Some Biological Characteristics of Mycoplasmatales
Virus-laidlawii 1

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SUMMARY

Mycoplasmatales virus-laidlawii 1 (MV-L1) produced plaques on lawns of 11 out of 17 strains of Acholeplasma laidlawii but not on 12 other mycoplasma species examined. Clones of A. laidlawii resistant to lysis by the virus could readily be obtained from survivors of the susceptible BNI strain. These resistant clones carry virus apparently serologically similar to MV-L1 as does the susceptible BNI strain itself.

The virus was sensitive to the action of u.v. light and chloroform but relatively insensitive to ether. Nonidet-P 40, heat and pH levels of between 8 and 10. MV-L1 passed readily through Millipore VS filters of 25 nm. pore diameter with loss of titre of 0.5 log p.f.u./ml. or less.

In one-step growth experiments the latent period was between 30 and 60 min. and the burst size varied from 4 to 213. Maximum yield was obtained when virus was added early in the logarithmic stage of acholeplasma growth. MV-L1 appeared to cause gradual lysis of the acholeplasma culture.

INTRODUCTION

A recent report (Gourlay, 1970) described the isolation and some properties of a virus which infects a member of the Order Mycoplasmatales, namely Acholeplasma laidlawii. In a subsequent article (Gourlay, Bruce & Garwes, 1971) this virus, designated Mycoplasmatales virus-laidlawii 1 (MV-L1), was shown to be bacilliform with a mean diameter of 14.6 nm. and a mean length of 89.8 nm. Its nucleic acid appeared to be DNA. In this paper we present the results of work on the virus sensitivity, replication and host range. Some of the results of the virus sensitivity were reported briefly in the earlier communications.

METHODS

Acholeplasma growth. The medium used for the culture of Acholeplasma was glucose-serum (GS) broth or agar (Gourlay & Leach, 1970). Later the DNA was omitted, 10% foetal calf serum (FCS) was used instead of 20%, and in solid medium, 0.5% agarose was used instead of 0.65%. This solid medium was poured on a base composed of PPLO broth (Difco), agarose, penicillin, thallium acetate and phenol red. Acholeplasma cultures were stored at −70°.

Acholeplasma assay. The titre of acholeplasma cultures was estimated on GS agar plates by the method of Miles & Misra (1938).

Virus production. Virus was propagated by inoculating a small amount of stock suspension of the virus into either broth cultures of the BNI strain of A. laidlawii (Gourlay, 1970) or on to solid medium plates in which acholeplasmas were incorporated in the top layer.
Frequently virus was also incorporated in the top layer together with the acholeplasmas. After incubation at 37° for 18 to 24 hr, virus was harvested as broth in the case of broth cultures or by flooding solid medium plates with phosphate buffered saline (PBS) pH 7.3 for 3 to 18 hr at room temperature (22°) after which the PBS containing the virus was removed. Stock virus suspensions were usually heated at 60° for 30 min. to inactivate viable acholeplasmas and were stored at −70°.

**Virus assay.** The titre of virus suspension was estimated either by a pour-plate method or by a modification of the Miles & Misra (1938) method. In the former, serial ten-fold dilutions of virus were prepared in PBS containing 5% FCS; equal volumes, 0.1 ml., of the virus dilutions and an acholeplasma culture (titre 1 to 6 × 10⁸ c.f.u./ml.) were mixed with 2 ml. melted GS agar at 45° to 50° and rapidly poured on to a PPLO base. In the Miles & Misra method, dilutions of the virus were applied by means of a standard 0.02 ml. dropper to a GS agar plate prepared and seeded with acholeplasmas as above. Plaques were counted after incubation at 37° for 24 to 48 hr. All titrations were performed in duplicate.

**Virus yield in relation to acholeplasma growth.** One hundred ml. of GS broth were inoculated with 1.65 × 10⁸ c.f.u. of the BN1 strain of *A. laidlawii* removed from storage at −70°. The broth was incubated at 37° and at various times after inoculation 1 ml. and 3 ml. samples were removed. The 1 ml. samples were used for titration of the acholeplasmas and for recording extinction at 660 nm, while the 3 ml. samples were inoculated with MV-L1 (m.o.i. about 0.1) and reincubated at 37°. After 24 hr the 3 ml. sample was titrated for virus activity. This experiment was repeated once using slightly different times.

