologically distinct from MV-L3 (designated BN1 virus).

METHODS
Strains of micro-organisms. Strains of A. laidlawii used were M1305/68 and BN1 (Gourlay, 1972). Two passage levels of BN1 were used, designated A14 and A17 respectively. The virus used was Mycoplasmatales virus-laidlawii 3 (MV-L3) isolated by Gourlay & Wyld (1973).

Preparation of virus and virus-infected cells for electron microscopy. A. laidlawii strains were grown at 37 °C in filtered (220 nm Millipore) GS broth (Gourlay & Wyld, 1972). A 24 h broth culture (120 ml) of A. laidlawii strain M1305/68 or strain BN1 (A14) was added to 600 ml of warm (37 °C) GS broth and incubated for a further 1 h at which time the acholeplasma titre was 1 × 10^6 c.f.u./ml. The culture was then dispensed into 4 samples
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and MV-L3 (4.3 x 10⁷ p.f.u.) or BN1 virus (2.9 x 10⁹ p.f.u.) was added to three of them.
The remaining sample was left uninoculated. These infected cultures were incubated at
37 °C, one for 6 h, one for 16 h and a third for 24 h. Cultures were titrated for virus (Gourlay,
1974), centrifuged at 2000 g for 15 min and the deposits of cells stained with ruthenium red
as described earlier. Thin sections were prepared for electron microscopy (Howard &
Gourlay, 1974). Free virus particles were applied to carbon-Formvar coated grids and
negatively stained with 2% phosphotungstic acid (PTA, pH 6·6). Measurements of particles
were made using photographic plates, the magnification of which had been calibrated with
reference to the lattice spacing of beef liver catalase.

Virus isolation. Virus was isolated from the BN1 (A17) strain of A. laidlawii by the method
described for MV-L3 by Gourlay & Wyld (1973). Four serial tenfold dilutions of a culture
of BN1 were prepared in buffered saline. Drops of each dilution were placed on GS agar
plates and the plates examined for plaques after incubation at 37 °C for 48 h.

Preparation of antiserum. Antiserum was prepared in rabbits by the method described by
Gourlay & Wyld (1972) using purified virus.

Virus characterization. BN1 virus was characterized by morphology, plaque characteristics,
sensitivity to detergent (Nonidet P40), ether and heat, formation of plaques on clones of
A. laidlawii which were resistant to the other mycoplasma viruses, and serologically by the
plaque inhibition and serum neutralization tests. Details of the methods can be found in

RESULTS

Acholeplasma laidlawii M1305/68 cells infected with MV-L3

Thin sections of samples of A. laidlawii cells taken 6 h after infection with MV-L3 showed
MV-L3 type particles, both free and apparently adsorbed to the acholeplasmal cell mem-
branes by their tails. Most of these particles appeared empty. In preparations from samples
taken 16 and 24 h after infection, both free and adsorbed MV-L3 type particles (43 to 55 nm
diam.) were observed, many of which stained darkly while others were unstained (Fig. 1 a).
Many approximately spherical particles were also seen within some of the acholeplasma
cells. These particles were 42 to 53 nm in diam. and densely stained (Fig. 1 a, b, c) although
occasionally some unstained particles were observed in the cell (Fig. 1 b). Frequently, how-
ever, a densely staining central core could be seen surrounded by a less dense periphery
(Fig. 1 a, c).

These intracellular particles were usually aligned along the periphery of the cell, adjacent
to the membrane (Fig. 1 a, b). In some acholeplasma cells the virus-like particles were
arranged in a paracrystalline array (Fig. 1 c).

Samples from control cultures to which MV-L3 had not been added showed no MV-L3
type particles nor any of the intracellular spherical particles referred to above.

The titre of MV-L3 in the culture of A. laidlawii 24 h after infection was 1.7 x 10⁸ p.f.u./ml.
The control culture contained no MV-L3.

