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Characteristics of *Anabaena variabilis* Influencing Plaque Formation by Cyanophage N-1

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Phage N-1 grown in *Anabaena* strain 7120 [N-1·7120] forms plaques on *A. variabilis* about 10^{-7} to 10^{-6} as efficiently as on *Anabaena* 7120. By manipulating different characteristics of the interaction between phage and host, it was possible to increase the relative efficiency of plaque formation to 0.38. Growth of *A. variabilis* at 40°C for at least three generations resulted in an increase in the rate of phage adsorption and a 10-fold increase in the efficiency of plaque formation. The efficiency of plaque formation was further increased about 42-fold, with little or no further increase in rate of adsorption, in a variant strain, *A. variabilis* strain FD, isolated from a culture of *A. variabilis* which had grown for more than 30 generations at 40°C. The low relative efficiency of plaque formation by N-1·7120 on *A. variabilis* could be partially accounted for if *A. variabilis* contains a deoxyribonucleic acid restriction endonuclease which is absent from *Anabaena* 7120. Indirect evidence for such an endonuclease included the following: (i) phage N-1 grown in *A. variabilis* (N-1·Av) had approximately a 7×10^3-fold higher relative efficiency of plaque formation on *A. variabilis* than had N-1·7120; and (ii) the efficiency of plaque formation by N-1·7120 on *A. variabilis* strain FD was increased by up to 146-fold after heating the latter organism at 51°C.

Auxotrophic mutants have been isolated from the N_2-fixing, filamentous cyanobacterium *Anabaena variabilis* (5), and the biochemical lesions in two uracil-requiring mutants have been identified (6). A possible vector for the transfer of genes in *A. variabilis* is cyanophage N-1, a virus which can lyse (1) or lysogenize (9) *Anabaena* strain 7120 (formerly called *Nostoc muscorum*). The latter strain, in contrast to *A. variabilis* (13), often plates with low efficiency when fragmented to single cells by cavitation, and may for that reason be less desirable than *A. variabilis* as an object for genetic analysis.

It was originally reported that phage N-1 did not attack *Anabaena variabilis* (*A. flos-aquae* IU 1444 [1]). However, we subsequently observed that *A. variabilis* would produce N-1 when small amounts of the cyanobacterium were inoculated on agar with high input of phage. The efficiency of plaque formation on *A. variabilis* was 10^{-7} to 10^{-6} of the efficiency of plaque formation on *Anabaena* 7120. In this paper, we show that the low and variable relative efficiency of plaque formation is attributable to a combination of factors, one of which may be DNA restriction. By manipulating the characteristics of the interaction between phage and host, it was possible to increase the relative efficiency of plaque formation to 0.38.

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MATERIALS AND METHODS

Organisms, media, and conditions of growth. *A. variabilis* Kützing (ATCC 29413, derived from IU 1444) was grown by using the medium of Allen and Arnon (2), which was either solidified with 1% Difco agar purified by the method of Braun and Wood (4) or, for liquid cultures, was diluted eightfold. The media for growth of *Anabaena* 7120, a cyanobacterium obtained from R. Haselkorn (University of Chicago), were the same except that they were supplemented with 2.5 mM $NaNO_3$ and 2.5 mM KNO_3. Cultures in liquid and on solid media were normally grown in the light at 30°C as described previously (5). Certain liquid cultures were grown similarly, but at 40°C in a rotatory shaker (125 rpm) illuminated by cool-white fluorescent lamps at an intensity of 480 ergs/cm^2 per s.

Phage suspensions. Plaque-forming units (PFU) per milliliter of suspension were assayed by overlaying 30 ml of solid medium in petri plates. The overlay was prepared by mixing 3 ml of a 1% solution of molten agar, at 51°C, with 3 ml of cell suspension containing 4×10^7 to 5×10^7 cells. To this mixture 0.1 ml of phage suspension was added. In the case of *Anabaena* 7120, the agar overlay contained 5 mM $NaNO_3$ and 5 mM KNO_3. Inclusion of nitrate in the latter case led to higher and more reproducible titers of phage, presumably because the phage adsorb better to nitrate-grown than to N_2-grown *Anabaena* 7120 (results not shown). Inclusion of nitrate in agar overlays of *A. variabilis* had no effect upon the efficiency of plaque formation. Plates were incubated at 30°C with illumination from

cool-white fluorescent lamps at an intensity of 350 ergs/cm^2 per s. Plaques could be counted after 3 and 5 days when the hosts were *Anabaena* 7120 and *A. variabilis*, respectively.