**Preparation of antiserum.** Antiserum was prepared in rabbits, which received an initial inoculation with 4.0 × 10¹⁰ p.f.u. purified virus (Gourlay et al. 1970) with sodium alginate and calcium gluconate (Medical Alginates Ltd., Perivale, Greenford, Middlesex) as adjuvant, followed 2 weeks later by four intravenous injections of equivalent amounts of virus, without adjuvant, at weekly intervals. The rabbits were bled for serum 4 days after the last injection.

**Virus filtration.** Virus harvested from lawns of the host acholeplasma was diluted 1/10 with PBS + 5% FCS and then serially filtered through Millipore membrane filters of decreasing pore size under 5 to 10 lb. positive pressure. The initial virus dilution and the filtrates were titrated.

**Host range of virus.** The host range of the virus was determined by placing a drop of high titre virus suspension (about 2 × 10⁹ p.f.u./ml.) on to a lawn prepared from each of the mycoplasmas being tested. The lawn was examined for plaque formation after incubation at 37° for 24 to 48 hr. Each mycoplasma was tested on at least three separate occasions.

**Chloroform sensitivity.** Stock virus suspension was diluted 1/10 in PBS + 10% FCS. Chloroform (Analar) was added to half the virus dilution to give a final concentration of 5% while an equal volume of PBS was added to the other half. The virus dilutions were then shaken for 10 min. at 4° or at room temperature and then the chloroform was removed by centrifugation at 850 g for 10 min. or by evaporation at 37°. The chloroform-treated and control virus suspensions were then titrated.

**Ether sensitivity.** In two experiments stock virus suspension was diluted 1/10 in PBS containing 10% FCS, and 20% diethyl ether (Analar) was added to half of it and 20% PBS was added to the other half as control. The mixtures were left at 4° for 18 hr after which the ether was allowed to evaporate at 37°. The ether-treated and control virus suspensions were titrated. Two further experiments were performed in a similar manner except that the virus was diluted in PBS alone.

**Sensitivity to Nonidet-P 40.** Stock virus suspension diluted 1/10 in PBS was mixed with an equal volume of 0.8% Nonidet-P 40 (B.D.H. Ltd.) for 15 min. at 37°. A similar virus dilution
mixed with an equal volume of PBS was used as a control. The Nonidet-treated and control
materials were then titrated.

Sensitivity to u.v. light. Five ml. samples of the virus suspension, diluted 1/10 in PBS,
were exposed in 5 cm diameter plastic Petri dishes (Nunclon, Sterilin Ltd., Richmond,
Surrey) at a distance of 12 cm. from an u.v. light source (Universal UV lamp, 254 nm.
Gelman-Camag. Model 54102) and titrated after exposure for various lengths of time. The
titrations were performed under ordinary light conditions and the plates were then incubated
at 37° in the dark.

Thermal inactivation of virus. Universal containers with 10 ml. PBS were immersed in
water baths at various temperatures. Ten min. were allowed for temperature equilibration,
after which 1 ml. of virus suspension was added to each tube to give 5 x 10⁸ p.f.u./ml. At
intervals 0.5 ml. samples were transferred to small bottles chilled in ice water. All samples
were titrated at the end of the experiment.

pH sensitivity. Samples of normal saline were adjusted to pH values varying from 1.2 to
12.2 by the addition of 1 M-HCl or 1 M-NaOH and 4.5 ml. amounts were added to universal
containers held at 37°. After 15 min., 0.5 ml. of virus suspension diluted 1/10 in normal saline
to give about 2 x 10⁸ p.f.u./ml. was added to each and the container shaken vigorously.
After a further 24 hr 0.2 ml. samples were titrated from each container.

