Characterization of a Mycoplasma Virus (MV-O1) Derived from and Infecting *Acholeplasma oculi*

By HIROKO ICHIMARU OGAWA* AND M. NAKAMURA

Department of Microbiology, Kurume University School of Medicine, Kurume 830, Japan

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A new Mycoplasmatales virus, referred to as MV-O1, was isolated during cloning of *Acholeplasma oculi* 19L. The virus formed plaques only on strains of *A. oculi*, i.e. the original clone, *A. oculi* 19L, a subclone of *A. oculi* 19L (*A. oculi*-i), *A. oculi* Goat 5 and a wild isolate (K-2) of *A. oculi*, but not on other acholeplasmas, including strains of *A. laidlawii*, nor on five human mycoplasma species tested. The virus required horse serum for multiplication as well as for plaque formation and passed through a 100 nm filter. Electron microscopy revealed enveloped, spherical particles 80–130 nm in diameter. The buoyant density of purified virus was 1.23 g ml⁻¹ in CsCl, and agarose gel electrophoresis indicated that the viral nucleic acid was DNA.

INTRODUCTION

The term 'Mycoplasmatales virus' designates those phages which infect prokaryotes without cell walls, i.e. *Mollicutes*. Since the discovery of acholeplasmavirus MV-L1 (Gourlay, 1970), several Mycoplasmatales viruses have been isolated from species of *Acholeplasma* (Gourlay, 1971; Liss & Maniloff, 1971; Gourlay & Wyld, 1973; Congdon *et al.*, 1979), *Spiroplasma* (Cole & Mitchell, 1977; Liss & Cole, 1981) and *Mycoplasma* (Jansson *et al.*, 1982; Clyde, 1974; Howard *et al.*, 1980; Gourlay *et al.*, 1983). Some of these viruses have been classified into five taxonomic divisions (Maniloff *et al.*, 1972): family *Inoviridae*, *Plasmaviridae*, *Podoviridae*, *Myoviridae* and unclassified. Three morphologically and serologically distinct taxonomic groups MV-L1 (group L1), MV-L2 (group L2) and MV-L3 (group L3) of Mycoplasmatales viruses can be propagated on strains of *Acholeplasma laidlawii*. They have been termed *Inoviridae* genus *Plectovirus*, *Plasmaviridae* genus *Plasmavirus* and *Podoviridae* respectively. Viruses have also been isolated from or observed in spiroplasmas (Cole *et al.*, 1974; Cole & Mitchell, 1977; Liss & Cole, 1981) and have been designated SpV1, SpV2, SpV3 and SpV4. SpV1 has been assigned to the *Podoviridae*, while the others have yet to be classified. Howard *et al.* (1980) isolated MV-Br1 from *Mycoplasma bovirhinis*, and Gourlay *et al.* (1983) reported the isolation of MV-Hr1 from *Mycoplasma hyorhinis*. These viruses were the first to produce plaques on *Mycoplasmataceae*. All of these viruses except MV-Br1, Hr1 and MV-M1 (Congdon *et al.*, 1979) (MV-modicum 1) form plaques on lawns of *A. laidlawii*. Therefore, *A. laidlawii* has been routinely employed as an indicator/host for mycoplasma viruses.

During cloning of *A. oculi* 19L, we noted the spontaneous appearance of plaque-like spots on lawns of a subclone, *A. oculi*-i. A preliminary report (Ichimaru & Nakamura, 1983) was presented at the IVth Congress of the International Organization for Mycoplasmaology. We subsequently identified this plaque-forming agent as a mycoplasma virus by electron microscopy following concentration and partial purification. In the present paper, some biological characteristics of the virus, referred to as MV-O1, are described.

Abbreviation: FCS, foetal calf serum.