Phage N-1 grown in *Anabaena* 7120, the phage obtained from R. Haselkorn, was cloned three successive times on this cyanobacterium on agar. The phage from a single plaque were then suspended in liquid medium and titrated. Lysates were prepared by infecting cultures of this cyanobacterium containing 5×10^6 cells per ml at a multiplicity of infection (PFU/cell) of 0.1 to 0.5. Phage grown on *Anabaena* 7120 will be denoted N-1·7120.

Because of the low infectivity of N-1 on *A. variabilis*, lysates of this host were more difficult to obtain. Isolated plaques were obtained by infecting lawns of *A. variabilis* with N-1·7120 at a multiplicity of infection greater than 5. Phage from a single plaque were streaked with a loop to a fresh lawn; single plaques appeared along the streak. The phage were serially subcultured from single plaques three additional times. A single plaque was then excised, and the phage were extracted by incubating the excised material overnight in 2 ml of liquid medium. These phage were used to obtain confluent lysis of 2×10^7 cells in an overlay. Phage extracted by gently squeezing the liquid from the agar through three layers of Miracloth (Chicopee Mills Inc., Milltown, N.J.) were designated N-1·Av. Lysates of *Anabaena* 7120 by N-1·7120 and of *A. variabilis* by N-1·Av attained titers, as assayed on *Anabaena* 7120, of approximately 10^8 PFU/ml.

Phage adsorption experiments. Exponentially growing cultures of the cyanobacteria were harvested at a culture density of 5×10^6 to 1×10^7 cells per ml. The cells were suspended in 0.5 ml of phage suspension (5×10^5 PFU/ml) to a final cell density of 5×10^8 cells per ml. At various times thereafter, 0.05 ml was removed and diluted into 10 ml of medium. The cells were removed by centrifugation at $1,000 \times g$ for 5 min, and 0.1 ml of the supernatant fluid was used in the plaque assay with cells of *Anabaena* 7120.

Heat treatment of *A. variabilis*. Cells were harvested and suspended in fresh medium. A 0.1-ml amount of suspension was added to 9.9 ml of liquid medium prewarmed to 51°C. At various times, portions were removed and diluted fivefold with medium precooled to 4°C.

RESULTS

Isolation of a variant strain of *A. variabilis* that replicates phage N-1 with high relative efficiency. The relative efficiency of plaque formation by phage N-1·7120 on *A. variabilis*, compared here and elsewhere with the efficiency of plaque formation on *Anabaena* 7120, was low and decreased as the amount of *A. variabilis* inoculated was increased. That relative efficiency was approximately 10^{-6} with an inoculum of 5×10^6 cells of *A. variabilis* per lawn (data not shown), and 1.2×10^{-7} with an inoculum of 5×10^7 cells (Table 1).

For reasons described below, it was desirable for certain experiments to grow *A. variabilis* at 40°C rather than at room temperature (22 to 24°C). Both N-1·7120 and N-1·Av formed more plaques on the cells which had been grown at 40°C. From one such culture grown at 40°C, a strain of *A. variabilis* (denoted *A. variabilis* strain FD) was isolated by three sequential single-colony isolations. The efficiencies of plaque formation by N-1·7120 and by N-1·Av were increased 470- and 420-fold, respectively, on *A. variabilis* strain FD grown at 40°C compared with wild-type *A. variabilis* grown at room temperature (Table 1).

These increases in the efficiency of plaque formation were at least partially due to increased adsorption of phage N-1, because *A. variabilis* strain FD grown at 40°C did adsorb phage N-1·Av much better than did wild type *A. variabilis* grown at room temperature (Fig. 1). The kinetics of adsorption of phage N-1·Av to strain FD are essentially identical to those for phage N-1·Av to *Anabaena* strain 7120. However, the greater

TABLE 1. *Relative efficiencies of plaque formation by phage N-1·Av, N-1·7120, and N-1·Av·7120 on various hosts*[a]

Host for plaque assay	Growth temp	Relative efficiency[a]		
		N-1·Av	N-1·7120	N-1·Av·7120
Anabaena strain 7120	22–24°C	1	1	1
Wild-type *A. variabilis*	22–24°C	9.1×10^{-4}	1.2×10^{-7}	1.0×10^{-6}
A. variabilis strain FD	40°C	0.38	5.5×10^{-5}	3.4×10^{-4}
Wild-type *A. variabilis*	22–24°C, then 3 generations at 40°C	0.009	ND	ND
A. variabilis strain FD	40°C, then 3 generations at 22–24°C	0.11	ND	ND
A. variabilis strain FD	40°C, then >20 generations at 22–24°C	0.14	ND	ND

[a] Numbers are calculated on the basis of a relative efficiency of plaque formation equal to unity for *Anabaena* strain 7120. ND, Not determined.

