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Diel Infection of a Cyanobacterium by a Contractile Bacteriophage

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Light was found to strongly influence the infection of a freshwater cyanobacterium (*Synechococcus elongatus* PCC 7942) by a contractile DNA phage named AS-1. Phage progeny production was correlated with the amount of light in the laboratory and occurred in a diel pattern under natural light. At least one effect of light on AS-1 infection is at the level of adsorption.

Viruses are present in marine and freshwater systems at more than 10^7 particles per milliliter (4, 18). This large number argues strongly for an important role in global biological and geochemical processes, including the recycling of nutrient stores due to virus-induced lysis (4, 6, 13, 20). In addition, phylogenetic analysis has revealed that viruses in aquatic ecosystems are genetically diverse, suggesting that they are under significant selection pressure, perhaps in response to host defense systems and/or variations in the environment (22). Understanding how environmental factors and/or host genetics affect the success of viral infection is therefore relevant to the understanding of aquatic ecosystems.

A previously characterized contractile DNA phage of *Synechococcus elongatus* named AS-1 is a member of the family *Myoviridae*, which includes the well-characterized T-even phages (11, 14–16) (Fig. 1A). AS-1 infects the well-characterized freshwater obligate phototroph *Synechococcus elongatus* PCC 7942 (14). A key feature of *S. elongatus* biology is that its gene expression is regulated by an intrinsic ~24-h circadian clock (5, 7, 8). The products of three key genes, *kaiA*, *kaiB*, and *kaiC*, are required for maintaining the normal gene expression rhythm (7, 8). Deletion of any of these three genes will result in a loss of rhythmicity but will not significantly affect growth rates (5). We were initially interested in examining whether mutations in circadian rhythm regulators of *S. elongatus* would affect AS-1 infection. We found that deletions in the genes that regulate *S. elongatus* circadian rhythm had only minor effects on AS-1 progeny production, whereas light had a profound influence on the AS-1 infection process.

MATERIALS AND METHODS

S. elongatus strain AMC541 is the parental strain for the circadian mutants. Strains AMC702, AMC703, and AMC704 have deletions in the *kaiA*, *kaiB*, and *kaiC* genes, respectively. Additional genetic information concerning the strains used can be found in the report of Ditty et al. (5). Cultures were usually grown under constant illumination at 45 μmol photons m^{-2} s^{-1} in a modified BG-11 medium (2). Light intensity was measured with a LiCor luminometer. The original preparation of AS-1 was obtained from the American Type Culture Collection. A stock of at least 10^9 phage per ml was prepared by infecting *S. elongatus* grown in the presence of light for more than 18 h, followed by two clarifications of the supernatant of the culture lysate by centrifugation. The clarified lysate was stored at 4°C with a drop of chloroform. As previously determined by Safferman et al. (14), the lysate was stable for a minimum of several weeks at 4°C.

Phage titers were determined by a plaque assay that used BG-11 agar (1.5% agar) overlaid with 4 ml of 0.75% agar in BG-11 medium, 0.3 ml of log-phase *S. elongatus* AMC541, and 5 to 200 μl of diluted phage lysate. Entrainment of cultures to synchronize their circadian clocks was performed by completely wrapping an early-log-phase culture of *S. elongatus* with aluminum foil for 6 h. Other incubation conditions (shaking speed, temperature, etc.) were not changed.

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RESULTS AND DISCUSSION

Circadian rhythm mutants and AS-1 infection. To examine the effects of mutations in the regulators of the circadian clock, we entrained early-log-phase cultures of wild-type *S. elongatus* and mutants with mutations in the *kaiA*, *kaiB*, and *kaiC* genes (5, 8, 10, 12). Upon release from a 6-h entrainment period, aliquots of the cultures were taken over time and plated in plaque assays with approximately 50 PFU of AS-1. The plates were incubated in continuous light until the number of plaques was enumerated approximately 2 days later. AS-1 plaques were generally uniform and were easily distinguished from the background (Fig. 1B). The number of plaques produced by the mutant deleted for *kaiB* was not significantly different from that formed on the parental strain, AMC541 (Fig. 1C). Similar results were obtained with cultures containing mutations in the *kaiA* and *kaiC* genes and with unentrained cultures that were grown under continuous light of ~45 μmol m^{-2} s^{-1} (data not shown). These results suggest that deletions in the *kai* genes do not significantly influence AS-1 infection.

