Characterization of an *Acholeplasma laidlawii* Variant with a REP⁻ Phenotype

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An *Acholeplasma laidlawii* variant has been isolated that has a REP⁻ phenotype. The properties of this variant, relative to parental cells, are: (i) it exhibits no change in cell growth kinetics; (ii) it does not propagate single-stranded deoxyribonucleic acid (DNA) mycoplasmaviruses but does propagate double-stranded DNA mycoplasmaviruses; (iii) it converts parental circular single-stranded mycoplasmavirus DNA to double-stranded replicative forms that are not replicated further; (iv) it exhibits no change in host modification and restriction; and (v) it has an increased ultraviolet light sensitivity. The REP⁻ isolate is the first stable mycoplasma variant to which a physiological defect has been attributed.

A variant of *Acholeplasma laidlawii* has been isolated that no longer allows growth of single-stranded deoxyribonucleic acid (DNA) mycoplasmaviruses but that retains the ability to propagate double-stranded DNA mycoplasmaviruses. This variant also exhibits an increased sensitivity to ultraviolet light (UV), relative to parental cells. Therefore, the variant is designated REP⁻, according to the convention (discussed by Denhardt et al. [4]) that cells that have lost the ability to support the growth of certain virus groups be referred to as having a REP⁻ phenotype.

The single-stranded DNA virus used in these studies was mycoplasmavirus MVL51, which has a circular chromosome of 2×10^6 daltons (10). Virus replication begins with the conversion of the parental single strand, as it penetrates the cell membrane, to a covalently closed double-stranded circular replicative form (RFI) (1, 1a). A nick or gap in the RFI molecule converts it to a relaxed form (RFII), which replicates at two to three membrane sites per cell to produce progeny RFI and RFII molecules. Progeny single-stranded chromosomes (SSI) are synthesized subsequently in the cytoplasm on these replicative forms, and maturation presumably occurs at the membrane as the virion is assembled and extruded. Infection is nonlytic; i.e., infected cells continue to grow, although at a reduced rate (11).

It is likely that host cell proteins are essential for the replication of MVL51 DNA, as has been found for the small single-stranded DNA *Escherichia coli* bacteriophages (e.g., reference 7). The existence of a REP⁻ *A. laidlawii* is consistent with the requirement for host cell functions in viral replication. The REP⁻ isolate is the first stable mycoplasma variant to which a physiological defect has been attributed.

MATERIALS AND METHODS

Cells and viruses. *A. laidlawii* JA1 was described previously (11). *A. laidlawii* 1305 was obtained from R. N. Gourlay (Institute for Research on Animal Diseases, Compton, England).

The REP⁻ derivative of strain JA1 was isolated from an MVL51 mottled plaque on a JA1 lawn and subsequently cloned. The mottled-plaque morphology is seen infrequently for MVL51 and is not a heritable characteristic. Whether REP⁻ cells are unique to such plaques was not investigated. The REP⁻ phenotype of the isolate has remained unchanged over nearly a year of daily passage.

The viruses used were: MVL51, a group 1 mycoplasmavirus (bullet-shaped virions with single-stranded circular DNA) (10, 11); MVL2, a group 2 mycoplasmavirus (double-stranded DNA virions virions; unpublished data); and MVL3, a group 3 mycoplasmavirus (double-stranded DNA virions with a complex morphology) (6). The isolation of MVL51 was described by Liss and Maniloff (9); and MVL2 and MVL3 were obtained from R. N. Gourlay. In addition, 11 other group 1 isolates were tested for growth on JA1 and JA1-REP⁻ lawns. These 11 viruses were isolated as described previously (8, 13) and are serologically similar, but not identical.

Cells were assayed as colony-forming units (CFU) on tryptose agar plates, and viruses were assayed as plaque-forming units (PFU) on strain JA1 as the indicator host.

Media and buffers. Tryptose broth and agar

plates were used for cultivating cells and viruses as described previously (8). The Tris-EDTA-NaCl buffer (pH 8.0) contained ‘0.01 M tris(hydroxymethyl)aminomethane (Tris), 0.001 M ethylenediaminetetraacetate (EDTA), and 0.1 M NaCl. The Tris-EDTA-NaCl-NaCN buffer was Tris-EDTA-NaCl buffer containing 10 mM NaCN. The high-salt buffer used was Tris-EDTA-NaCl buffer containing 1 M NaCl. Phosphate buffer contained 0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, and 0.85% NaCl (pH 7.5).

Preparation of labeled virus. Radioactively labeled MVL51 was prepared as described previously (1).

