Release of a Group 1 Mycoplasma Virus from *Acholeplasma laidlawii* After Treatment with Mitomycin C

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Received 6 April 1981/Accepted 27 May 1981

A rod-shaped group 1 mycoplasmal virus was released from *Acholeplasma laidlawii* strain JA2 after treatment with 2.5 μg of mitomycin C per ml. Similar treatment of *A. laidlawii* strain Bju failed to stimulate release of any PFU.

A mycoplasma virus was first isolated by Gourlay in 1970 (20). Since then, over 50 additional isolates have been reported (reviewed in reference 12). These isolates have been placed into three morphologically distinct groups: bullet-shaped particles (group 1); spherical and enveloped viruses (group 2); and icosahedral particles with short tails (group 3) (12). Recently, a virus of a fourth morphologically distinct group has been isolated from *Mycoplasma bovirhinis* (6). All isolates have been recovered from spontaneous plaques, washes of lawns of mycoplasmas, or filtrates of broth cultures (12, 13). These experiments suggested that viruses may be carried, in some manner, by a variety of mycoplasmas. Several people have attempted, unsuccessfully, to induce virus from mycoplasmas by using UV irradiation, mitomycin C (MC), and nitroso-guanidine (13, 15). This paper reports the stimulation of group 1 mycoplasma virus release from *Acholeplasma laidlawii* strain JA2 after treatment with MC.

MATERIALS AND METHODS

*Mycoplasmas and viruses used.* Strains of *A. laidlawii* used were JA1, Bju, BC1-13 (described in reference 18), JA2, AL1, AL3, and AL9 (described in this report). Group 1 virus MVL51 (11), group 2 virus MVL2 (3), and group 3 virus MVL3 (5, 9) were also used. MVL51 and MVL3 were propagated in JA2, a spontaneous mutant of JA1 (11) which is resistant to 5 μg of rifampin per ml. MVL2 was propagated in JA1.

*Media and growth conditions.* All *A. laidlawii* strains were grown in tryptose broth (containing 1% glucose and 10% heat-inactivated donor horse serum; GIBCO Laboratories, Grand Island, N.Y.) (10, 11). The number of viable cells was determined by counting colony-forming units (CFU) on tryptose agar plates (formulated as above with the addition of 1.5% agar); viruses were assayed as PFU on lawns of *A. laidlawii* strain BC1-13 unless otherwise noted. All incubations were carried out at 37°C.

Mycoplasma cultures were maintained by daily inoculation of 100-fold dilutions into fresh medium. Viruses were characterized by host range and by heat inactivation, as previously described (4, 10, 11).

**MC treatment.** A modification of the MC induction protocol of Otsuji et al. (16) was used. A 22-h broth culture of *A. laidlawii* was diluted 1:1 with an equal volume of prewarmed broth. After the culture was incubated for 60 min at 37°C, an equal volume of tryptose broth containing various concentrations of MC (Sigma Chemical Co., St. Louis, Mo.) was added. After variable periods of incubation at 37°C, MC-treated cells were diluted 1:10 into warm, fresh tryptose broth and then assayed for CFU and PFU. As a control, broth cultures without added MC or MC solutions without added mycoplasmas were treated in the same manner.

RESULTS AND DISCUSSION

**Conditions of optimal virus stimulation.** The optimal MC concentration and duration of treatment were determined in experiments that used (i) a constant concentration of MC for various times and (ii) various concentrations of MC for a fixed time period. From these experiments (data not shown), the following conditions were established for optimal virus stimulation: about 10⁸ CFU of cells per ml and 2.5 μg of MC per ml (final concentration) for 30 min at 37°C in the dark. Under these cell number and time constraints, higher and lower MC concentrations led to less virus release (Table 1).

**Release of viruses from *A. laidlawii.*** Strains JA2 and Bju were treated with MC under the regimen noted above. After 30 min, the cultures were diluted into fresh medium and assayed for CFU and PFU. Throughout the 12 h of this experiment, no PFU were found in any control samples lacking MC. MC-treated cultures of strain Bju underwent a 2-h "lag" period and then resumed a growth rate similar to that of untreated cells (data not shown), but in no cases were PFU found. JA2, treated similarly, displayed a 3- to 4-h lag and then resumed growth (Fig. 1). Concomitant with resumption
of growth in MC-treated JA2 cultures, there was a release of PFU which formed plaques on lawns of BCI-13 (Fig. 1). Release of virus did not appear to result in cell death, although the proportion of cells actually releasing PFU may have been too small to result in a detectable loss in CFU.

As already noted, PFU assays were initially made on BCI-13 indicator lawns. Subsequently, I found that MC-stimulated virus plaqued at the same apparent efficiency on JA2 as on BCI-13 (data not shown).

**Characterization of the virus released.**

Examination of electron micrographs of negatively stained, MC-treated JA2 revealed bullet-shaped group 1 type particles on the surface of cells (Fig. 2). In addition to its morphological characteristics, a virus can be classified as a group 1 mycoplasma virus on the bases of its resistance to heating and its ability to form plaques on particular mycoplasma strains. It has been previously shown that group 2 viruses are sensitive to heating (60°C for 30 min), whereas group 1 and 3 viruses are resistant to this heat treatment (reviewed in references 12 and 13). The virus isolated from induced JA2 (tentatively called MVL80) was heat resistant (Table 2).