Acholeplasma laidlawii BN1 cells to which no virus had been added

During separate studies, thin sections were prepared from A. laidlawii-strain BN1 (A17)
cells that had not been experimentally infected with virus. In these preparations about 14 %
of the cells contained particles 40 to 45 nm in diam., resembling the spherical particles seen
in the MV-L3-infected A. laidlawii strain M1305/68 cells. These particles were usually
densely stained throughout (Fig. 2 a, b) but occasionally they had a densely stained central
core (Fig. 2 b). In some sections these intracellular particles appeared polyhedral and those
Intracellular virus in Acholeplasma laidlawii

Fig. 1. Section of cells of A. laidlawii M1305/68 from a culture inoculated 24 h previously with MV-L3. (a) Free and adsorbed virus particles are seen. Some are darkly stained while others appear empty. (b) Many densely stained and a few apparently empty particles are seen within the cell. (c) Particles within the cell showing densely stained cores surrounded by a less dense periphery.

aligned along the cell membrane were attached to the inner surface of the membrane by a short tail (Fig. 2a).

Isolation of virus from A. laidlawii strain BN1 (A17)

After numerous attempts to isolate the agent from the BN1 (A17) strain of A. laidlawii, about 15 to 20 plaques were observed on the first dilution of the wash of the BN1 (A17)
strain (which contained many acholeplasmas) grown on GS agar plates. A few plaques were picked with a wire into phosphate buffered saline (pH 7.3) containing 5% foetal calf serum and the plaque-forming agent was propagated on the BN1 (A14) strain of *A. laidlawii*. The virus was cloned by picking single plaques on four successive occasions. Four separate clones were obtained in this way and all behaved similarly in subsequent characterization studies.
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Acholeplasma laidlawii BN1 (A14) cells infected with BN1 virus

Thin sections of A. laidlawii BN1 (A14) cells taken 24 h after infection with the BN1 virus revealed intra- and extracellular roughly spherical particles 41 to 48 nm in diam. (Fig. 2c). These resembled the particles observed in the BN1 (A17) strain of A. laidlawii from which the BN1 virus was isolated. The titre of BN1 virus in the culture of A. laidlawii 24 h after infection was $1 \times 10^{10}$ p.f.u./ml.

Characterization of the BN1 virus

The BN1 virus produced plaques about 1 to 2 mm in diam. on lawns of the BN1 (A14) strain. Confluent plaques frequently possessed a distinct halo.

When the BN1 virus, diluted 1/1000 in buffered saline, was exposed on three occasions to a u.v. light source, the titres (4.7, 5.9 and 6.1 log p.f.u./ml) were reduced by at least 3.3, 4.5 and 4.7 log p.f.u./ml respectively. Exposure of the virus, diluted 1/10 in buffered saline, to a temperature of 60 °C for 30 min on three occasions reduced the titres (6.1, 7.8 and 7.8 p.f.u./ml) by 2.2, 2.5 and 2.5 p.f.u./ml respectively.

In two separate experiments the BN1 virus was shown not to be sensitive to the detergent Nonidet-P40 or diethyl ether. Treatment of the agent (titres 5.9 and 7.5 log p.f.u./ml) with Nonidet-P40, 0.4% for 15 min at 37 °C resulted in titres of 6.1 and 7.1 log p.f.u./ml respectively. When one part of diethyl ether was added to four parts of the virus diluted 1/10 in buffered saline and placed at 4 °C for 18 h, the titres (6.7 and 7.0 log p.f.u./ml) were increased by 0.1 and 0.4 log p.f.u./ml respectively.

In three separate attempts, serial filtration through Millipore filters of decreasing pore sizes of a sample of the virus diluted in serum saline (titre 7.9 to 8.6 log p.f.u./ml) under positive pressure of 5-10 lb/in² revealed that the virus passed through the filter of 220 nm pore size with average loss of 0.5 log p.f.u./ml. There was, however, an average loss in titre of 2.0 log p.f.u./ml when passed through a filter of 100 nm pore size. No plaque forming activity was detected in the 50 or 25 nm filtrates.