Infection and lysis. Log-phase cultures of JA1 or JA1-REP⁻ cells were infected with ^{32}P-labeled MVL51, and after 5 min unadsorbed viruses were removed by centrifugation and washing. The pellet of infected cells was suspended in prewarmed tryptose broth and incubated at 37°C. At desired intervals, infected cells were harvested by centrifugation and suspended in Tris-EDTA-NaCl buffer. For isolation of fast-sedimenting complexes (membrane-associated viral DNA), lysis was accomplished by three cycles of freezing in liquid nitrogen and thawing. For isolation of viral DNA replicative intermediates, cells were lysed by adding Sarkosyl NL97 to a final concentration of 0.4%.

Sucrose gradient centrifugation. For isolation of the fast-sedimenting complex, 1 ml of the freeze-thaw lysate was layered onto a 34-ml 5 to 20% (wt/vol) linear sucrose gradient, buffered by Tris-EDTA-NaCl, over a 4-ml CsCl shelf containing 1.2 g of CsCl per ml in 40% sucrose. Centrifugation was for 3 h at 24,000 rpm in a Beckman L3-50 ultracentrifuge using an SW27 swinging-bucket rotor. The fast-sedimenting complex was recovered on the CsCl shelf (1a).

Replicative intermediates were analyzed by sedimentation through 38-ml 5 to 20% (wt/vol) linear high-salt sucrose gradients as described previously (1). Lysate (1 ml) was layered on top of the gradient and centrifuged for 16 h at 5°C at 24,000 rpm in an SW27 rotor.

Both types of gradients were collected from the top in 1.2-ml fractions by using an ISCO density gradient factionator.

Measurement of radioactivity. Gradient fractions were precipitated by adding an equal volume of ice-cold 10% trichloroacetic acid. The precipitated samples were filtered, washed, dried, and assayed for radioactivity as described by Das and Maniloff (1).

UV inactivation. Log-phase cultures were harvested by centrifugation and suspended in phosphate buffer. Cell aggregates were dispersed in a tissue homogenizer, and 3.0 ml of the suspension was pipetted into a 60-mm petri dish and UV irradiated (Westinghouse germicidal lamp; 257 nm). Irradiation was at ambient temperature in the dark and with constant agitation. At desired intervals, 0.1-ml samples were removed and assayed for CFU by plating on tryptose agar plates. The UV source was calibrated with a germicidal dosimeter. The inactivation parameters, the survival curve slope, and zero-dose intercept (extrapolation point) were calculated by using the computer program described by Das et al. (2).

RESULTS

Virus growth in REP⁻ cells. The REP⁻ variant was identified by its inability to serve as an indicator host for the single-stranded DNA mycoplasmavirus MVL51. Figure 1 shows one-step growth curves for MVL51 grown on JA1 and JA1-REP⁻ cells. Two hours after infection, the number of PFU in an infected culture of JA1 had increased over 100-fold. In that same period, the number of PFU in an infected JA1-REP⁻ culture had not increased above the background level of unadsorbed virus. As will be discussed below, virus adsorption to JA1 and JA1-REP⁻ was similar.

MVL51-infected JA1 cells grew more slowly than uninfected cells (Fig. 2), as previously reported by Liss and Maniloff (11). The JA1-REP⁻ cells grew slightly faster than JA1 cells, and the JA1-REP⁻ growth rate was not affected by MVL51 infection (Fig. 2).

Eleven other group 1 (single-stranded DNA) virus isolates were tested, and all formed plaques on JA1 lawns but not on JA1-REP⁻ lawns. To examine the ability of the REP⁻ variant to grow other mycoplasmaviruses, group 2 and 3 (double-stranded DNA) mycoplasmaviruses were tested on JA1-REP⁻. Both groups made plaques on lawns of both JA1 and JA1-
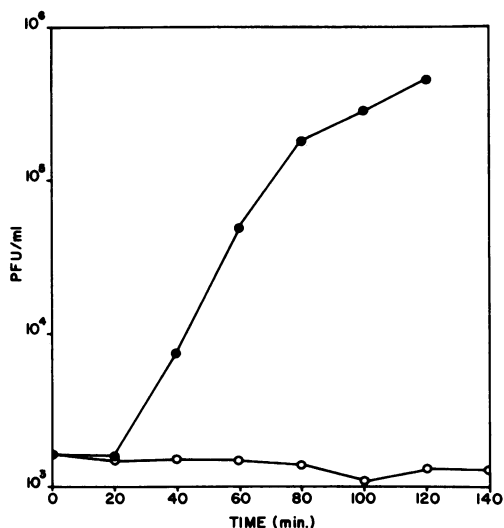