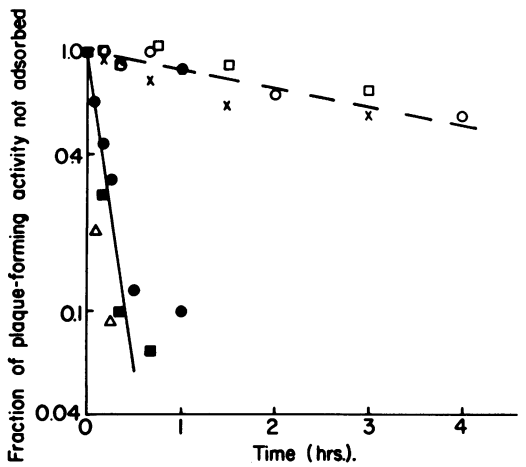

FIG. 1. *Adsorption of phage N-1·Av to Anabaena 7120 and to wild-type A. variabilis and A. variabilis strain FD. Adsorption experiments were performed as described in the text with cells which were grown as follows: wild-type A. variabilis grown at room temperature for more than 10 generations* (○), *and then at 40°C for 3 generations* (●); *A. variabilis strain FD grown at 40°C for more than 10 generations* (■) *and then at room temperature for 3 generations* (□); *A. variabilis strain FD grown for more than 20 generations at room temperature (X); and Anabaena strain 7120* (△) *grown at room temperature in the presence of nitrate.*

rate of adsorption by strain FD appears to account for only part of the enhanced efficiency of plaque formation by that strain. When wild-type *A. variabilis,* grown at room temperature, was transferred to 40°C, it attained within three generations essentially the same rate of adsorption of N-1·Av as shown by strain FD. However, it formed plaques with an efficiency only about 10-fold greater than the efficiency of plaque formation by wild-type *A. variabilis* grown at room temperature, i.e., approximately 42-fold less than the efficiency of plaque formation by *A. variabilis* strain FD (Table 1).

In a reciprocal experiment, *A. variabilis* strain FD was grown for more than 20 generations at room temperature. Its rate of adsorption of N-1·Av was thereby decreased to a rate essentially the same as the rate of adsorption by wild-type *A. variabilis* grown at room temperature (Fig. 1); however, its efficiency of plaque formation with N-1·Av was decreased only about 2.8-fold, to a level that was still 150-fold more efficient than was plaque formation by wild-type *A. variabilis* grown at room temperature (Table 1). In fact, the rate of adsorption (data not shown) and the efficiency of plaque formation (Table 1) by *A. variabilis* strain FD were changed similarly after only three generations of growth at room temperature. Adsorption curves essentially identical to those shown in Fig. 1 were obtained with phage N-1·7120. Efficiencies of plaque formation by N-1·7120 were not determined on temperature-shifted *A. variabilis.*

After 20 generations at room temperature, a suspension of *A. variabilis* strain FD was streaked at 25°C. Five of the clones which arose were transferred to liquid culture at room temperature. All five cultures exhibited efficiencies of plaque formation with N-1·Av that were 150- to 350-fold greater than the efficiency of plaque formation by wild-type *A. variabilis* (cf. 120-fold after three generations at room temperature, Table 1).

Influence of the host in which N-1 was grown. Phage N-1·7120 and N-1·Av formed plaques on wild-type *A. variabilis,* grown at room temperature, with efficiencies far less than their efficiency of plaque formation on *Anabaena* 7120 (Table 1). These two types of phage formed plaques on *A. variabilis* strain FD, grown at 40°C, with efficiencies in between those on wild-type *A. variabilis* and on *Anabaena* 7120. Regardless of which strain of *A. variabilis* was used, N-1·Av formed plaques with a relative efficiency approximately 7×10^3-fold greater than did N-1·7120 (compared with the efficiency of formation of plaques by that same phage on *Anabaena* 7120).