Table 1. *Strains of mycoplasma*

Species	Strain*	Species	Strain*
<i>Acholeplasma laidlawii</i>	A ¹	<i>Mycoplasma pneumoniae</i>	FH-Liu ¹
	B ¹	<i>M. salivarium</i>	PG 20 ¹
	BN1 ¹	<i>M. hominis</i>	PG 21 ¹
	1041 ¹	<i>M. fermentans</i>	PG 18 ¹
<i>A. modicum</i>	PG49 ²	<i>M. orale</i>	CH19299 ¹
<i>A. granularum</i>	BTS # 39 ²		
<i>A. axanthum</i>	S-743 ²		
<i>A. hippikon</i>	C1 ²		
<i>A. equifetale</i>	C112 ²		
<i>A. oculi</i>	19L ¹		
	19L ²		
	i ³		
	Goat 5 ¹		
	75151-L-5A ¹		
	3557 TC ¹		
	K-1 ⁴		
	K-2 ⁴		

* Sources of strains: 1, Dr M. F. Barile, Mycoplasma Branch, Bureau of Biologics, US Food and Drug Administration; 2, Dr M. Ogata, Department of Veterinary Microbiology, Faculty of Agriculture, University of Tokyo; 3, a clone of *A. oculi* 19L which produced plaques spontaneously; 4, isolates from a slaughter-house in Kurume.

METHODS

Mycoplasma strains. These are listed in Table 1.

Culture media. For multiplication of and experiments with mycoplasmas and acholeplasmas, as well as MV-O1, Chanock agar or broth (Chanock *et al.*, 1962) was used. When glucose or arginine fermentative strains were used, Chanock broth was supplemented with 0.002% (w/v) phenol red and 0.5% (w/v) glucose (pH 7.8), or 0.002% (w/v) phenol red and 0.5% (w/v) L-arginine (pH 7.0). For the one-step viral growth experiment, tryptose broth was used according to the method of Maniloff (1969).

Preparation of MV-O1 virus. MV-O1 virus was first recognized by chance as spontaneous plaques on a lawn of a clone (i) of *A. oculi* 19L. Originally the culture fluid of *A. oculi*-i was used as viral material. After it was found that MV-O1 virus could infect *A. oculi* 19L, the virus was prepared using *A. oculi* 19L as the host.

A. oculi-i (approximately 10^4 cells ml⁻¹) was inoculated into flasks containing Chanock broth, and incubated for 2–3 d at 37 °C. Culture fluids were obtained by filtration through a 220 nm filter (Millipore), and their plaque-forming activity was assayed on lawns of *A. oculi*-i. Active samples were used as the starting material for virus production. A suspension of plaque-forming agent (MV-O1 virus) was inoculated onto a 2 h lawn of *A. oculi* 19L (85 mm in diameter) and incubated for at least 6 h at 37 °C. Approximately 7 ml of phosphate-buffered saline (PBS) (pH 7.3) was added to the lawn and allowed to stand at room temperature for 6 h. The PBS was then removed and filtered through a Millipore filter (220 nm) to eliminate host cells. The final virus suspension thus obtained was stored at 4 °C.

Virus assay. *A. oculi* 19L host cells were propagated in Chanock broth supplemented with 0.5% (w/v) bovine serum albumin (albumin, bovine plasma fraction V, Armour) instead of horse serum and 0.002% (w/v) phenol red (pH 8.0), harvested at late exponential phase (yellowish orange) and then stored at –80 °C. Thawed cultures were inoculated onto the whole surface of Chanock agar plates (85 mm in diameter) and incubated for 2–3 h at 37 °C. Series of 10-fold dilutions of each virus sample (20 µl) were applied to the lawns and incubated for 24 h at 37 °C. To visualize plaques, the lawns were overlaid with 5 ml soft agar containing 2,3,5-triphenyltetrazolium chloride and 20 ml 1% (w/v) agar containing 1.5% (w/v) PPLO broth (Difco) and incubated at 37 °C until the lawns appeared red (Fraser & Crum, 1975).