One-step growth experiment. This was performed by the one-step growth method of

Acholeplasma lysis by MV-L1. Ten ml. broth cultures of A. laidlawii BNI were incubated
overnight at 37° and treated with an M.S.E. ultrasonic disintegrator for 45 sec. at maximum
output and returned to 37° for a further 1½ hr at which time their viable titres were 3.8 x 10⁷
to 3.8 x 10⁸ c.f.u. acholeplasmas/ml. Sufficient virus was then added to achieve a m.o.i. of
about 1. PBS or virus inactivated with 5% chloroform was added to a control culture. The
cultures were then held at 37° and the mycoplasma concentration estimated at intervals by
optical absorbance at 660 nm. and by titration after treatment with MV-L1 antiserum for
10 min. at 37°. Growth curves were constructed.

Effect of ribonuclease on plaque formation. The activity of MV-L1 on the host acholepla-
smas grown on solid medium plates containing 1 mg./ml. of ribonuclease-A (Sigma, type I-
A) in the top layer of the plate, was tested (Bradley, 1966).

RESULTS

Host range of MV-L1

MV-L1 was first detected on a lawn prepared from a broth culture of a mycoplasma
which was subsequently cloned, identified as Acholeplasma laidlawii and designated the
BNI strain (Gourlay, 1970). MV-L1 was examined for its ability to lyse 16 other strains of
A. laidlawii and 12 other species of mycoplasma details of which are given in Table 1.
Plaques were produced on 10 cloned strains of A. laidlawii, namely M1304/68, M1305/68,
M1307/68, M1301/68, M1302/68, 03, TGE(C), 151, 152 and 179. The plaques varied, from a few
faint discrete plaques to distinct confluent zones covering the whole area of the drop. On
some lawns plaques were produced only after the acholeplasma constituting the lawn had
undergone one or two subcultures in GS broth. No plaques were observed on the lawns of
any of the other species of mycoplasma examined.

Plaque morphology

The plaques produced by MV-L1 on solid medium plates were larger than those described
in the earlier work (Gourlay, 1970). At high dilutions single isolated plaques up to 6 mm. in
diameter were observed, they were turbid and frequently possessed an indistinct halo around the periphery (Fig. 1). Plaques could be produced on lawns incubated either at 37° or at room temperature. On one occasion, following three successive passes of virus in which the inoculating suspension was not heated to 60°, large plaques up to 12 mm. in diameter were produced.

