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Evidence for Lysogeny and Viral Resistance in the Cyanobacterium *Phormidium uncinatum*

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Abstract. Cyanophage LPP-1-induced lysogens and a resistant mutant of the cyanobacterium *Phormidium uncinatum* were isolated and characterized. In lysogens, spontaneous lysis occurred and increased with the growth of the host cyanobacterium. The virus-liberating property of the lysogens was not lost with the viricidal concentration of EDTA, and the titer obtained was $>3 \pm 10^3$ PFU ml^{-1}. Heat and UV treatment of lysogens failed to induce lysis, but mitomycin C induced lysis by fivefold. The adsorption rate of the virus on the lysogens was slower than on the sensitive parent host. The resistant mutant lost its virus-adsorbing property.

Cyanophages are capable of entering into a lysogenic relationship with their hosts [5, 7–9, 13, 15]. However, induction of the lytic state under lysogenic conditions appears to be more difficult with cyanophages than with bacteriophages [3, 13, 15]. Chloramphenicol-treated cells, infected with the temperate viruses LPP-1D and LPP-2, resulted in 100% lysogeny [3]. A temperate virus, LPP 2–SP I, capable of entering into lysogenic state in *Plectonema boryanum* was also isolated [11]. Furthermore, lysogeny is reported in P-2 virus as a turbid plaque-forming isolate in *Pl. boryanum*, by AR-1 virus in *Anabaenopsis raciborskii* [17, 18], and by N-1 virus in *Nostoc muscorum* [12]. However, there is no previous report on lysogeny and its induction in a bloom-forming cyanobacterium *Phormidium uncinatum* by the virus LPP-1. The present article deals with the isolation and growth pattern of lysogens of cyanobacterium *Ph. uncinatum*. Attempts were also made to induce lysis in lysogens after various physicochemical treatments.

Materials and Methods

The axenic culture of *Phormidium uncinatum* (IU 1462/7) and cyanophage LPP-1 used in the present investigation were obtained through the courtesy of R. S. Safferman (National Environmental Center, Cincinnati, OH). The host cyanobacterium was maintained on agar slants enriched with modified Chu no. 10 medium [6] with trace elements [2]. From the agar slants, *Ph. uncinatum* (CU 1462/7) filaments were transferred under sterile conditions to liquid medium. The cultures were always maintained at 26° ± 2°C in a culture room and illuminated with cool white fluorescent light at an intensity of 480 erg/cm^2/s for 10 h/day. The host cyanobacterium was subcultured at 15-day intervals by transferring 5-ml aliquots of an actively growing culture to 200 ml fresh medium and shaking it twice daily by hand to keep it in an exponential condition for the experiments.

LPP-1 cyanophage was obtained for the experiments by infecting batch cultures of *Ph. uncinatum* (6.5×10^6 cells ml^{-1}) with the phage at a multiplicity of infection of 0.1. After lysis, the lysates were centrifuged for 10 min at 10,000 *g* to remove cell debris. The virus concentration was determined throughout the experiments by using the plaque assay technique [14]. Aliquots of 0.5 ml diluted lysate were thoroughly mixed with 2 ml of concentrated liquid cyanobacterial filaments and 2.5 ml of melted nutrient agar (medium with 1% agar) and plated on agar plates having a base of 0.8 agar-containing medium (30 ml in 100 × 17-mm Petri dishes). The inverted plates were counted from the resulting plaque counts after four days of incubation. The clonal population of the virus was raised from a single plaque isolate and lysates were always filtered through a sintered glass filter (2.5 cm GF/C; Whatman, England) before being used in experiments.

Exponentially grown cultures of parent host and lysogenic and virus-resistant isolates were placed in fresh sterile medium after two washings at almost equal cell densities. The cell concentration and input multiplicity (IM) of adsorption mixtures as shown in Table 1 were inoculated in each of the tubes and thoroughly mixed; 5-ml portions of adsorption mixtures were placed in sterile tubes and incubated, unshaken, in the culture room. An aliquot of 0.1-ml samples was pipetted out at intervals of 15 min and diluted to 10 ml with sterile growth medium for measuring the extent of adsorption. After thorough shaking, the dilutions were centrifuged at 5000 *g* for 5 min and 0.5 ml supernatant was mixed with the host cyanobacterium and plated by the double-agar layer technique for plaque assay [14]. The adsorption rate constant K was calculated using the formula $K = 2.3/(B)t \times \log_{10}$