Infection of A. oculi 19L with MV-O1 virus. Virus suspension (1 ml), prepared by the washing method (Liss & Maniloff, 1971), was inoculated into a mixture of 0.5 ml tryptose broth and 0.5 ml freshly prepared *A. oculi* 19L broth (16 h at 37 °C) containing 10^8 c.f.u. ml⁻¹. After incubation at 37 °C for 60 min, 0.5 ml of this mixture was transferred to 50 ml fresh tryptose broth and incubated at 37 °C. Samples were harvested at intervals and c.f.u. of host cells and viral p.f.u. were assayed.

One-step growth experiment. An *A. oculi* 19L culture which had been kept at –80 °C was inoculated into tryptose broth supplemented with 20% (v/v) trypsin-treated horse serum (instead of bovine serum albumin), incubated overnight and centrifuged at 16000 g for 30 min. The pellet was resuspended in PBS to approximately 1/20 of the original volume. A 900 µl portion of this cell suspension (3.1×10^9 c.f.u. ml⁻¹) was mixed with 100 µl

concentrated MV-O1 virus suspension ($10^{9.5}$ p.f.u. ml⁻¹), and incubated for 3 h at 37 °C for virus adsorption (m.o.i. = 1.0). Cells were separated from free virus by centrifugation at 11 000 *g* for 15 min in Eppendorf tubes, washed with 1.0 ml PBS and resuspended in 1.0 ml PBS. This suspension was inoculated into 50 ml tryptose broth containing trypsin-treated horse serum and incubated at 37 °C. At intervals, 1 ml of the culture was transferred to an Eppendorf tube (1.5 ml), centrifuged and viruses in the supernate were assayed.

Physical treatments. In the heat stability test, 0.5 ml volumes of MV-O1 virus suspension were distributed into screw-capped tubes (15 × 150 mm) and incubated in a water bath. One tube was removed from the water bath at each time interval and cooled immediately in an ice bath. Virus titres were assayed at the end of the experiment. UV sensitivity was tested as follows. Samples (0.5 ml) of MV-O1 virus suspension were placed in open Petri dishes (35 mm diam.) and exposed at a distance of 12 cm to a 20 W germicidal lamp (Toshiba Electric Co.), with occasional agitation. After specified periods of irradiation, the suspensions were tested individually for plaque formation.

Chemical treatments. MV-O1 virus was treated with organic solvents, based on the method of Gourlay (1972).

(i) MV-O1 virus suspension (0.5 ml), previously diluted 10-fold in PBS containing 1% (v/v) foetal calf serum (FCS), was mixed with 0.025 ml chloroform. After vigorous mixing, the mixture was kept for 10 min at 4 °C and then the chloroform was removed under vacuum, and the virus titres were assayed.

(ii) Diethyl ether (0.1 ml) was added to 0.5 ml MV-O1 virus suspension previously diluted 10-fold in PBS containing 1% (v/v) FCS. After 15 min at 37 °C, the virus was assayed.

(iii) A 0.4% (w/v) aqueous solution of Nonidet P-40 (Sigma) was mixed with an equal volume of MV-O1 virus suspension, previously diluted 10-fold with PBS containing 1% (v/v) FCS, and the virus was assayed after 15 min incubation at 37 °C.

Enzyme treatment. The following enzymes were used: trypsin (bovine pancreas, 12000 BAEE units per mg powder; Sigma) and α -chymotrypsin (bovine pancreas, 45 units mg⁻¹; ICN Pharmaceuticals) in 50 mM-Tris/HCl, 5 mM-CaCl₂ (pH 8.0), protease (*Streptomyces griseus*, 5.8 units per mg solid; Sigma) and protease (*Bacillus subtilis*, 12 units per mg solid; Sigma) in 50 mM-Tris/HCl (pH 8.0), lipase (porcine pancreas; Sigma) and lipase (wheat germ, Sigma) in 50 mM-Tris/HCl, 5 mM-CaCl₂ (pH 8.0), phospholipase D (cabbage; P-L Biochemicals) in 50 mM-Tris/HCl, 50 mM-CaCl₂ (pH 5.5), and DNAase I (bovine pancreas; Sigma) in 150 mM-Tris/HCl, 30 mM-MgCl₂ (pH 6.0). Concentrations of enzymes used were 500 µg ml⁻¹, except for DNAase I (100 µg ml⁻¹).