To examine further the effects of deletion of *kaiA* and *kaiB* on AS-1 infection, we quantified progeny phage production in cultures of *S. elongatus* inoculated with 3×10^6 PFU of AS-1 and incubated overnight for 18 h (sufficient time to allow approximately two cycles of infection). Each mutant and its maternal strain were grown with light at both 3 and 45 μmol m^{-2} s^{-1}. Infections of Δ*kaiA* and Δ*kaiB* mutants and the maternal strain AMC541 all produced significant bursts of AS-1 at 45 μmol m^{-2} s^{-1}, and the final burst was more than 3 log units higher than the input (Fig. 1D). However, at 3 μmol m^{-2} s^{-1}, the burst size was only ninefold higher than the input amount of AS-1 for AMC541 and was not significantly different from input for the Δ*kaiA* and Δ*kaiB* mutants. While in-

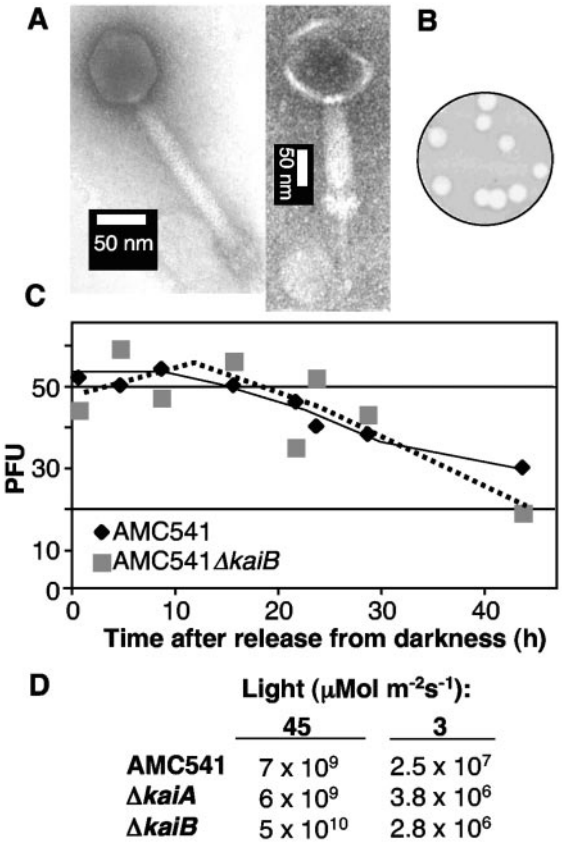

FIG. 1. Bacteriophage AS-1 and progeny phage production in *S. elongatus* PCC 7942. (A) Electron micrographs of AS-1, showing an intact particle (left) and an empty one (right) that has contracted its injection tubule. Viruses were negatively stained with uranyl acetate. (B) Demonstration of the plaques produced by AS-1. (C) Comparison of the abilities of AS-1 to form plaques on a mutant with a deletion in the *kaiB* gene (Δ*kaiB*) and on its parental strain, AMC541, over time. Approximately 50 PFU was added to the cultures after they were released from entrainment (time zero), and the results were enumerated approximately 2 days later. (D) Comparison of the number of progeny phage released by AMC541 and circadian clock regulatory mutants in the light or the dark. Progeny phage titers were determined 18 h after the introduction of AS-1.

