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GROWTH CHARACTERISTICS OF THE BLUE-GREEN ALGAL VIRUS LPP-1

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ABSTRACT

SAFFERMAN, ROBERT S. (Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio), AND MARY-ELLEN MORRIS. Growth characteristics of the blue-green algal virus LPP- 1. J. Bacteriol. **88**:771-775. 1964.—The blue-green algal (BGA) virus, strain LPP-1, formed two distinct plaque variants. During subsequent propagation, each of the isolated variants eventually reverted to a mixture of both plaque types. The large-plaque-former, multiplying at a somewhat faster rate, showed maximal virus production within 60 hr after infection; maximal titer of the small-plaque mutant was reached after a 76-hr incubation period. No differences were observed in either their host spectra or their pH and thermal stability. Evidence was presented which demonstrated that BGA virus samples could be assayed with reasonable accuracy from plaque counts. Variations resulting from this plating technique were within the range of experimental error that has been generally reported for such a system.

In a recent study, Safferman and Morris (1963) reported the isolation of a new viral agent from the filamentous blue-green alga *Plectonema boryanum*. From electron micrographs, it was observed that this agent had a polyhedral structure with an average diameter of about 66 mμ. More recently, micrographs of purified viral preparations showed that the particles have short tails, which are approximately one-fourth as long as the diameter of the head (Schneider, Diener, and Safferman, 1964). Although the host spectrum of the blue-green algal (BGA) virus included strains belonging to the genera *Lyngbya*, *Plectonema*, and *Phormidium*, these infected algae are nevertheless very similar in their morphological characteristics. Plaques produced by the BGA virus (strain LPP-1) were reported to vary from less than 0.1 mm to more than 8.0 mm in diameter. These variations have since been attributed to the presence of two virus types within the strain. In this communication, physicochemical characteristics of the plaque types are presented, together with data on the reproducibility of the plating technique adopted for measuring the activity of the BGA virus.

MATERIALS AND METHODS

The virus was cultured under aseptic conditions in 4-liter bottles containing 3,000 ml of a modified Chu No. 10 broth [$Ca(NO_3)_2 \cdot 4H_2O$, 0.232 g; K_2HPO_4, 0.01 g; $MgSO_4 \cdot 7H_2O$, 0.025 g; Na_2CO_3, 0.02 g; $Na_2SiO_3 \cdot 5H_2O$, 0.044 g; ferric citrate, 0.0035 g; citric acid, 0.0035 g; distilled water, 1,000 ml]. Each bottle was inoculated with 200 ml (40 to 80 mg, dry weight) of a 3-week-old culture of *Plectonema boryanum* strain IU 594 (Indiana University Culture Collection) and incubated for 3 days prior to the addition of the virus (10 ml at a titer of 10^7 plaque-forming units per ml). After inoculation with the virus, the cultures were incubated for an additional 3 to 4 days at 20 C with constant agitation. A gas mixture of 5% carbon dioxide in air was bubbled through the medium, and continuous illumination was provided by "cool white" fluorescent tubes at a light intensity of 500 to 600 ft-c. Titers regularly varied between 10^8 and 10^9 plaque-forming units per ml. The lysates were centrifuged and then passed through a sterile, ultrafine, sintered-glass filter. The resulting materials were stored at 4 C for periods not exceeding 60 days.

To produce small quantities of virus or to determine the susceptibility of other algal strains to the virus, 1 ml of the BGA virus and 2 ml of a 2- to 3-week-old algal culture were added to 250-ml Erlenmeyer flasks containing 100 ml of a modified Chu No. 10 broth. Conditions used for culturing the virus were described previously (Safferman and Morris, 1963).

The plating technique developed for assaying the algal virus was a modification of a method that had been described for phage investigations

TABLE 1. *Distribution of plaque counts per plate at 1-in-10^6 dilution of stock suspensions of BGA virus*

Expt no.	Plaque count groups	No. of plates in each group
1	96–105	4
	106–115	10
	116–125	13
	126–135	4
	136–145	3
2	36–40	5
	41–45	9
	46–50	15
	51–55	14
	56–60	2

TABLE 2. *Plaque counts in parallel dilution series from a 10^6 virus stock*

Expt no.	Plate no.	Replicate dilution*								Arithmetic mean counts ± SD
		1	2	3	4	5	6	7	8	
1	1	43	38	40	39	36	41	40	47	41 ± 4.4
	2	42	48	36	39	45	37	41	51	
2	1	52	48	55	47	50	—	—	—	49 ± 3.8
	2	47	52	42	45	51	—	—	—	

* Results are shown as plaque counts per plate.