The inhibitors used were: trypsin inhibitor (300 µg ml⁻¹; Sigma) for trypsin, 50 mM-Tris/HCl, 5 mM-ZnCl₂ for lipase and 100 mM-Tris/HCl, 0.2 mM-EDTA for DNAase I. Proteases and phospholipase D were diluted with 50 mM-Tris/HCl.

Virus purification. Saturated ammonium sulphate (767 g l⁻¹, pH 7.0) was gradually added with mixing to MV-O1 virus suspension to 30% saturation (very fine sediments were produced), and the suspension was kept at 4 °C overnight. After centrifugation at 16000 *g* for 15 min, the sediment was resuspended in PBS to 1/20 to 1/50 of the original volume, desalted with CF-25 (Amicon) and then membrane filtered (450 nm pore size) to concentrate the virus. Equal volumes (3 ml) of three caesium chloride solutions (1.5, 1.3, 1.1 g ml⁻¹) were sequentially loaded in a polyallomer tube (10 ml) and then 1 ml of concentrated virus suspension was layered on top. After centrifugation at 240000 *g* (Hitachi, RP65T rotor) for 16 h, 1.0 ml fractions were collected from the bottom of the tube and p.f.u. were assayed in each fraction. The highest titre was found in the fraction of buoyant density 1.23 g ml⁻¹.

Scanning electron microscopy. In order to see the mode of virus adsorption to cells, tryptose broth supplemented with 10% (v/v) yeast extract, 20% (v/v) trypsin-digested horse serum and 0.5% (w/v) glucose was put into a Petri dish (3.5 ml, 35 mm) containing a glass cover slip (approx. 5 × 5 mm) and 1/10 the medium volume of a stock culture of *A. oculi* 19L was inoculated. After overnight incubation at 37 °C, the culture medium was removed and 3 ml MV-O1 virus suspension (10^7 p.f.u. ml⁻¹) was added and incubated for 1 h at 37 °C for virus adsorption. Then the virus suspension was removed and the cover-slip carrying *A. oculi* cells was washed three times with PBS and then pre-fixed with 0.25% glutaraldehyde in 0.06 M-cacodylate buffer (pH 7.4) for 2 h at 4 °C. After washing with PBS, the glass cover slip was doubly fixed with 1% (w/v) osmium tetroxide in cacodylate buffer for 1 h at 4 °C and dehydrated with an alcohol series followed by isoamyl acetate. Electron micrographs were taken (Hitachi, HFS-2) after critical-point drying and gold-sputter coating.

Transmission electron microscopy. For observation of virus morphology, partially purified virus obtained by centrifugation in a caesium chloride density gradient was subjected to ultrafiltration (Amicon, CF-25) and washed with 2% (w/v) ammonium acetate several times to remove caesium chloride. The pellet thus obtained was resuspended in approximately 0.1 ml 2% (w/v) ammonium acetate. A sample was applied to a Formvar-coated grid and negatively stained with 2% (w/v) potassium phosphotungstate (pH 7.4) and then observed by transmission electron microscopy (Hitachi, HU-12AS).