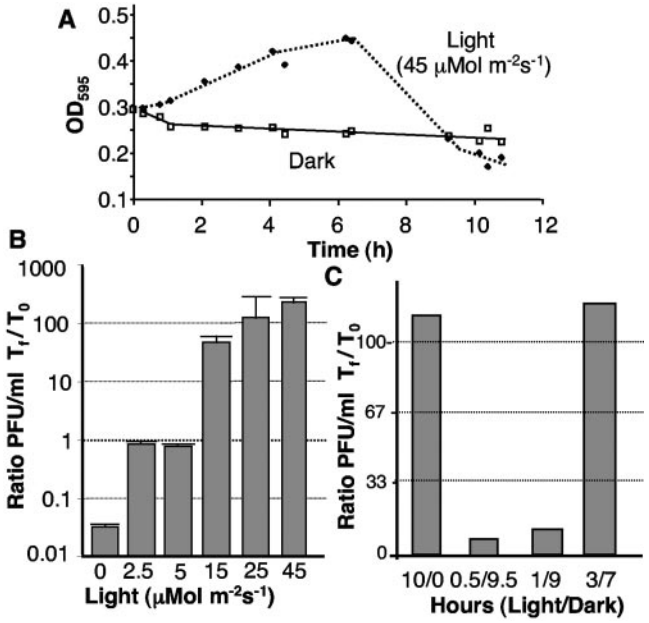

FIG. 2. Light affects lysis and progeny formation by AS-1. (A) Cell lysis curve for *S. elongatus* AMC541 infected with AS-1 (MOI, 5) under high and low light conditions. The optical density of the cell culture at 595 nm (OD_{595}) was determined on a ThermoSpectronic instrument by using the medium as a blank for each measurement. (B) PFU of phage produced by cultures grown at various intensities of light. The final titer (T_f) was divided by the titer in the initial inoculum (T_0) to determine the phage burst size (T_f/T_0). Each light condition was tested in three independent cultures, and the error bar indicates 1 standard deviation. (C) Effect of incubation time in the light on the ability to complete the infection process in the dark. Numbers to the left and right of the slash indicate the amount of time (in hours) for which a culture was grown in the light or the dark, respectively. Each condition was tested in two independent cultures with highly similar results.

fected AMC541 produced more phage than did the Δ*kaiA* and Δ*kaiB* mutants, we note that all AS-1 progeny production was severely reduced at low light. We interpret these results to indicate that the circadian regulatory proteins had only minor effects on AS-1 infection. Furthermore, we hypothesize that AS-1 infection of *S. elongatus* is correlated with light intensity, likely due to changes in the physiology of the host cell.

Light and AS-1 infection. To confirm that phage infection is dependent on light, we inoculated an early-log-phase culture of *S. elongatus* grown at 45 μmol m^{-2} s^{-1} with AS-1 at a multiplicity of infection (MOI) of 5. The culture was divided into two aliquots 30 min later. One was maintained at 45 μmol m^{-2} s^{-1}, and the second was covered with aluminum foil. The optical densities of the two cultures at 595 nm were monitored over time. Sampling of the covered culture was done in a darkened room. The covered culture stopped increasing in its optical density almost immediately and maintained a fairly constant density throughout the experiment. Even after 18 h, the cells retained their characteristic green color and showed no visible signs of lysis. In contrast, the culture grown in light increased in density for several hours, then lysed between 6.5 and 9.5 h. The timing of the lysis was consistent with previous reports of AS-1 infection (14, 15, 16). This result indicates that cells unable to photosynthesize will not support normal lysis by AS-1.

Next, we tested the effects of different light intensities on AS-1 progeny production. *S. elongatus* was grown at 45 μmol m^{-2} s^{-1}, infected with AS-1 for 1 h, and then divided into aliquots that were incubated at light intensities ranging from no detectable light to 45 μmol m^{-2} s^{-1}. The yield of phage titers after 10 h was quantified and normalized as a ratio of the input (Fig. 2B). Increased light intensity resulted in a corresponding increase in the production of progeny phage. These results provide further evidence that light directly influences the success of AS-1 infection.

Next, we examined whether *S. elongatus* infected during growth in light could complete the infection in the dark. One flask of *S. elongatus* grown at 45 μmol m^{-2} s^{-1} of light was infected with AS-1. Aliquots were then removed at 0.5, 1, and 3 h postinoculation and transferred to flasks covered with aluminum foil. The original sample and the aliquots were incubated for a total of 10 h, after which the burst size was deter-

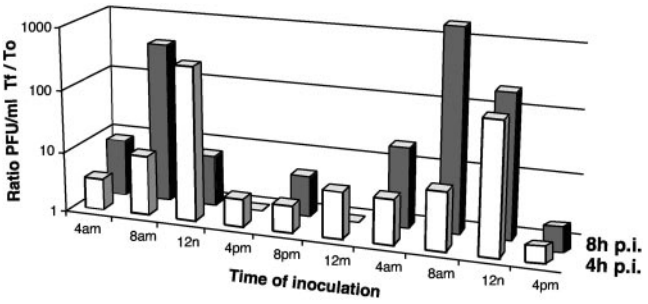

FIG. 3. Time course examining AS-1 progeny production in cultures exposed to natural light. The horizontal axis indicates the timing of the initial introduction of AS-1 phage (MOI, ca. 1). Aliquots of the culture were harvested at 4 and 8 h after inoculation and were then kept in the dark for 8 to 16 h to allow completion of that round of infection. The burst size is calculated from the PFU at the end of the experiment (T_f) divided by the PFU in the initial inoculum (T_0).