Table 1. *Species and strains of mycoplasma tested for susceptibility of MV-L1*

<table>
<thead>
<tr>
<th>Species or strain of mycoplasma</th>
<th>Source</th>
<th>Received from</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. laidlawii (PG8)</td>
<td>Sewage</td>
<td>N.C.T.C. Colindale, London</td>
</tr>
<tr>
<td>A. laidlawii M1304/68*</td>
<td>Cattle</td>
<td>Dr. R. H. Leach</td>
</tr>
<tr>
<td>A. laidlawii M1305/68*</td>
<td>Cattle</td>
<td>Dr. R. H. Leach</td>
</tr>
<tr>
<td>A. laidlawii M1306/68</td>
<td>Cattle</td>
<td>Dr. R. H. Leach</td>
</tr>
<tr>
<td>A. laidlawii M1307/68*</td>
<td>Cattle</td>
<td>Dr. R. H. Leach</td>
</tr>
<tr>
<td>A. laidlawii 0–324</td>
<td>Sheep</td>
<td>Dr. R. H. Leach</td>
</tr>
<tr>
<td>A. laidlawii M1301/68*</td>
<td>Swine</td>
<td>Dr. R. H. Leach</td>
</tr>
<tr>
<td>A. laidlawii M1302/68*</td>
<td>Swine</td>
<td>Dr. R. H. Leach</td>
</tr>
<tr>
<td>A. laidlawii M1308/68</td>
<td>Swine</td>
<td>Dr. R. H. Leach</td>
</tr>
<tr>
<td>A. laidlawii 03*</td>
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<td>I.R.A.D. Compton</td>
</tr>
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<td>Cattle</td>
<td>I.R.A.D. Compton</td>
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<td>A. laidlawii 012</td>
<td>Cattle</td>
<td>I.R.A.D. Compton</td>
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<td>A. laidlawii 3GE(C)*</td>
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<td>I.R.A.D. Compton</td>
</tr>
<tr>
<td>A. laidlawii 151*</td>
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<td>Dr. D. A. Haig</td>
</tr>
<tr>
<td>A. laidlawii 152*</td>
<td>Cattle</td>
<td>Dr. D. A. Haig</td>
</tr>
<tr>
<td>A. laidlawii 179*</td>
<td>Cattle</td>
<td>Dr. D. A. Haig</td>
</tr>
<tr>
<td>A. granularum</td>
<td>Swine</td>
<td>Dr. R. T. Hodges</td>
</tr>
<tr>
<td>A. granularum M217/69</td>
<td>Swine</td>
<td>Dr. R. H. Leach</td>
</tr>
<tr>
<td>Acholeplasma sp. M221/69</td>
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<td>Dr. R. H. Leach</td>
</tr>
<tr>
<td>M. hyorhinis</td>
<td>Swine</td>
<td>Dr. R. T. Hodges</td>
</tr>
<tr>
<td>M. bovirhinis (PG42)</td>
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<td>M. dispar (462/2)</td>
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<td>I.R.A.D. Compton</td>
</tr>
<tr>
<td>Serological group 6 (Squire)</td>
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<tr>
<td>Serological group 7 (N29)</td>
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<td>Dr. R. H. Leach</td>
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<tr>
<td>M. mycoides var. mycoides (v5)</td>
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<td>M. mycoides var. mycoides (Gladydale)</td>
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<td>I.R.A.D. Compton</td>
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<td>I.R.A.D. Compton</td>
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<tr>
<td>M. mycoides var. mycoides (KH31)</td>
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<td>N.C.T.C. Colindale, London</td>
</tr>
<tr>
<td>M. orale type 1 (CH19299)</td>
<td>Human</td>
<td>N.C.T.C. Colindale, London</td>
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</tbody>
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* Strains susceptible to MV-L1.

Fig. 1. Plaques formed by MV-L1 on a plate of *Acholeplasma laidlawii* strain BN1. Stained neutral red. × 1.7.
Selection of clones of *A. laidlawii* BNI resistant to MV-L1

After 24 hr growth of MV-L1 in a broth culture of the BNI strain of *A. laidlawii*, a sample of the culture was inoculated on to a GS agar plate. From the acholeplasma colonies that grew, four were selected and cloned by picking single colonies on three successive occasions. The four clones were propagated in GS broth and lawns were prepared subsequently from each. Drops of MV-L1 suspension (titre $1 \times 10^8$ p.f.u./ml.) were placed on each lawn and on a control lawn of the parent BNI strain. After incubation the lawns were examined for plaques.

Distinct plaques were visible on the control lawn and on one of the other lawns; but none were visible on the three remaining lawns, which were apparently resistant to infection with MV-L1.

Examination of *A. laidlawii* strain BNI and clones of BNI resistant to MV-L1 for the presence of carrier virus.

*A. laidlawii* BNI, and the 3 BNI clones resistant to MV-L1 together with a non-susceptible *Acholeplasma* sp. M221/69 were each seeded onto GS agar plates. When the lawns were well grown the plates were washed with PBS for 3 to 5 hr at room temperature (22°). Samples from each washing were then dropped on to fresh lawns of each organism. MV-L1 suspension was used as a control.

The results (Table 2) show that plaques were produced on a lawn of the BNI strain by washings from the 3 clones of BNI resistant to MV-L1 and also by the washing from the parent BNI strain but not by the washing from the M221/69 strain. No plaques were produced on any of the other lawns.