The BN1 virus was purified in a manner identical to that used for MV-L3 (Garwes et al. 1975) except that a second isopycnic equilibrium centrifugation was performed each time. The mean buoyant density at which the BN1 virus bands in CsCl solution, estimated by optical absorbance at 260 and 280 nm and by infectivity assay from six runs, was 1.50 g/ml (range 1.50 to 1.53).

Antisera prepared in rabbits to MV-L1, MV-L2, MV-L3 and the BN1 virus inhibited the formation of plaques produced by the homologous viruses on lawns prepared from the BN1 (A14) or M1305/68 (for MV-L2) strains of A. laidlawii, but MV-L1, MV-L2 and MV-L3 antisera failed to inhibit the formation of plaques by the BN1 virus on the BN1 (A14) lawn. Conversely, sera against the BN1 virus failed to inhibit the formation of plaques by the other three viruses. In a serum neutralization test (Clyde, 1974), the ability of the antisera to the four viruses to neutralize both homologous and heterologous viruses was determined. The results, given in Table 1, show that serologically the BN1 virus is quite distinct from MV-L1, MV-L2 and MV-L3.

Lawns were prepared from two clones of the BN1 (A14) strain of A. laidlawii, one clone resistant to MV-L1 and MV-L3, and the other to MV-L1 and the BN1 agent by the method described by Gourlay (1972). Control lawns were also prepared from fully susceptible BN1 (A14). Drops of tenfold dilutions of MV-L1, MV-L3 and BN1 virus were placed on each lawn. After incubation at 37 °C for 48 h, all the viruses and the BN1 virus produced plaques on the control lawns and on all the other lawns apart from those that were resistant to the
Table 1. Cross-neutralization of mycoplasma viruses and the BN1 agent

<table>
<thead>
<tr>
<th>Virus</th>
<th>MV-L1</th>
<th>MV-L2</th>
<th>MV-L3</th>
<th>BN1 agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV-L1</td>
<td>&gt; 2560</td>
<td>ND†</td>
<td>ND</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>MV-L2</td>
<td>ND</td>
<td>&gt; 2560</td>
<td>ND</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>MV-L3</td>
<td>ND</td>
<td>ND</td>
<td>&gt; 2560</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>BN1 agent</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>2560</td>
</tr>
</tbody>
</table>

* Reciprocal of the greatest dilution which produces plaque reduction of 60% or more related to virus controls.
† ND, Not done.

Table 2. Ability of MV-L1, MV-L3 and BN1 agent to produce plaques on lawns prepared from clones of A. laidlawii BN1 (A14) susceptible and resistant to each virus

<table>
<thead>
<tr>
<th>Host A. laidlawii strain</th>
<th>MV-L1</th>
<th>MV-L3</th>
<th>BN1 agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible BN1 (A14)</td>
<td>9.51</td>
<td>8.35</td>
<td>8.48</td>
</tr>
<tr>
<td>BN1 (A14) resistant to MV-L1+ MV-L3</td>
<td>&lt; 2.4</td>
<td>&lt; 2.4</td>
<td>8.18</td>
</tr>
<tr>
<td>BN1 (A14) resistant to MV-L1+ BN1 agent</td>
<td>&lt; 2.4</td>
<td>8.87</td>
<td>&lt; 2.4</td>
</tr>
</tbody>
</table>

The plaques produced by the BN1 virus on the MV-L1 plus MV-L3 resistant lawns were not inhibited by antisera to MV-L1, MV-L2 or MV-L3, whereas the plaques produced by MV-L3 on the lawns prepared with the clone resistant to MV-L1 plus the BN1 virus were inhibited by MV-L3 antiserum.

Electron microscopy of purified BN1 virus negatively stained with phosphotungstic acid revealed uniform particles with a polyhedral head, a collar and a short tail. The head frequently resembled an octahedron in profile and tail fibres were often observed (Fig. 3). Measurements made of 25 particles gave the mean dimensions of 52 nm (s.d. 3.1) between opposing planes and 56 nm (s.d. 2.7) between opposing vertices. Measurement of 18 tails indicated a length of about 23 nm.