Extraction of viral nucleic acid. A 3 ml volume of polyethylene glycol 6000 (20%, w/v, Wako Co.) was added to 100 ml MV-O1 virus suspension and the mixture kept overnight at 4 °C. Pellets obtained by centrifugation at 16000 *g* for 30 min were resuspended in 1 ml 10 mM-Tris/HCl buffer (pH 8.0). Extraction of DNA was attempted by a modification of the method of Liss & Maniloff (1972). Briefly, 1 ml 10 mM-Tris/HCl buffer (pH 8.5) saturated with phenol was added to 1 ml virus suspension and incubated for 30 min at 37 °C with gentle agitation. A solution

of 5 M-NaCl was added to the aqueous layer to achieve a final concentration of 1 M and the mixture was then placed in an ice bath for 5–6 h, after which 2 vols cold ethanol (-20°C) were added followed by centrifugation at 240000 *g* (Hitachi RP 65 rotor) for 15 min. The sediment was resuspended in 100 μl 10 mM-Tris/HCl buffer containing 1 mM-EDTA, disodium salt (pH 8.1). These extracts were analysed by 1% (w/v) agarose gel electrophoresis using Tris/borate buffer. After running at 100 V for 1 h, the gel was stained with ethidium bromide (400 ng ml^{-1}) for 15 min and illuminated by UV light (UV trans-illuminator model TM-36) to detect DNA. Uninfected cells of *A. oculi* 19L were used as a control.

RESULTS

Discovery of spontaneous plaque formation

During cloning of *A. oculi* 19L, spontaneous plaque formation was observed by chance on a lawn of a subclone of *A. oculi* 19L, which was thereafter referred to as *A. oculi*-i. The small size of the plaques made enumeration difficult unless the lawn was stained with tetrazolium chloride (Fig. 1).

The plaque-forming agent (termed MV-O1 virus) was also spontaneously released into the culture fluid from cells of *A. oculi*-i during cultivation. *A. oculi*-i was cultivated in Chanock broth and culture fluid was harvested at intervals, filtered through a 220 nm pore membrane and applied to lawns of *A. oculi*-i and *A. oculi* 19L. Plaques were produced on both strains (Fig. 2). The lytic activity of the culture fluid of *A. oculi*-i, due to spontaneous release of the virus, increased with the increase in cell numbers during growth (Fig. 3).

Attempt to induce lysogenic virus in A. oculi 19L

Since the MV-O1 virus was released spontaneously from a clone of *A. oculi* 19L, it was possible that there were some cells of *A. oculi* 19L lysogenic for the virus. An attempt to induce lysogenic virus from *A. oculi* 19L was made using mitomycin C (1 $\mu\text{g ml}^{-1}$, 24 h at 37°C) and UV irradiation (15 W at a distance of 25 cm for 1–5 min). Plaques were not observed on lawns of treated cells, and the washing solution prepared from treated cells did not have plaque-forming activity.

Propagation of the virus

Large-scale propagation of the virus was initiated using a washing method. The success of this procedure is influenced by four important factors: (1) the age of the *A. oculi* 19L lawn, (2) the composition of the PPLO agar, (3) the infection period for adsorption and multiplication of the virus, and (4) the incubation period between addition of PBS and harvest. These factors were investigated separately.

The optimal yield of virus was obtained when the virus was inoculated onto 2 h lawns of *A. oculi* 19L (Fig. 4). Inoculation of 12 h lawns did not result in plaque formation.

The presence of horse serum in PPLO agar was essential for multiplication of the virus as well as for production of plaques. Some horse sera contained an inhibitor of virus multiplication and plaque formation, but pretreatment with trypsin (2000 units ml^{-1} , 20 min, 37°C) apparently inactivated the inhibitor (M. Nakamura & Y. Yoshitake, unpublished data). Therefore, horse serum pretreated with trypsin was used in subsequent experiments.

The optimal incubation period at 37°C between inoculation of the virus and addition of the washing solution was approximately 6 h.

The optimal time between addition of PBS and harvest was approximately 6 h.

Infection experiment using the virus material obtained by the washing method

The virus material obtained by the washing method was inoculated into tryptose broth containing *A. oculi* 19L and the relationship between virus yield and the growth of acholeplasma cells was observed as described in Methods. The virus concentration increased exponentially and reached a plateau at about 18 h post-inoculation. Inactivation of host cells occurred up to 12 h after inoculation; thereafter the cells began to divide, and the maximum titre of the virus was reached soon afterwards (Fig. 5). Clones of *A. oculi* 19L isolated from 48 h culture broth were susceptible to the virus.