(Adams, 1950). Plaque counts were determined on plates in which 5 ml of an inoculated agar had been evenly distributed over a 15-ml solidified layer of 1.5% modified Chu No. 10 agar. The surface layer was prepared in test tubes and consisted of 0.5 ml of an appropriately diluted virus suspension, 2.0 ml of a 3-week-old culture of *Plectonema boryanum* IU 594 (0.4 to 0.8 mg, dry weight), and 2.5 ml of a 1% modified Chu No. 10 agar, which had been previously melted and cooled to about 47 C. The virus was diluted at tenfold intervals in a salt solution containing 0.2 g of $MgCl_2 \cdot 6H_2O$, 5.85 g of NaCl, and 1,000 ml of distilled water. The plates, illuminated at 160 to 180 ft-c, were incubated for 3 to 4 days at 20 C before plaque counts were made.

RESULTS

Reproducibility of the plating technique. Of the algae susceptible to the BGA virus, *P. boryanum* IU 594 proved to be best suited for studies on this new virus-host system. The relatively uniform development of this alga in both liquid and solid media warranted the adoption of the previously described plaque-counting method. Experiments were, therefore, designed to determine both the reproducibility of the plating technique and the effects of virus concentrations on the relative number of plaque units formed.

Table 1 presents the results obtained by preparing a large number of replicate plates from one suitable virus dilution. To test the degree of normality of the data in Table 1, the arithmetic mean and the standard deviations (SD) were computed for both experiments by the method of Geary (1936) and were found for experiment 1 and 2 to be 118.2 ± 10.1 and 45.4 ± 4.95, respectively.

From the table prepared by Geary on the 1, 5, and 10% points of sampling distribution of α (average deviation/SD) it was found that the Geary ratios of 0.85 and 0.84 for the data in experiments 1 and 2, respectively, are quite reasonable for a normal distribution. Furthermore, the Lexis ratios of $10.1/\sqrt{118.2}$ and $4.95/\sqrt{45.4}$, which amount to 0.92 and 0.69, are well within the limits usually obtained for this ratio in carefully conducted bacteriological and virological tests. These Lexis ratios are indeed lower than the theoretical value of unity for a Poissonian population!

For ascertaining the reliability of the dilution procedure in producing reproducible plate counts, eight dilution series were prepared by parallel dilution of a single virus suspension in one experiment, and five were prepared in a similar manner in another experiment. The counts obtained in these experiments are shown in Table 2. Here again, the relatively high reproducibility of the counts is borne out by the relatively low values of standard deviation.

To determine the relationship between virus concentration and the relative number of plaque counts obtained, a series of successive dilutions made from a single virus stock were plated under standard conditions. In Fig. 1 it can be seen that the plaque-forming units are in linear proportion to the virus concentration within the concentration range tested, thus satisfying a necessary condition for quantitative studies on virus development.

In some slowly adsorbing virus-host systems, plaque counts are affected if contact time between the host and the virus has been varied before plating (Groman and Lockart, 1953). To test for

this effect, samples of *P. boryanum* IU 594, incubated in a liquid medium with a suitable dilution of the BGA virus, were assayed at intervals of 0, 10, 20, and 30 min. No measurable difference in plaque counts was observed to result from varying the exposure time.

Storage, filtration, and ionic effects on virus infectivity. The BGA virus retained about 70% of its initial activity when stored in its own lysate for 30 days at 4 C. Use of distilled water as a diluent subjected the virus to rapid inactivation. Adams (1959) reported that a similar problem was prevalent among the bacterial viruses, but this could generally be prevented by the use of a solution containing 0.1 M sodium chloride and 0.001 M magnesium chloride. When incubated in this solution for 3 days at 20 C, the BGA virus showed no substantial loss in its titer. Virus stocks were freed from contaminating bacteria by filtration through an ultrafine sintered-glass filter. This generally resulted in a less than twofold decrease in viral activity. The same virus preparation, passed through a membrane filter (0.45 μ), showed at least a tenfold loss, whereas assays made after Seitz filtration revealed that the filtrate contained virtually no infectious viral particles.

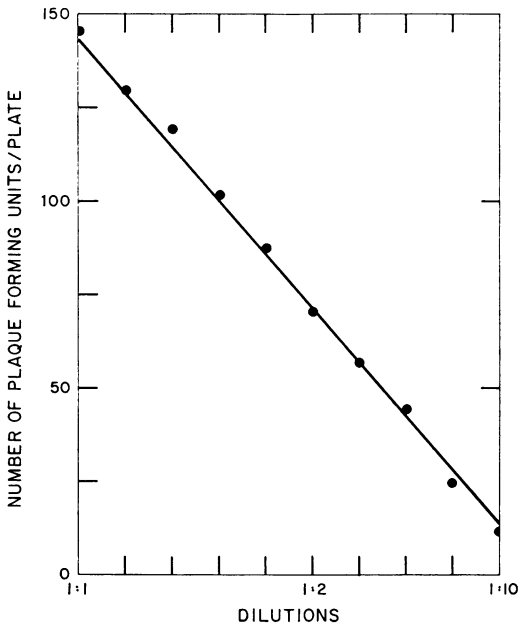

FIG. 1. *Proportionality of relative virus concentrations of strain LPP-1 to plaque-forming units.*