DISCUSSION

The results of titrations showed that at least $3.3 \times 10^{10}$ p.f.u. of MV-L3 were present in the A. laidlawii strain M1305/68 cultures, infected 24 h earlier with MV-L3, that revealed intracellular virus-like particles, while the un inoculated control culture revealed no such particles. It would appear therefore that the particles observed were, in fact, MV-L3. Similarly $1 \times 10^{10}$ p.f.u./ml of the BN1 virus was present in the A. laidlawii strain BN1 (A14) cells, 24 h after infection with the BN1 virus, indicating that the observed particles were BN1 virus.

Very little information is available on the replication of MV-L3 but from examination of these electron micrographs it would appear that MV-L3 attaches by its tail and presumably injects its nucleic acid in a manner similar to the tailed bacteriophages. The presence of apparently mature virus particles within the acholeplasma cells indicates that MV-L3 differs in its method of replication from both MV-L1 and MV-L2 in which no intracellular virus particles have been demonstrated (Maniloff et al. 1977). Electron micrographs of the cell-associated BN1 virus indicate that this virus probably behaves in a manner similar to MV-L3.
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Liss (1977) reported an average of only six progeny from every MV-L3 infected acholeplasma cell. However, it is obvious from our electron micrographs that many more intracellular virus particles can be present in an acholeplasma cell before release. Either many particles are not infectious or the number of progeny depends on the strain of acholeplasmas used as host.

The demonstration of apparently mature virus particles which were subsequently shown to be BN1 virus in the BN1 (A17) strain of A. laidlawii that had not been experimentally infected, is not really surprising as various mycoplasma viruses have been isolated from a number of different A. laidlawii strains (Gourlay, 1974). The nature of the mycoplasma virus carrier state is not known (Maniloff et al. 1977). In the case of the A17 passage level about 14% of the acholeplasma cells possessed intracellular virus particles, whereas electron microscopic examination of two other earlier passage levels of the BN1 strain (which had been stored at −70 °C) did not reveal any similar intracellular virus particles. Presumably the virus was present but in insufficient numbers to be detected.

The presence of densely staining central cores in some of the intracellular virus particles of MV-L3 and BN1 virus may be only the result of shrinkage due to the fixation process necessary in the preparation of sections for the electron microscope. The size of the intra-
cellular virus particles can only be approximate because of possible shrinkage due to fixation and because the intracellular virus was observed only in sections.

Isolation of a plaque-forming agent from the BNI (A17) strain of A. laidlawii was accomplished after many attempts. The characteristics of the BNI agent are consistent with those of a virus. This virus clearly differs from MV-L1 and MV-L2 but it does resemble MV-L3 in its general morphology, its resistance to detergent and diethyl ether and the size of plaques it produces. However, it is apparently more heat labile than MV-L3 and it forms plaques on lawns of A. laidlawii resistant to MV-L3 and, conversely, lawns of A. laidlawii resistant to the BNI virus are not resistant to MV-L3. Furthermore the BNI virus is serologically distinct from MV-L3 by both cross-plaque-inhibition and cross-neutralization tests. There are also minor morphological differences between BNI virus and MV-L3, the head of the former being perhaps an octahedron and the tail possessing fibres which we have not observed on MV-L3. The buoyant density of BNI virus in CsCl, 1.51 g/ml, also differs from that of MV-L3 (1.477 g/ml).

During this work, MV-L3 appeared to measure only 51.5 nm (s.d. 2.4) between opposing planes and 54 nm (s.d. 2.4) between opposing vertices from measurement of 25 negatively stained particles, compared with the earlier measurements of 57 × 61 nm (Garwes et al. 1975). This discrepancy is difficult to explain and may reflect a difference in the negative stain used (PTA instead of uranyl acetate), or a difference in the A. laidlawii host strain (BNI instead of M5305/6).

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REFERENCES


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