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Table 1. Cyanophage LPP-1 adsorption rate on parent, lysogenic, and resistant mutants

Cyanobacterial strain	Cell concentration $\times 10^6$ ml^{-1}	IM (PFU ml^{-1} $\times 10^{-3}$)	Adsorption rate (ml/min) $\times 10^{-10}$
Parent	3.6	2.5	8.5
Lysogen 1	3.8	2.5	4.6
Lysogen 2	4.0	2.4	4.1
Virus resistant	4.0	2.5	No adsorption

P_0/P_t [1], where P_0 is the virus assay at time zero, P_t is the virus not adsorbed at time t in minutes, and B is the concentration of host cells as number of cells ml^{-1}.

Growth was measured under standard conditions in 10 or 50 ml of medium contained in 18 × 150-mm test tubes or 150-ml Erlenmeyer flasks plugged with cotton. At two-day intervals, the optical density of the cultures was measured using a Spectronic-20 spectrocolorimeter at 665 nm. The growth was also measured in terms of number of cells ml^{-1} by using a Feinoptic hemocytometer.

Exponentially grown cultures of one of the lysogens were centrifuged at 5000 *g* for 5 min, washed twice with sterile growth medium, and suspended in a fresh sterile culture medium. An aliquot of 30 ml containing 6.5 × 10^6 cells ml^{-1} was incubated at 25°, 30°, 35°, 40°, 45°, and 50°C in an ultrathermostat-type water bath. After a 2-h interval, aliquots of 2.5 ml were removed and diluted to 10 ml in sterile growth medium at 25°C and 0.1 ml supernatant of the dilution flasks was plated in triplicate for the plaque assay. In another set of experiments, the heat-treated cultures were washed by repeated centrifugation, and pellets resuspended in 30 ml fresh sterile growth medium and incubated for growth under light. At two-day intervals, 0.5-ml portions of the supernatants were plated in triplicate for plaque assay after appropriate dilution.

Treatment of lysogens

UV irradiation. The lysogenic clones grown in log phase were washed by two cycles of centrifugation and suspended in fresh growth medium to a cell density of about 3 × 10^7 cells ml^{-1}. A 10-ml cell suspension was transferred to a sterile Petri dish and exposed to ultraviolet (UV) light from a 15-W General Electric germicidal lamp with most of its energy at 2537 A in open Petri dishes at a distance of 22.5 cm (85 erg/mm^2/s) from the lamp. The suspension was stirred constantly on a magnetic stirrer during the irradiation procedure. Aliquots of 0.1 ml were periodically withdrawn, diluted, and plated on the sensitive host. The plates were incubated in the dark for 15 h and then transferred to normal growth conditions. All operations were conducted in dim yellow or red light to avoid photoreactivation.

Mitomycin C. A solution of mitomycin C (Sigma Chemicals) was added to the actively growing cultures of lysogenic clones to a final concentration of 0.1–2.0 μg ml^{-1}. After 30 min incubation at room temperature in the dark, cells were separated by centrifugation, washed thrice with growth medium to remove traces of mitomycin C, and resuspended in a fresh growth medium to a cell density of 10^6 cells ml^{-1} and incubated under standard growth conditions. At 2-h intervals, small portions of the culture were centrifuged and the supernatant was assayed for cyanophage titer. Prophage induction rate constant, expressed as the specific constant of virus production (kv) under a set of defined conditions, was calculated by the formula $kv \cdot t = \log P_t - \log P_0$, where P_0 is the initial virus titer and P_t the virus titer at time t.

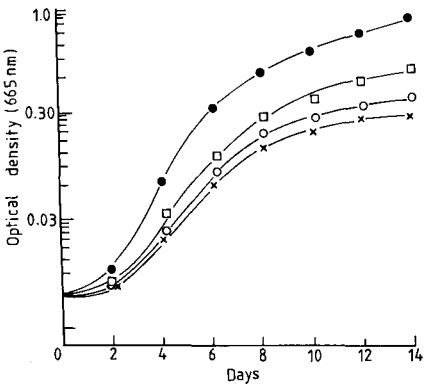

Fig. 1. Growth measured in terms of optical density of cultures of wild parent, lysogens, and virus-resistant strains of *Phormidium uncinatum:* ●, wild; □, Lys 1; ○, Lys 2; and ×, resistant.