Fig. 1

Fig. 2

Fig. 1. Plaques produced by MV-O1 virus on a lawn of *Acholeplasma oculi* 19L. Small plaques could be visualized by treatment with tetrazolium chloride (0.05%, w/v) according to the method of Fraser & Crum (1975). Bar, 250 μ m.

Fig. 2. Plaques produced by serial 10-fold dilution of MV-O1 virus on a lawn of *Acholeplasma oculi* 19L. The lawn was stained with 0.05% (w/v) tetrazolium chloride.

Fig. 3

Fig. 4

Fig. 3. Relationship between growth of *A. oculi*-i (○) and the spontaneous release of MV-O1 virus into the culture fluid (●). A stock culture of *A. oculi*-i was inoculated into Chanock broth supplemented with 20% (v/v) horse serum, 10% (v/v) yeast extract, 0.5% (w/v) glucose and 0.002% (w/v) phenol red and incubated at 37 °C. C.f.u. of *A. oculi*-i and lytic activity of the culture fluid were assayed at intervals. The virus titre was expressed as the highest dilution of culture filtrate that gave confluent plaques.

Fig. 4. Influence of the age of the lawn of *A. oculi* 19L on the replication of virus MV-O1. Volumes (0.5 ml) of exponentially growing *A. oculi* 19L in Chanock broth supplemented with 0.5% (w/v) bovine serum albumin and 0.002% (w/v) phenol red (pH 7.8) were each inoculated onto Chanock agar plates (85 mm) and incubated at 37 °C for various periods. Serial 10-fold dilutions of MV-O1 virus were assayed on the lawns.

Fig. 5

Fig. 5. Multiplication of MV-O1 virus (○) and growth of host (*A. oculi* 19L) cells (●). Virus suspension (1 ml) prepared by the washing method was inoculated into a mixture of 0.5 ml tryptose broth and 0.5 ml freshly prepared *A. oculi* 19L broth (16 h at 37 °C) containing 10^8 c.f.u. ml⁻¹. After incubation at 37 °C for 60 min, 0.5 ml of this mixture was transferred to 50 ml fresh tryptose broth and incubated at 37 °C. Samples were harvested at intervals and c.f.u. and p.f.u. were assayed.

Fig. 6

Fig. 6. One-step growth curve of MV-O1 virus. A suspension of *A. oculi* 19L (900 µl; 3.1×10^9 c.f.u. ml⁻¹) was mixed with 100 µl of a concentrated MV-O1 suspension ($10^{9.5}$ p.f.u. ml⁻¹), and incubated for 3 h at 37 °C for virus adsorption (m.o.i. = 1.0). Cells were separated from free viruses by centrifugation at 11000 *g* for 15 min in Eppendorf tubes, washed with 1.0 ml PBS and resuspended in 1.0 ml PBS. This suspension was inoculated into 50 ml tryptose broth containing trypsin-treated horse serum and incubated at 37 °C. At intervals, 1 ml of the culture was transferred to an Eppendorf tube, centrifuged and viruses in the supernate were assayed.

One-step growth of MV-O1 virus

Adsorption of MV-O1 virus to host cells was completed after 60 min at 37 °C. The percentage of host cells infected with virus was 0.1–1.0% when virus and cells were mixed at m.o.i. = 1. Following adsorption, free viruses were removed and the infected cells were transferred to freshly prepared culture medium. MV-O1 virus began to be released after a 3 h lag phase (Fig. 6), and multiplied exponentially up to 12 h incubation. The burst size ranged from 2 to 200 particles per cell under these experimental conditions: the net release of MV-O1 virus was 1.23×10^4 ml⁻¹ from $10^{2.8-3.8}$ infected host cells.