mined. The cultures transferred to darkness after 0.5 and 1.0 h were unable to produce a normal burst, while the culture incubated in light for 3 h released a significant amount of progeny phage (Fig. 2C). These results indicate that should AS-1 infection proceed for a critical period of time in light, the remainder of the infection process can take place while light is limited.

The amount of light required for successful AS-1 infection can be related to the progression of events during AS-1 infection (15, 16). AS-1 actively degrades the host DNA within 1 h after infection and will inhibit host protein synthesis 3 to 4 h after infection (16). AS-1 DNA could be detected from between 2.5 and 3 h until the end of the infection, with the packaging of phage particles taking place by 8 h. Interestingly, treatment of AS-1-infected cells with the RNA polymerase inhibitor rifampin before 2 h completely stopped AS-1 DNA synthesis, while treatment at 3 h after infection or later had only modest effects on DNA synthesis (16). These observations indicate that some changes in the infection process occur near the 3-h time point. Whether these changes can be related to the requirement for light in AS-1 infection remains to be determined.

Diel infection by AS-1. The correlation between light intensity and AS-1 burst size under laboratory conditions (Fig. 2B) suggests that AS-1 infection occurs in a diel pattern. To determine if this is true for AS-1 infection of *S. elongatus* under natural light, cultures grown by a west-facing window were inoculated every 4 h for a 36-h span. Aliquots were collected at 4 and 8 h after infection and were kept in the dark to allow time for the completion of one round of infection. Cultures inoculated during the day produced up to 2 log units more phage than those inoculated in the evening or night (Fig. 3). Phage production increased again at the start of the second day. Furthermore, of the cultures that were inoculated a few hours before dawn, those collected at 4 h, before there was significant light, produced fewer progeny than the same samples collected after 8 h, when there was more light. Thus, AS-1 infection appears to cycle in a diel manner.

Light and AS-1 adsorption. Virus infection can be functionally separated into specific stages, starting with adsorption. Cseke and Farkas (3) had reported that adsorption of AS-1 to