Table 2. Examination of washings from *A. laidlawii* strain BNI, clones of *A. laidlawii* BNI resistant to MV-L1 and *Acholeplasma* sp. M221/69 for the presence of plaque-forming agents on lawns prepared from each of them

<table>
<thead>
<tr>
<th>Washings from</th>
<th>M221/69</th>
<th>Resistant A</th>
<th>Resistant C</th>
<th>Resistant D</th>
<th>BNI</th>
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<tr>
<td>M221/69</td>
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<td></td>
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<tr>
<td>Resistant/A</td>
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<tr>
<td>Resistant/c</td>
<td></td>
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<tr>
<td>Resistant/d</td>
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<tr>
<td>BNI</td>
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<tr>
<td>MV-L1 control</td>
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</table>

The agents in the various washings responsible for plaque formation were then examined for sensitivity to inhibition by MV-L1 antiserum. Lawns of BNI were inoculated with 2 drops of a 1/10 dilution of each of the washings. When the drops were dry a loopful of MV-L1 antiserum was placed in the centre of one drop and normal rabbit serum in the centre of the other drop. After incubation at 37° for 24 hr the plaques were examined for inhibition indicated by a circle of acholeplasma growth within the plaque.

The plaques produced by the washing from BNI and by washings from the MV-L1 resistant clones of BNI were inhibited by antiserum prepared against MV-L1, indicating that the agents carried by these strains are serologically similar to MV-L1.

The titre of agent in the washing from one of the MV-L1 resistant clones was about $1 \times 10^8$ p.f.u./ml. and in the BNI washing was about $1 \times 10^6$ p.f.u./ml. These agents produced isolated plaques at high dilution and the addition of high dilution of the BNI agent to a culture of *A. laidlawii* BNI resulted in a 4 log increase in titre.
Clones of BNI, resistant to the virus in the BNI washing, were then obtained. Lawns prepared from four of these clones were then examined for resistance to MV-L1 stock suspension and also to the virus in both the washing from one of the MV-L1 resistant BNI clones and to the virus in the BNI washing. No plaques were produced on any of the lawns by any of the viruses, indicating that clones of BNI resistant to the virus which was obtained from BNI itself were also resistant to MV-L1 and to the virus from the MV-L1-resistant clones.

Further attempts to obtain a virus from washing lawns of *A. laidlawii* BNI did not always succeed, but similar virus was isolated, on at least seven separate occasions.

![Fig. 2. Inactivation of MV-L1 at various temperatures.](image)

**Sensitivity to physical and chemical treatments**

When held at 60° for 30 min. activity of MV-L1 suspension decreased by about 1.0 log p.f.u./ml. At higher temperatures activity decreased further until no activity could be detected in a sample held at 80° for 30 min. (Fig. 2). MV-L1 was stable between pH 8 and pH 10 when held at 37° for 24 hr. At higher and lower pH levels, viral activity was progressively lost. In two experiments, u.v. light inactivated 90% of the virus in 19 and 23 sec. respectively. Three separate filtration experiments were performed and the results of titrations of the virus before and after filtration are given in Table 3. From these results it can be seen that MV-L1 passes through Millipore filters of 25 nm. pore diameter with a loss of titre of 0.5 log p.f.u./ml. or less. In five separate experiments chloroform caused a drop in titre of between 6.5 and 7.5 log p.f.u./ml. Ether caused a drop in titre of 1.4 and 1.97 log p.f.u./ml. when PBS was used as diluent, whereas when PBS containing FCS was used as diluent the drop in titre was nil and 1.25 log p.f.u./ml. In five experiments in which the virus was treated with Nonidet-P40 the titre either remained unchanged (in two experiments) or was reduced by a maximum of 1.0 log p.f.u./ml.
Yield of MV-L1 in relation to mycoplasma age

The results of the two experiments are shown in Fig. 3. Maximum yield of virus was obtained when the virus was added early in the logarithmic stage of acholeplasma growth, i.e. 6 to 12 hr after incubation commenced. If virus was added late in the log phase or in the retardation or stationary phase of growth, the yield of virus was reduced drastically and considerably less virus was obtained than was added. After 18 hr, an equilibrium was reached.
when almost the same number of virus particles were yielded as were added. The mean generation time of *A. laidlawii* in the GS broth during these experiments was calculated from the growth curves as about 130 min.

**Yield of virus**

The titre of virus obtained from growth on solid medium plates was usually higher than that obtained from growth in broth culture. In the former the titre was frequently over $1 \times 10^{10}$ p.f.u./ml and occasionally over $1 \times 10^{11}$ p.f.u./ml.