Infection mode

Scanning electron microscopy of *A. oculi* 19L cells infected with MV-O1 virus revealed many roughly spherical particles, 50–60 nm in diameter, on the surface of infected host cells; no particles were observed in the case of non-infected cells (Fig. 7). These particles were therefore presumed to be viruses.

Biological and biochemical characteristics of MV-O1 virus

Host range. Clear plaques were formed on lawns of *A. oculi* 19L (University of Tokyo) and its subclone, *A. oculi*-i, and turbid plaques on lawns of *A. oculi* 19L (NIH), *A. oculi* Goat 5 and the field isolate *A. oculi* K-2. Plaques were not produced on lawns of *A. oculi* strains 75151-L-5A, 3557 TC and K-1 nor on the lawns of several other mycoplasmas and achleplasmas tested (Table 2).

Sensitivity to physical and chemical treatments. The results are summarized in Table 3. MV-O1 virus was completely inactivated at 60 °C within 5 min. At 56 °C, the titre [$\log_{10}$ (p.f.u. ml⁻¹)] of MV-O1 virus was reduced by 4.8 after 30 min incubation. Plaque formation occurred at temperatures ranging from 22 to 40 °C. The virus was not significantly sensitive to UV irradiation: a 13% reduction in titre was observed after irradiation for 256 s. Titres of MV-O1

Fig. 7. Scanning electron micrographs of (a) infected cells of *A. oculi* 19L (note virus particles on the cell surface) and (b) non-infected cells of *A. oculi* 19L. Bars, 100 nm.

Table 2. *Host range of MV-O1 and other Mycoplasmatales viruses*

Species	MV-O1	Viruses reported*		
		MV-L1	MV-L3	MV-M1
<i>A. laidlawii</i> A	—	ND	ND	ND
<i>A. laidlawii</i> B	—	—	ND	ND
<i>A. laidlawii</i> BN1	—	+	+	—
<i>A. laidlawii</i> 1041	—	ND	ND	ND
<i>A. modicum</i> PG49	—	—	+	+ or —
<i>A. granularum</i> BTS #39	—	ND	ND	ND
<i>A. axanthum</i> S-743	—	ND	ND	ND
<i>A. hippikon</i> C1	—	ND	ND	ND
<i>A. equifetale</i> C112	—	ND	ND	ND
<i>A. oculi</i> 19L	+	—	+	ND
Human mycoplasma species†	—	—	—	—

—, No plaques; +, plaque formation; ND, not done.

* Congdon *et al.* (1979).

† *M. pneumoniae* FH-Liu, *M. salivarium* PG20, *M. hominis* PG21, *M. fermentans* PG18 and *M. orale* CH19299 were used.

Table 3. *Influence of physical and chemical treatments on MV-O1 virus*

Treatment	Change in titre [log ₁₀ (p.f.u. ml ⁻¹)]*
Filtration	
220 nm	0.0 (6.7)
100 nm	-0.5 (6.7)
50 nm	-0.4 to -0.2 (6.7)
25 nm	Non-filterable
Heat	
56 °C, 10 min	-3.7 (7.9)
20 min	-4.3 (7.9)
30 min	-4.8 (7.9)
60 °C, 5 min } 10 min }	Completely inactivated
Detergent	
Nonidet P-40 (0.2%, 37 °C, 15 min)	-4.4 (7.4)
Organic solvents	
Chloroform (5%, 4 °C, 10 min)	-2.3 (7.7)
Ether (20%, 4 °C, 18 h)	-3.0 (7.5)
Enzymes (500 µg ml ⁻¹ , 37 °C, 1 h)	
Trypsin	-0.4 (6.9)
α-Chymotrypsin	-0.2 (6.9)
Protease (<i>B. subtilis</i>)	-6.9 (6.9)
Protease (<i>S. griseus</i>)	-6.9 (6.9)
Lipase (pancreas)	+0.3 (6.7)
Lipase (wheat germ)	-0.1 (6.7)
Phospholipase D	-0.1 (6.9)
DNAase I (100 µg ml ⁻¹ , 37 °C, 1 h)	+0.1 (6.7)

* The titre of the non-treated control is shown in parentheses. All the values are the mean of duplicate assays at least.

virus were reduced by approximately 90% and 99% after passing a suspension through 100 nm and 50 nm filters, respectively. No virus was detected in the filtrate after passage through a 25 nm membrane filter.