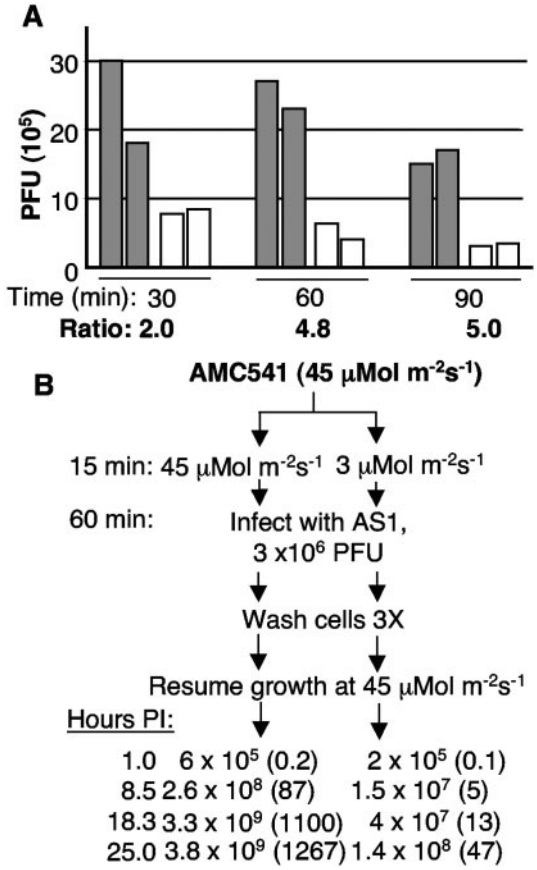

FIG. 4. Effect of light on the initiation of adsorption by AS-1. (A) Number of PFU present in the culture supernatant after 1 h of adsorption with cultures grown at 45 μmol m^{-2} s^{-1} or 3 μmol m^{-2} s^{-1}. Each bar represents an independent culture inoculated with AS-1 for the amount of time indicated on the horizontal axis. Gray bars, PFU for cultures incubated in the dark; white bars, PFU for cultures inoculated in the light. (B) Progeny phage production of cultures inoculated for 1 h at either 45 μmol m^{-2} s^{-1} or 3 μmol m^{-2} s^{-1} of light. After the initial inoculation, cells in the culture were washed to remove unbound phages and allowed to resume infection in the light at 45 μmol m^{-2} s^{-1}, as shown in the flow diagram. The results of the plaque-forming assays after resumption of infection (shown at the bottom) are expressed as PFU at the specified time postinfection (PI), with the ratio to the input PFU given in parentheses. The initial inoculum contained 3×10^6 PFU.

Anacystis nidulans is modulated by light and sodium ions. We examined whether light affects AS-1 adsorption of *S. elongatus* (Fig. 4). Cultures were grown in the presence of light and aliquoted, and a portion was transferred to light at 3 μmol m^{-2} s^{-1} for 15 min prior to the addition of AS-1 to both the light and dark cultures. Thirty, 60, and 90 min later, aliquots were removed and centrifuged for 1 min at 10,000 × *g* to remove the cells. The number of PFU present in the supernatant was quantified. Figure 4A shows the results from two independent experiments, each with two replicates that yielded consistent results (data not shown). Cultures at 3 μmol m^{-2} s^{-1} had approximately four- to sixfold-higher PFU in the supernatant fraction than cultures grown at 45 μmol m^{-2} s^{-1} light, indicating that light could affect AS-1 adsorption. There was a general decrease in the total number of PFU in both cultures

over time, suggesting a gradual loss of AS-1 infectivity in this experiment, possibly due to nonspecific binding by AS-1 or inactivation by UV light (19).

Since the number of unadsorbed AS-1 phage changed by only a few fold in the previous experiment, we examined whether AS-1 adsorption is affected by light by measuring progeny phage production. Cells were first grown with normal light (45 μmol m^{-2} s^{-1}) and then divided into two cultures, one of which was shifted to 3 μmol m^{-2} s^{-1} for 15 min prior to addition of AS-1 to both cultures. After a 60-min incubation period to allow for possible adsorption, the cells in the two cultures were pelleted by centrifugation and then washed three times with the culture medium in an attempt to remove unbound phage. The cells were then resuspended in the medium and grown in the light. Aliquots of the cells were removed at various times and analyzed for plaque formation (Fig. 4B). The samples removed immediately after the washes had low PFU, providing a starting point for quantification of phage production. Over the next 8 to 25 h, an abundance of progeny phage was released from the culture in which AS-1 could adsorb in the presence of normal light. In contrast, the culture that had the initial phage adsorption period at 3 μmol m^{-2} s^{-1} produced fewer progeny phage (Fig. 4B). These results confirm that light had an effect very early in the AS-1 infection process, most likely at the level of adsorption.

Summary and conclusions. Viruses need to be well tuned to the physiology of the host cell in order to maximize their fitness. *S. elongatus* gene expression is known to be controlled by a circadian clock, a process based on the diurnal light/dark cycle on Earth. We observed that infection by the contractile DNA phage AS-1 is more affected by the availability of light than by the circadian cycle.

Light appears to be intimately linked to the success of AS-1 infection. This is not surprising, since the physiology of *S. elongatus* and other obligately photosynthetic bacteria will change significantly in response to the diel cycle (see reference 1 for a review). However, the fact that AS-1 could produce a normal burst after infection was allowed to proceed in the light for 3 h suggests that AS-1 could overcome the normal restrictions after a certain stage in the infection process. Being able to complete the infection process in the dark could also provide an adaptive advantage to the phage, since progeny release could occur in the dark, thus decreasing exposure to UV light, which could deactivate phage (reference 19 and references within). An alternative interpretation of the results, which we consider less likely, is that light had a direct effect on phage proteins that could trigger the ability to infect *S. elongatus*.

Light is often limiting in aquatic systems, and the light/dark cycle is known to affect a number of cellular and ecological processes, including hormonal regulation in vertebrates (21), carbon partitioning in plants (23), and the migration of phytoplankton (9, 17). Should these results from AS-1 reflect the requirements of additional photosynthetic microbes, then the regeneration of nutrients in aquatic ecosystems mediated by viral lysis could be correlated with light intensity.

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