Isolation of a large and small plaque type in strain LPP-1. In a previous study, it was reported that the BGA virus strain LPP-1 produced plaques that varied widely in size (Fig. 2A). Since this strain had been purified from plates on which very few well-isolated plaques occurred, these observations were first thought to be due to a slowly adsorbing virus-host system. However, no appreciable differences in plaque size were found when susceptible algal preparations that had been kept in contact with the virus for periods of up to 1 hr were centrifuged and the infected algae were plated. The plaques could be divided into two types on the basis of size. Several plaques of each type were picked and cultured in 250-ml Erlenmeyer flasks according to the procedure previously described. Upon plating, two distinct plaque types were isolated. Since neither variant predominated, the large-plaque former in Fig. 2B was arbitrarily designated as the wild type (r^+); thus, the small plaque type in Fig. 2C has been referred to as the mutant strain (r).

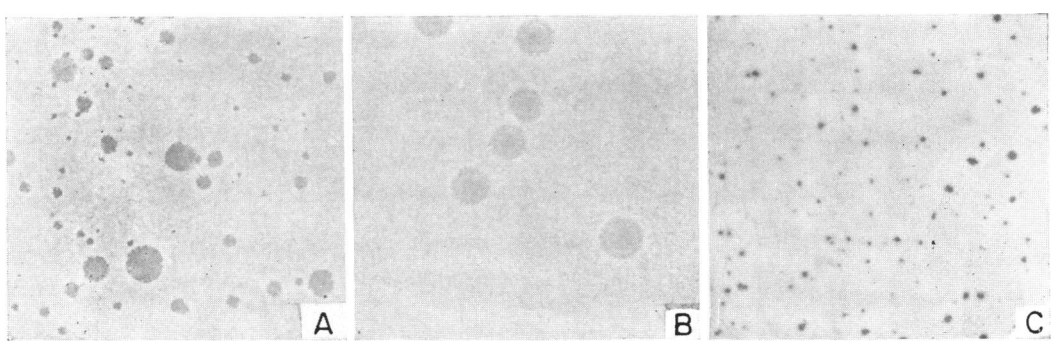

FIG. 2. *Plaques of the blue-green algal virus strain LPP-1. (A) Mixed plaque types; (B) r^+ virus (wild-type (C) r virus (mutant). The photographs, taken 4 days after plating, represent the actual size of the plaques. Further incubation of both the r and r^+ viruses would result in a substantial increase in plaque sizes.*

TABLE 3. *Effect of temperature and pH on the stability of the blue-green algal virus*

Plaque type	Per cent survival for 1 hr*												
	Temp (C)					pH							
	25	40	45	50	55	4	5	6	7	8	9	10	11	12
r	100	100	57	5	0	0	83	100	96	100	100	94	84	0
r^+	100	98	62	3	0	0	88	96	97	100	98	100	91	0

* Survival rates below 0.001% are reported as 0. The controls that were incubated at 25 C and pH 8 served as reference points for calculating the comparative data into per cent survival.

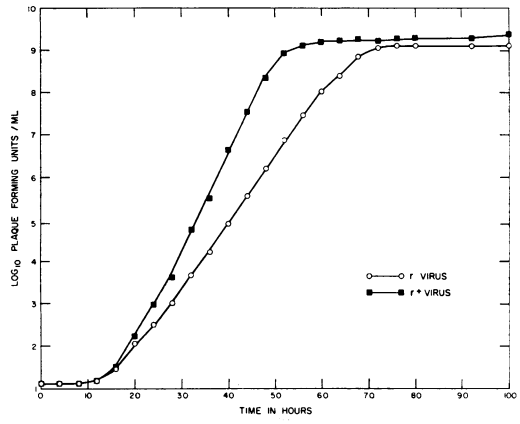

FIG. 3. *Growth curves of the r and r^+ viruses from strain LPP-1.*

The r and r^+ viruses, picked and replated twice from single plaques, were passed through sterile, ultrafine sintered-glass filters and stored at 4 C. Generally, after three or more transfers each plaque type was found to revert to a mixture of large and small plaques. All experiments were, therefore, carried out with stored virus stocks that had been assayed for plaque purity just prior to their use. The host spectrum of each plaque type was compared with that of the original LPP-1 strain (r and r^+) and was found to be identical.