FIG. 1. *Growth of MVL51 in JA1 and JA1-REP⁻. Log-phase cultures of JA1 (●) or JA1-REP⁻ (○) were infected at an MOI of about 10 and incubated at 37°C for 10 min. The cultures were then diluted 10^6-fold to prevent further adsorption and assayed for PFU on JA1 lawns at the indicated times.*
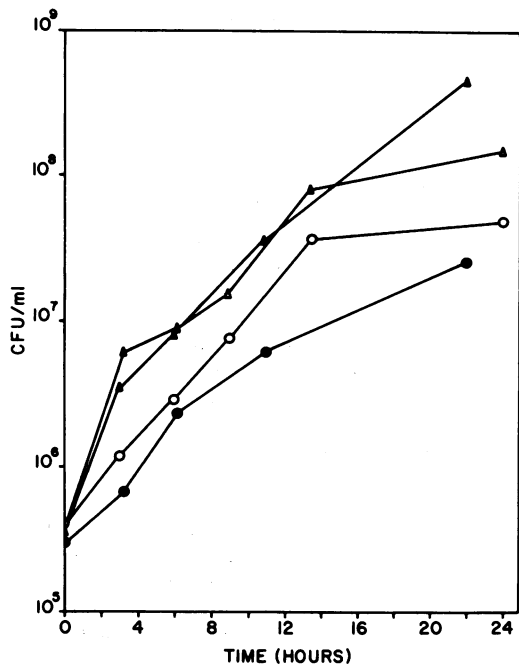
FIG. 2. *Growth of uninfected and MVL51-infected JA1 and JA1-REP⁻ cells. Duplicate cultures were either uninfected or infected with MVL51 at an MOI of about 2. Cultures were diluted 1,000-fold and assayed for CFU at the indicated times: (○) uninfected JA1, (●) infected JA1, (△) uninfected JA1-REP⁻, (▲) infected JA1-REP⁻.*

REP⁻. The growth curve for the group 2 virus was the same in JA1 and JA1-REP⁻ cells (Fig. 3). Hence, JA1 can allow the growth of all three groups of mycoplasmaviruses, but JA1-REP⁻ only propagates the two double-stranded DNA virus groups.

Host modification and restriction of group 2 mycoplasmavirus by *A. laidlawii* strains JA1 and 1305 have been reported (12). JA1-REP⁻ was examined for the retention of this specificity, consistent with it being a derivative of JA1. As shown in Table 1, JA1 and JA1-REP⁻ are identical in their host modification and restriction properties.

Abortive viral replication in REP⁻ cells. Normal MVL51 infection, in JA1 cells, begins with the association of the parental viral DNA with the host cell membrane (1a). To examine whether this process occurs in JA1-REP⁻ cells, ³²P-labeled MVL51 viruses were added to log-phase JA1 and JA1-REP⁻ cultures at a multiplicity of infection (MOI) of about 5. After 5 min of adsorption, unadsorbed viruses were removed by centrifugation. Five minutes later, the cells were gently lysed by freezing and thawing and were centrifuged through a high-salt sucrose gradient over a CsCl shelf to sepa-

rate fast (membrane-associated) and freely sedimenting viral DNA (described in Materials and Methods).

In JA1 cells (Fig. 4a), as reported previously (1a), about 60% of the infecting viral DNA was found as fast-sedimenting material on the CsCl shelf, and the remaining 40% was found as slower, freely sedimenting material. A similar distribution was found in infected JA1-REP⁻ cells (Fig. 4b). This implies that virus adsorption and penetration are the same in JA1 and JA1-REP⁻ cells.

Since no difference was found in the association of infecting viral DNA with the subcellular fast-sedimenting component in JA1 and JA1-REP⁻ cells, the extent of replication of viral DNA was then examined in JA1 and JA1-REP⁻ cells. These experiments were performed as de-
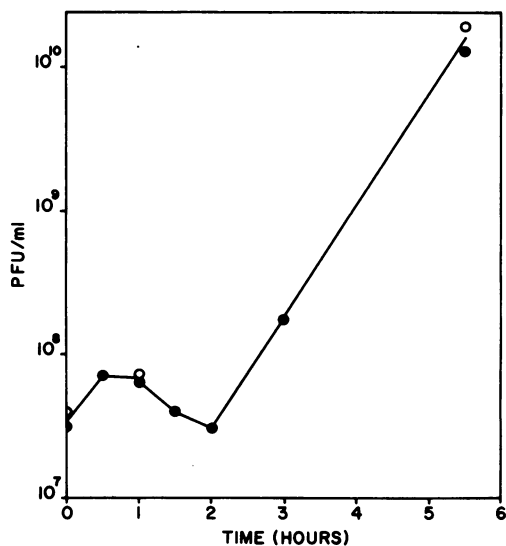