When phage N-1·Av formed plaques in lawns of *Anabaena* 7120, the phage which were produced (denoted N-1·Av·7120) formed plaques on wild-type *A. variabilis* and on *A. variabilis* strain FD at relative efficiencies much more similar to the corresponding values obtained for phage N-1·7120 than to those for phage N-1·Av on the same hosts (Table 1).

Effect of heat treatment of *A. variabilis* on the efficiency of formation of plaques by N-1·7120. The efficiency with which N-1·7120 formed plaques on *A. variabilis* strain FD was increased up to 146 times by heating the latter organism to 51°C for various periods of time (Table 2). Similar increases in the efficiency of plaque formation upon heating of *A. variabilis* strain FD were observed when the cells were exposed to phage N-1·Av·7120, but little or no such increase was observed with phage N-1·Av. An effect of heating was also observed with wild-type *A. variabilis* grown at room temperature, but these cells were not routinely used in these experiments because they survived the heat treatment poorly.

DISCUSSION

Aside from an effect possibly attributable to restriction endonucleases (see below), the efficiency of formation of plaques by both N-1·7120 and N-1·Av on *A. variabilis* could be in-

TABLE 2. *Effect on the relative efficiencies of plaque formation by phage N-1·Av, N-1·7120, and N-1·Av·7120 of heat treatment of A. variabilis strain FD*[a]

Duration of heating at 51°C (min)	Relative efficiency[a]		
	N-1·Av	N-1·7120	N-1·Av·7120
0	1	1	1
1.0	2	3	1
2.5	2	8	5
5.0	1	146	73
7.5	0.2	35	17
10.0	0.06	46	5

[a] Numbers are relative to each other only in vertical columns.

creased in two ways. First, the rate of adsorption was increased by growing the cells at 40°C for three or more generations. Because heterotrophic bacteria are known to incorporate different lipids in their envelope layers in response to changes in the temperature of growth (7), it is possible that changes in lipid composition occur in *A. variabilis* and are associated with the increased rate of adsorption. Second, use of a variant strain isolated from a culture of *A. variabilis* that had been grown for more than 30 generations at 40°C resulted in about a 45-fold increase in plating efficiency relative to use of the wild-type strain. The enhanced efficiency of formation of plaques by strain FD, although decreased about threefold after three generations of growth at room temperature, was not further reduced after prolonged growth of the strain at room temperature. Whether *A. variabilis* strain FD is a mutant or some sort of epigenetic variant is not known. Because the increase in efficiency of formation of plaques by N-1·Av on this strain, relative to the wild-type strain of *A. variabilis*, cannot be attributed to changes in adsorption rates, it appears that *A. variabilis* strain FD is changed in some manner that increases the efficiency of replication of adsorbed phage.

At some frequency, phage DNA from a foreign host is modified before it can be degraded by restriction endonucleases (3, 10, 11). Because of the modification, the DNA of the progeny phage is not subject to degradation upon reinfection of the same new host. DNA restriction endonucleases are often labile to heating; they can sometimes be inactivated partially or completely by heat treatments that do not kill the cells (12). Thus, if a phage is subject to DNA restriction, the efficiency with which it forms plaques can often be increased greatly by heating of the cells which are to receive the phage. It has been reported that a different strain of *Anabaena*, a strain which forms neither heterocysts nor spores and has therefore only provisionally been called *A. variabilis*, has two restriction enzymes (8).

It cannot be determined from the data presented whether *Anabaena* strain 7120 contains any DNA restriction-modification systems. However, the following evidence suggests that a restriction-modification system may be operative in our strain of *A. variabilis* which is not operative in *Anabaena* strain 7120. (i) Compared with its efficiency of plating on *Anabaena* strain 7120, N-1·Av forms plaques approximately 7×10^3-fold more efficiently on *A. variabilis* than does N-1·7120. (ii) N-1·Av·7120 forms plaques with an efficiency much closer to the efficiency of N-1·7120 than of N-1·Av. (iii) The efficiency of formation of plaques in *A. variabilis* by N-1·7120 is increased by prior heating of the cells to 51°C. Heating of *A. variabilis* to 51°C may result in only a partial inactivation of the putative restriction endonuclease activity affecting N-1·7120 because the titer of phage N-1·7120 on cells of *A. variabilis* strain FD treated in this way is still reduced relative to the titer on *Anabaena* 7120; much higher relative titers are obtained with N-1·Av.

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