![Graph showing rate of growth of Acholeplasma laidlawii strain BNI in broth cultures](image)

**Fig. 4.** Rate of growth of *Acholeplasma laidlawii* strain BNI in broth cultures inoculated with viable or inactivated MV-L1. Growth measured by optical extinction. •—•, Culture infected with inactivated MV-L1; □—□, culture infected with viable MV-L1.

**One-step growth experiment**

A number of separate experiments were performed but the results were very variable. It was evident, however, that the latent period was between 30 and 60 min. The rise period was between 1 and 2 hr and the burst size varied from 4 to 213.

**Effect of ribonuclease on plaque formation**

In two experiments, the addition of ribonuclease to the medium had no effect on the formation of plaques, as the titre of the virus grown on ribonuclease-containing plates and normal plates was similar.

**Acholeplasma lysis by MV-L1**

A typical growth curve chosen from a number of experiments is shown in Fig. 4. The optical extinction and viable mycoplasma titre of the inactivated virus control culture rose steadily over a period of hours, while the extinction of the culture inoculated with live virus rose in a similar manner for the first 30–60 min. after which the extinction fell steadily until it was below that at which it started. The viable acholeplasma titre followed a similar pattern.
DISCUSSION

The sensitivity of 19 strains of *A. laidlawii* to lysis by MV-L1 varied from complete insensitivity to almost full sensitivity. Amongst the sensitive strains it was observed that there was a gradation of sensitivity, as shown by the number of plaques produced on the relevant lawns by a standard number of virus particles. It was also noticed that the sensitivity of a number of these *A. laidlawii* strains varied from time to time. The reasons for this are not clear but they appear to be associated with growth of the acholeplasmas.

The production of turbid plaques and the evidence that resistant clones of the acholeplasma can readily be obtained, indicate that lysogeny occurs. Washings of lawns of the resistant clones and the susceptible parent BN1 strain of *A. laidlawii* contain agents which produce plaques on lawns of the BN1 strain. These agents are serologically similar to MV-L1 and at least two of them appear to be viral in nature as they produce single isolated plaques at high dilution and, in addition, the BN1 agent multiplies in a broth culture of the acholeplasma. Clones of the BN1 strain of *A. laidlawii* resist the agent in the BN1 washings were shown to be resistant also to MV-L1 and to the agent in the washings of the MV-L1 resistant clones of *A. laidlawii*. This is a further example of the similarity of these agents and MV-L1, and also suggests that MV-L1 does not contain more agents than are contained in the washings from BN1 or the MV-L1 resistant clones of *A. laidlawii*. This is a further example of the similarity of these agents and MV-L1, and also suggests that MV-L1 does not contain more agents than are contained in the washings from BN1 or the MV-L1 resistant clones of *A. laidlawii*. The implications of these agents and MV-L1, and also suggests that MV-L1 does not contain more agents than are contained in the washings from BN1 or the MV-L1 resistant clones of *A. laidlawii*. The lysogenic condition appears complex and the implications interesting but further work is required to clarify the situation.

The results of the experiments on the yield of virus in relation to the age of the acholeplasma culture are straightforward and show that maximum yield of virus is obtained when the virus is added early in the log phase of acholeplasma growth. The variable results of the burst size in the one-step growth experiments indicate that the method of acholeplasma enumeration employed is probably not entirely suitable for this type of work, since colony-forming units may consist of many clumped acholeplasma cells. The use of ultrasonic disintegration in an attempt to disperse these clumps was used in the experiments on acholeplasma lysis. These lysis experiments showed that the addition of viable virus to a growing culture of *A. laidlawii* led to a drop in the optical extinction of the culture compared with its starting extinction and compared to a control culture inoculated with inactivated virus. This suggests that the virus lyses the acholeplasmas. This lysis apparently occurs gradually over a period of time rather than as a sudden event.

We wish to thank Dr R. H. Leach, Dr R. T. Hodges, Dr D. A. Haig and the National Collection of Type Cultures, London for supplying the various mycoplasma and acholeplasma strains indicated in Table 1, and Dr D. J. Garwes for the purified MV-L1 used for preparation of antiserum.

REFERENCES


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