FIG. 3. *Growth of a group 2 virus on JA1 and JA1-REP⁻. Log-phase cultures (4 ml each) (about 5 × 10⁸ CFU/ml) of JA1 (○) and JA1-REP⁻ (●) were each infected with an equal volume of MVL2 stock (3 × 10¹⁰ PFU/ml) and incubated at 37°C for 10 min. Infected cells were harvested by centrifugation, washed and resuspended in 10 ml of prewarmed tryptose broth, and incubated at 37°C. At regular intervals, portions were removed and assayed for PFU on JA1 lawns.*

TABLE 1. *Relative number of virus plaques on A. laidlawii lawns*

Virus[a]	Relative PFU on *A. laidlawii* strain:		
	JA1	JA1-REP⁻	1305
MVL2·JA1	1.0	0.9	0.015
MVL2·JA1-REP⁻	1.1	1.0	0.015
MVL2·1305	0.011	0.014	1.0

[a] The symbols after the dot indicate the last host on which the virus was grown.

scribed for Fig. 4, except that, after removal of unadsorbed ^{32}P-labeled viruses, the infected cells were suspended for 5 min in medium containing [^{3}H]deoxythymidine, to label nascent DNA. Cells were then lysed by Sarkosyl NL 97 and sedimented through high-salt sucrose gradients as described in Materials and Methods. This procedure has been shown (1) to allow identification of the viral DNA replicative intermediates.

As reported previously (1), in lysates of 5-min-infected JA1 cells (Fig. 5a), the parental label was found in double-stranded RFI and RFII and nascent DNA was in RF components, mostly in RFI. No parental label was found at the position of single-stranded DNA (SSI); that time was too early for appearance of progeny SSI. Lysates of the infected JA1-REP⁻ cells showed that the single-stranded parental DNA had been converted to double-stranded RFI and RFII. However, essentially no nascent viral DNA was found in the JA1-REP⁻ cells. The small amount of DNA synthesis required to convert the single-stranded parental DNA to double-stranded forms was not detectable under these labeling conditions. The absence of nascent DNA at the RF positions, therefore, means that little or no further replication of the parental RF to progeny RF took place in JA1-REP⁻ cells. The conversion of parental viral single-stranded DNA to double-stranded forms in REP⁻ cells confirms that the early steps in infection (i.e., adsorption, penetration, and parental RF formation) are not impaired in the REP⁻ variant.

UV sensitivity of REP⁻ cells. JA1-REP⁻ was found to be more sensitive to inactivation by UV irradiation than JA1 cells (Fig. 6). Al-
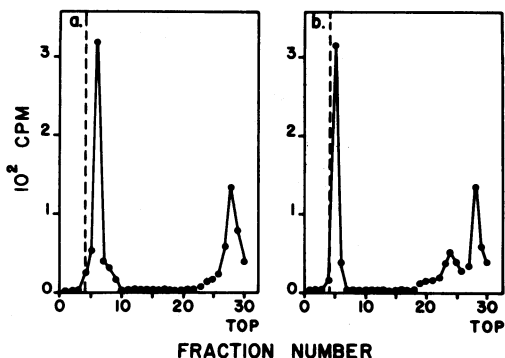

FIG. 5. *Velocity sedimentation analysis of viral DNA intermediates in infected JA1 and JA1-REP⁻ cells. Log-phase cultures of (a) JA1 and (b) JA1-REP⁻ cells were infected with ^{32}P-labeled MVL51 at an MOI of 10. Five minutes after infection, unadsorbed viruses were removed by centrifugation and washing. The infected cells were suspended in prewarmed tryptose broth containing 40 μCi of [^{3}H]deoxythymidine per ml. After 5 min of incubation at 37°C, an equal volume of ice-cold Tris-EDTA-NaCl-NaCN buffer was added. The cells were harvested, lysed, and layered onto high salt, 5 to 20% sucrose gradients as described in the text. The SSI position was determined from a parallel gradient analysis of purified viral DNA, and the positions of RFI and RFII were calculated from their sedimentation rates relative to SSI, as given by Das and Maniloff (1). Sedimentation is from left to right.*