pH and thermal stability of the r and r^+ viruses. To characterize the r and r^+ viruses more fully, studies were performed to determine the effects of temperature and pH on the survival of these plaque types. Stock cultures of the r and r^+ viruses prepared according to the above method had a pH of 7.8 $\pm$ 0.2 and a concentration of 10^8 plaque-forming units per ml. For pH experiments, the virus preparations were first brought to 25 C and then adjusted with either HCl or NaOH to the appropriate pH. After incubation for 1 hr, these preparations were readjusted to the original pH. Samples were then removed and assayed for viral infectivity. Both plaque types had nearly identical pH ranges (Table 3). Some of the minor differences can be explained on the basis of experimental error. In addition, it should be noted that the BGA virus is exceedingly stable in the alkaline range, particularly in comparison with the bacterial viruses, which are generally stable from pH 5 to 8 (Adams, 1959).

Thermal inactivation studies were made in water baths at temperatures ranging from 25 to 55 C. The procedure consisted of incubating stock cultures of the r and r^+ viruses for 1 hr at the appropriate temperature and then immediately plating suitably diluted samples. No adjustments were made in the initial pH of the virus preparations. As seen in Table 3, the r and r^+ viruses could not be differentiated on the basis of their thermal inactivation patterns.

Multiplication rates of the r and r^+ viruses. Growth curves of the r and r^+ viruses were determined at 20 C in 250-ml Erlenmeyer flasks containing 125 ml of a modified Chu No. 10 broth. Each flask was inoculated with a 5-ml sample (2 mg, dry weight) of a 3-week-old culture of *Plectonema boryanum* IU 594 and 1 ml of a virus stock solution suitably diluted to give an initial count of about 20 plaque-forming units per ml of medium. The viruses were propagated under static conditions for 100 hr at a light intensity of 180 ft-c. At 4-hr intervals, samples were withdrawn and assayed. Owing to the filamentous nature of the host cells, accurate counts of their cell numbers could not be determined; however, cell concentrations were estimated by approximating the total length of the algal filaments for a specific volume and then dividing this by the average cell length. On comparing these rough calculations with the virus counts, it is evident that the multiplicity of infection in this experiment is less than 0.001.

From the growth curves in Fig. 3, distinct differences can be seen in the multiplication rates of the two plaque types. Although no newly synthesized r and r^+ particles were observed until 12 hr after infection, a 16-hr difference was seen in their growth curves at the time of maximal virus production. The r^+ virus reached its maximal titer at 60 hr, whereas the r virus, multiplying at a somewhat slower rate, required a 76-hr incubation period. When these plaque types were

propagated under the same cultural conditions, the titer of the r^+ virus was consistently greater than that of the r virus.

DISCUSSION

The cultural characteristics of the host alga, *Plectonema boryanum* IU 594, led to the adoption of methods that are well established in phage investigations. Employment of a plaque assay was of particular significance in view of the obstacles encountered with methods developed for viruses from higher plants. In the experimental results, the reproducibility of the plaque counts has been shown to be within the range of variation that has been observed in phage systems. No attempt was made to determine the relationship between plaque counts and the total number of virus particles in the sample; however, it seems reasonable to assume from recent estimates (Schneider et al., 1964) that the absolute efficiency of the plating procedure would be similar to that reported for bacterial viruses (Luria, Williams, and Backus, 1951).

From the findings presented in Fig. 3, it can be theorized that during a specific incubation period the two plaque sizes evolved as a result of the differences in the respective multiplication rate of the variants. Nevertheless, caution should be exercised in interpreting the significance of this factor, since small plaque variants found in several animal viruses have been attributed to the presence of an inhibitor in the agar (Takemoto and Liebhaber, 1961; Takemori and Nomura, 1960; McClain and Hackett, 1959). Takemoto and Liebhaber (1961) found that a sulfated polysaccharide was the agar component responsible for the small plaque type observed in the encephalomyocarditis (EMC) virus. Reversal of this inhibitory effect could be obtained by adding diethylaminoethyl (DEAE) dextran to the agar as a binding agent for the sulfated polysaccharide (Liebhaber and Takemoto, 1961). In view of the similarities between the plaque types observed in the BGA and EMC viruses, a sample of DEAE dextran was tested. It was found to have no effect on the small-plaque variant in the BGA virus when added to the agar at a concentration of 1.0 or 2.0 mg/ml.

It has been noted that in the alkaline range the BGA virus has a pH stability considerably greater than that generally reported for bacterial viruses. This is of particular interest since the algal host, *P. boryanum* IU 594, develops readily from pH 7 to 11 and shows little or no development below neutrality. Although most of the other host strains have not been thoroughly studied with regard to optimal culture conditions, relatively good development is obtained in a modified Chu No. 10 broth that has a pH of 9.1 after sterilization. Thus, one might speculate about the ecological significance of the pH range on the parasitic relationship that exists in this virus-host system.

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