Free viral inactivation by EDTA treatment. A disodium salt of ethylenediamine tetraacetic acid (EDTA) was added to the suitable diluted lysogens to obtain a final concentration of 100 μg ml^{-1}. Aliquots of 0.1 ml were withdrawn at regular intervals, rapidly diluted, and plated.

Results

Isolation of lysogens and virus-resistant mutant. The lysogenic isolates together with the virus-resistant mutant of the cyanobacterium *Phormidium uncinatum* were isolated with an average mutation frequency of 2.2 × 10^{-5} from liquid cultures as well as from agar plates. Some of the cells of the filaments or short trichome were left unlysed in the titer or in plaques and grew. These were purified by at least three alternating growth cycles in liquid and solid media.

The lysogenic and resistant clones were treated with 100 μg ml^{-1} EDTA (pH 7.2) for 1 h, which completely inactivated free virus; these were multiplied and tested further for plaque. The viral titer was more than 10^3 PFU ml^{-1} in the supernatant of lysogenic cultures whereas the same was lost in the virus-resistant clone. The plaques were produced around the lysogenic filaments in the lawn of sensitive cyanobacterium and appeared continuously at different times, confirming that the virus is produced during the growth period of the lysogenic filaments.

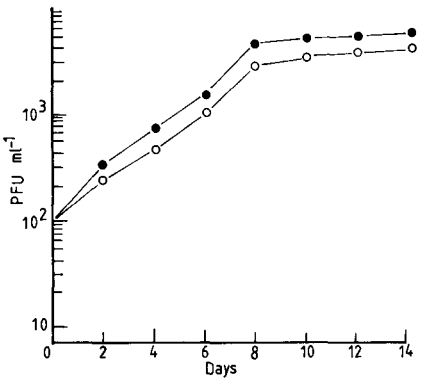

Fig. 2. Liberation of free virus particles during the growth of lysogenic cultures of *Phormidium uncinatum:* ○, Lys 1; and ●, Lys 2.

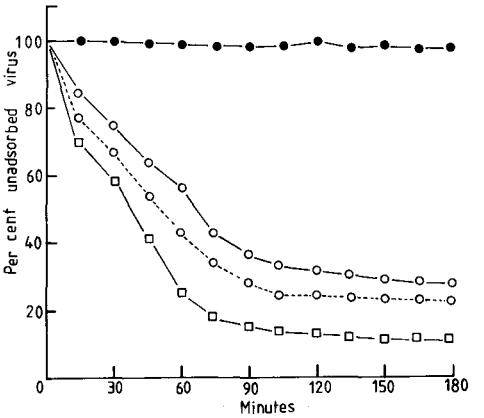

Fig. 3. Adsorption of LPP-1 virus to parent wild, lysogens, and virus-resistant strains of *Phormidium uncinatum:* □—□, wild; ○—○, Lys 1; ○---○, Lys 2; and ●—●, resistant.

Growth properties. Some of the purified clones (Lys clone 1, Lys clone 2, and virus Res clone 1) were selected for further investigation. Growth experiments revealed (Fig. 1) that the lysogens and virus-resistant clone grew slower compared with the wild strain, and that the rate of viral release was maximum during the exponential growth phase. The final viral titer obtained in the cultures of the two lysogens varied between 2.8×10^3 and 4.4×10^3 PFU ml^{-1} (Fig. 2), respectively. To test whether every cell in the lysogen cultures is capable of liberating phage, isolated colonies were inoculated onto parent host *Ph. uncinatum* lawn and tested for phage production. Over 78% of the colonies were found to produce phage as judged by a halo of lysis surrounding the lysogenic colonies. The ability of lysogen trichomes to produce phage was not completely inactivated by EDTA treatment.

Viral adsorption to lysogenic and virus-resistant isolates. An adsorption experiment conducted with two different lysogens revealed slow adsorption of virus when compared with the sensitive parent host. There was no adsorption on virus-resistant mutant (Fig. 3). Further, it is evident from Table 1 that the rate of virus adsorption to the lysogenic isolates was less than that of the sensitive parent host.