Three A. laidlawii strains (AL1, AL3, and AL9) were selected because of their differing susceptibilities to previously defined mycoplasma viruses. Strain AL1, isolated from JA1 infected with MVL51, was sensitive to group 2 and 3 virus isolates. AL3, isolated from a BCI-13 culture infected with both MVL51 and MVL2, would plaque only group 3 viruses. AL9, isolated from a JA1 culture infected with MVL2, was sensitive to group 1 and 3 viruses. Although all three special indicator strains consistently released endogenous viruses, these viruses did not produce spontaneous plaques on control plates. As a result, these strains were useful differential indicators but would not be useful for propagating viruses. With the virus indicators listed in Table 2, MVL80 appeared to be group 1-like.

**Conclusions.**

Treatment of A. laidlawii JA2 with MC resulted in the release of a group 1 mycoplasma virus, MVL80. This virus formed plaques on several “indicator” A. laidlawii strains. The finding that strain Bju did not produce PFU by the same MC treatment and plating on the same indicator strains confirmed that the treatment used does not stimulate release of endogenous virus (as measured by PFU) in the indicator strains.

The virus released from JA2 also formed plaques on JA2 lawns. This event is similar to “cryptic lysogeny,” a phenomenon by which viruses can be carried by a cell without conferring immunity to infection by homologous viruses. Typically, cryptic lysogeny is found in some coliphage mutants (1, 14) or with wild-type phages of *Achromobacter* (19) and *Proteus mirabilis* (7).

Although the protocol used here is similar to that used for induction of bacteriophage from eubacteria (8, 16), I have purposefully avoided the use of the term induction: induction implies a complex series of responses of a microbe to a variety of stimuli. One result is the release of bacteriophage which was previously integrated into the host chromosome (as reviewed in ref-

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**TABLE 1. Effect of various MC concentrations on viability and virus stimulation from A. laidlawii JA2**

<table>
<thead>
<tr>
<th>Final MC conc (µg/ml)</th>
<th>CFU/ml&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PFU/ml&lt;sup&gt;b&lt;/sup&gt;</th>
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</tr>
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</tr>
<tr>
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<td>3.6 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
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<tr>
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<td>0</td>
</tr>
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<td>1.75 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>0</td>
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<tr>
<td>0.0</td>
<td>2.3 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
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</tr>
</tbody>
</table>

<sup>a</sup> Cell titer assayed 30 min after addition of MC.

<sup>b</sup> Virus titer assayed 5 h after addition of MC.

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**FIG. 1. Result of treating A. laidlawii JA2 with 2.5 µg of MC per ml (final concentration). Treatment was for 30 min in the dark at 37°C, after which the culture was diluted 100-fold and then assayed for CFU and PFU. Plaque assay used lawns of A. laidlawii BCI-13.**
Fig. 2. (A) MC-treated JA2 cells negatively stained with ammonium molybdate (3%). Virus particles of variable length (noted by arrow) are seen on the cell's surface. Bar, 0.5 μm. (B) Untreated control JA2 cells treated as in (A). Bar, 0.5 μm.

Table 2. Host range and heat sensitivity of MVL80 and reference viruses

| Virus   | Bju | JA2 | BC1-13 | AL1 | AL3 | AL9 | Virus titer (PFU/ml) after heating at 60°C for 30 min | % of unheated control samples
|---------|-----|-----|--------|-----|-----|-----|------------------------------------------------------|---------------------------
| MVL51   | -   | +   | -      | -   | -   | +   | 4.0 × 10⁶                                                | 25                        |
| MVL2    | -   | -   | +      | +   | -   | -   | 0                                                    | 0                        |
| MVL3    | -   | +   | +      | +   | +   | +   | 4.25 × 10⁶                                              | 106                       |
| MVL80   | -   | +   | +      | -   | -   | +   | 4.25 × 10⁶                                              | 12.5                      |

* (PFU/ml of heated samples)/(PFU/ml of unheated samples) × 100.

a Control samples were treated in tryptose broth at 37°C for 30 min.
b Original titer was 8 × 10⁷ PFU/ml. After heating, no PFU were detected in two 25-μl samples.

erence 20). After doing these experiments, I found that the induction protocol resulted in release of a virus presumably containing single-stranded, covalently closed DNA (as reviewed in reference 12). To attempt explanation of the phenomenon described here in terms of classic bacteriophage induction, I would be forced to make assumptions about many of the intracellular characteristics of the group 1 mycoplasma viruses. Rather than prematurely construct the alleged nature of the carrier state of group 1 mycoplasma viruses, I shall refrain from using the term induction and use the word stimulation instead.
The mechanism of stimulation of MVL80 by MC is unknown. Preliminary data (A. Liss and T. Brown, unpublished data) indicate that late logarithmic- or stationary-growth-phase mycoplasmas must be used to repeatedly yield virus after MC treatment. This may explain why other workers have failed to find viruses released from mycoplasmas after MC treatment (13, 15, 17). In all reports I can find, it appears that midlogarithmic or younger mycoplasmas were used for induction attempts. The use of actively growing cells is optimal if one expects induction of bacteriophage but, as noted above, the direct application of bacteriophage induction protocols may be inappropriate in the group 1 mycoplasma virus system. Attempts at characterizing the MC stimulation of virus release in a variety of mycoplasmas are in progress.

ACKNOWLEDGMENTS

I thank John Swanson, Len Mayer, Penny Hitchcock, and Alan Barbour for help in writing this paper. I also thank Bill Todd for helping with the electron microscopy and Teresa Brown for fine technical assistance. Special thanks go to Susan Smaus for final preparation of this manuscript.

LITERATURE CITED