Treatment for 15 min with the non-ionic detergent Nonidet P-40 (0.2%, v/v) reduced the virus titre to less than 10⁻⁴. Among various enzymes tested, only the proteases completely inactivated the virus (Table 3).

Morphology of MV-O1 virus

Transmission electron microscopy of purified virus revealed many roughly spherical viral particles, ranging from 80 to 130 nm in diameter, with a mean of 100 nm (Fig. 8a). Higher magnification revealed that MV-O1 is an enveloped virus, although the fine structure of the capsid could not be determined (Fig. 8b).

Viral nucleic acid

Agarose gel electrophoresis of the washing solution of infected *A. oculi* 19L cells revealed a distinct band, whereas no band was found in samples from the washing solution of non-infected cells. In addition, cell extracts of *A. oculi* 19L revealed no band corresponding to that from the washing solution of infected cells. The band detected in infected cells was subsequently confirmed as DNA by the diphenylamine method and by disappearance upon digestion with DNAase I. These results strongly suggest that the nucleic acid of MV-O1 is DNA.

DISCUSSION

The virus described in this paper was referred to as MV-O1, 'O' referring to *A. oculi* and '1' indicating the first virus isolated from this species. It was curious that no plaques were produced if *A. oculi*-i was subcultured once in a newly prepared culture medium. In other words, the virus-producing ability of *A. oculi*-i was lost after subcultivation.

Fig. 8. Electron micrographs of MV-O1 virus purified by equilibrium density gradient centrifugation in caesium chloride and negatively stained with 2% (w/v) potassium phosphotungstate. (a) Roughly spherical virus particles, 80–130 nm in diameter. Bar, 1 μ m. (b) At higher magnification an envelope can be observed; however, no clear capsid structure is visible. Many fragments surround the viruses. Bar, 100 nm.

The properties of the virus differ distinctly from those of other mycoplasma viruses previously reported in the following respects: (1) the limited host range, and (2) the strict serum requirement for virus multiplication. There were some morphological and biochemical similarities between MV-O1 virus and *Plasmaviridae* *Plasmavirus* strain L2, although the host range and the temperature range for growth differed. The MV-O1 virus can replicate at 22 °C, whereas L2 acholeplasma virus L2 strain can not (Gourlay, 1973; Liss & Maniloff, 1971). The MV-O1 virus infects and replicates only in *A. oculi* species, but absolutely not in strains of *A. laidlawii*. In contrast, all other members of acholeplasmavirus groups can infect and form plaques on the lawns of at least some *A. laidlawii* strains (Gourlay, 1972).

Because MV-O1 virus infects and multiplies only in *A. oculi* 19L cells, the virus genome may integrate into the chromosome of *A. oculi* 19L. However, at present, it is difficult to detect the viral genome in cells of *A. oculi* 19L, and induction of latent virus in *A. oculi* 19L has yet to be achieved.

Evidence indicating that the MV-O1 virus has a strict serum requirement for multiplication as well as for plaque formation is extremely interesting, since neither the host cell, nor other acholeplasma viruses, show this requirement. The substances in serum which are essential for multiplication of the virus have yet to be identified. Some lots of horse serum contain an inhibitor for virus multiplication which can be inactivated by trypsin digestion, and plaques can be produced when an albumin-like fraction extracted from horse serum is added to culture medium in place of horse serum (unpublished data). Although it is certain that horse serum contains both an inhibitor and an essential factor for virus multiplication, it is not yet clear whether these factors are involved in adsorption or multiplication of the virus.

It is suggested that the MV-O1 virus be classified as a new group in the genus *Plasmavirus*.

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