FIG. 4. *Association of infecting viral DNA with a fast-sedimenting component. Log-phase cultures of (a) JA1 and (b) JA1-REP⁻ were infected with ^{32}P-labeled MVL51 at an MOI of about 5. Cells were harvested and lysed as described in the text. Sedimentation is from right to left. The dotted line indicates the position of the CsCl-sucrose shelf.*
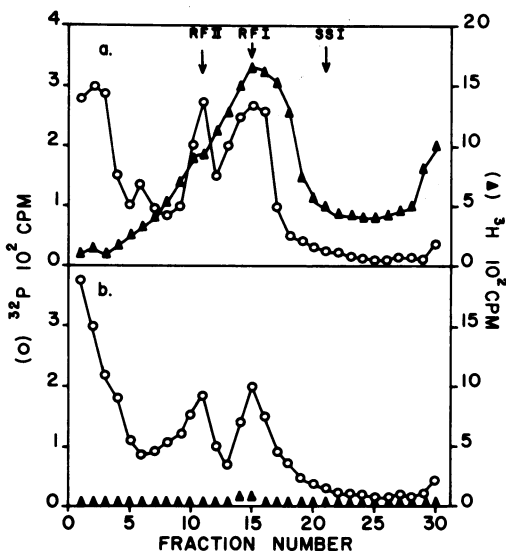
though the intercept (extrapolation of the linear part of the curve back to zero dose) was the same for both cell strains (2.66 for JA1 and 2.57 for JA1-REP⁻), the survival curve slope (a measure of the inactivation cross section) was increased for REP⁻ cells (9×10^{-3} mm^2/erg for JA1 and 12.2×10^{-3} mm^2/erg for JA1-REP⁻).

DISCUSSION

This paper has described the initial characterization of an *A. laidlawii* JA1 variant that has lost the ability to propagate group 1 mycoplasmaviruses; hence, the variant has a REP⁻ phenotype for this virus group. The JA1-REP⁻ cells continue to be hosts for the propagation of group 2 and group 3 viruses. JA1-REP⁻ has the same host modification and restriction specificity and frequency (Table 1) as JA1, consistent with it being a JA1 variant.

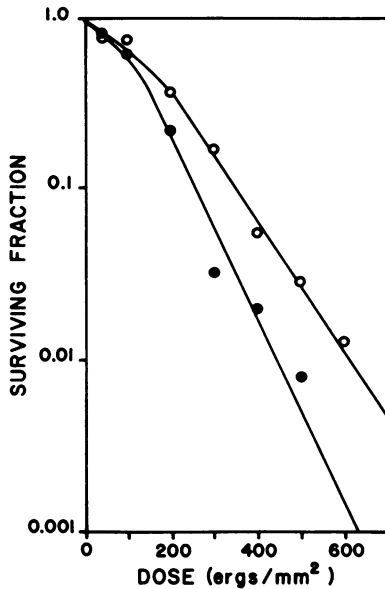The *A. laidlawii* JA1-REP⁻ phenotype is similar to that observed with *Escherichia coli*

FIG. 6. *UV inactivation of JA1 (O) and JA1-REP⁻ (●) cells. Each point represents the average of five experiments, except for JA1-REP⁻ cells where the values at 100 and 200 ergs/mm² are the average of four experiments and the value at 500 ergs/mm² is the average of two experiments.*

rep-3 mutants. *E. coli rep-3* mutants are not impaired in cell growth, do not allow growth of single-stranded DNA bacteriophages, and have increased sensitivity to UV and X-ray irradiation (3–5).

Group 1 viral replication has been shown to involve the formation of a membrane-associated complex with the parental viral DNA strand, which is converted to double-stranded replicative forms (RFI and RFII), and semiconservative replication of the parental RF to produce progeny RF molecules (1, 1a). It is these progeny RF that are released into the cytoplasm to form progeny single-stranded viral DNA. There have been, as yet, no reported data on the relative roles of viral and cellular gene products in the different replication steps.

In JA1-REP⁻ cells, the initial steps of MVL51 infection are not impaired: a parental viral DNA-cell membrane complex is formed (Fig. 4), and the single-stranded DNA is converted to double-stranded replicative forms (Fig. 5). However, little or no formation of progeny RF molecules is found. This finding implicates the requirement for a cell function in the replica-

tion of parental RF to produce progeny RF. Interestingly, *E. coli rep-3* mutants are also blocked in an analogous step in the replication of single-stranded DNA coliphages (4).

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