Prophage induction by heat treatment. The induction pattern obtained by temperature treatments to the lysogens and resistant mutant is shown in Table 2. It is evident that the lysogen was heat insensitive. The phage-liberating property of the lysogens was completely inactivated at 50°C.

Table 2. Effect of heat treatment on the lysogen of *Phormidium uncinatum*

Temperature (°C)	Viral titer at the end of 14 h (PFU/ml)	Virus yield	
		% Increase	% Reduction
20	2.65×10^3	0	0
25	3.0×10^3	11.6	0
30	4.2×10^3	36.9	0
35	2.8×10^3	5.35	0
40	1.8×10^3	0	32.07
45	2.2×10^2	0	91.69
50	0	0	100.0

Initial viral titer PFU/ml.

Prophage induction by UV irradiation. Attempts were unsuccessful at inducing lysis with UV light. Exposure to UV light resulted, at most, in a 2.5-fold increase in the infective center.

Prophage induction by mitomycin C. Mitomycin-C induction experiments were performed with the exponentially grown cultures of the lysogenic and virus-resistant clones with different concentrations; 0.6 μg ml^{-1} mitomycin C was found to be the optimum concentration for inducing lysis at 10^6 cells ml^{-1} density. Further, it was observed that the viral titer was greater in mitomycin-C-treated cultures than in the control. The titer of virus at hour zero served as control in all treatments. The induction was evident in the 2-h treatment with 0.6 μg ml^{-1} mitomycin-C concentration, where a sharp increase in virus titer was observed; it continued to increase

Table 3. Rate of prophage induction in lysogenic and virus-resistant mutants of *Phormidium uncinatum* by mitomycin C

Cyanobacterial mutant	Cell concentration $\times 10^6$ ml^{-1}	kv with MC/h			
		0.1 μg/ml	0.3 μg/ml	0.6 μg/ml	1 μg/ml
Lysogen 1	3.6	0.25	0.46	0.75	0.65
Lysogen 2	3.6	0.22	0.52	0.82	0.52
Resistant	3.6	0	0	0	0

for up to 4 h and then declined. Plaques were absent in all the concentrations in cultures of virus-resistant clone. There was no increase in viral titer in cultures of untreated lysogenic clones with mitomycin C within 6 h.

Discussion

The active virus liberated in the medium during the growth period of the lysogens because of the continuous lysis of few lysogenic cells as a result of the accidental activation of the induction machinery that appears to be coupled with the metabolic state of the cells (Table 3). Our attempts to demonstrate lysogeny by induction of the lytic cycle with both UV irradiation and heat treatment were unsuccessful. However, there is a report that the temperature-sensitive lysogenized mutant of *Plectonema* grew stably at 26°C, but when the lysogenized cells were grown at 40°C, induction took place with the production of progeny phage [13]. On the contrary, the present system appears to be nonthermoinducible. The induction of lysis involves the inactivation and/or conformational changes of the immune repressor protein in lysogens [10]; the noninducible nature of the lysogen after heat/UV treatment indicated that the immune repressor protein is not likely to be thermoinactivated or undergo conformational changes by heat or UV shock. Instead, the lysogens lose the ability to liberate virus particles on long incubation at high temperature like N-1 cyanophage-induced lysogeny in *Nostoc muscorum* [12].

Mitomycin C is known to induce lysis in lysogens of *Plectonema* and *Anabaenopsis* [18], *Pl. boryanum* [4, 16], and in *N. muscorum* [12]. However, in the LPP 2–SP I-induced lysogen of *Pl. boryanum*, the lysis could not be induced [11]. Increase in viral titer after mitomycin-C treatment in the present system as a function of time suggested the induction of lysogeny as in any other system [4, 12, 16, 18].

The above observations have confirmed lysogeny in the bloom-forming cyanobacterium *Ph. uncinatum* due to LPP-1 virus-like bacteriophage lysogeny and suggested that lysogeny might permit survival of both the virus and cyanobacterial host at a low population density where environmental conditions were not ideal for either of them. Later, when conditions become ideal, virus particles may be released by either spontaneous or chemical induction.

Conclusions

1) Cyanophage LPP-1 induced lysogeny and resistance in the cyanobacterium *Phormidium uncinatum* spontaneously.
2) Temperature and UV treatments of lysogens failed to induce lysis.
3) Mitomycin C (0.6 μg ml^{-1}) induced lysis in the lysogen with an optimum titer in 4 h.

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