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Applied and Environmental Microbiology, Apr. 1997, p. 1551–1556 Vol. 63, No. 4
0099-2240/97/$04.00+0

Chlorella Virus PBCV-1 Encodes a Homolog of the Bacteriophage T4 UV Damage Repair Gene *denV*†

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Received 30 September 1996/Accepted 27 January 1997

The bacteriophage T4 *denV* gene encodes a well-characterized DNA repair enzyme involved in pyrimidine photodimer excision. We have discovered the first homologs of the *denV* gene in chlorella viruses, which are common in fresh water. This gene functions in vivo and also when cloned in *Escherichia coli*. Photodamaged virus DNA can also be photoreactivated by the host chlorella. Since the chlorella viruses are continually exposed to solar radiation in their native environments, two separate DNA repair systems, one that functions in the dark and one that functions in the light, significantly enhance their survival.

DNA exposure to UV radiation produces two major photoproducts, cyclobutane pyrimidine dimers and pyrimidine-pyrimidone 6-4 photoproducts (5). Surviving UV irradiation requires the capacity to repair both of these dipyrimidine lesions, and there are three repair processes for this purpose, as follows. (i) Photoreversal directly converts dimers and 6-4 photoproducts to monomers. (ii) Nucleotide excision repair removes both of the lesions and replaces them with the correct pyrimidines. (iii) Base excision repair initiates incision of the glycosyl bond of the 5′ pyrimidine of the dimers (5). Solar radiation damages DNA-containing viruses which are common in aquatic environments (6, 35). Virus survival in these environments depends on the ability to reverse DNA damage resulting from solar UV radiation. Typically, this UV damage is repaired by host enzymes (called host cell reactivation) (5). The bacteriophage T4 is unusual because it contains a gene, *denV*, which encodes a UV-specific DNA glycosylase-apyrimidine lyase enzyme, endonuclease V, that initiates repair of UV-formed pyrimidine dimers (5, 49). Endonuclease V cleaves the *N*-glycosyl bond of the 5′ thymidine in the thymine dimer site (pyrimidine dimer-DNA glycosylase) and then cleaves the phosphodiester bond at the abasic site (abasic lyase) (22, 24, 30, 46).

Endonuclease V homologs have not been reported even among other T-even bacteriophages, although several microorganisms, including *Escherichia coli* (2), *Micrococcus luteus* (8, 23), and *Saccharomyces cerevisiae* (9), contain endonuclease V-like enzyme activities. The sizes and amino acid sequences of the enzymes from these other organisms differ substantially from those of endonuclease V. We recently discovered an open reading frame (ORF) with 41% amino acid identity to T4 endonuclease V while sequencing the double-stranded DNA (dsDNA) genome of virus PBCV-1 (16).

PBCV-1 is the prototype of a family (*Phycodnaviridae*) of large (175- to 190-nm-diameter), polyhedral, plaque-forming viruses that replicate in certain unicellular, eukaryotic chlorella-like green algae present as endosymbionts in some isolates of *Paramecium bursaria* (37, 41). PBCV-1 virions contain at least 50 proteins and a lipid component located inside the outer glycoprotein capsid (32, 45). The PBCV-1 genome is a linear, nonpermuted, 330-kb dsDNA molecule with covalently closed hairpin ends (7, 26). Phycodnaviruses are ubiquitous in fresh water collected worldwide. Typically, the virus titer in fresh water is 1 to 100 PFU ml^{-1}, but titers as high as 40,000 PFU ml^{-1} have been obtained (39, 50). The virus titer is dynamic and exhibits seasonal fluctuations (43, 48).

We report here that the PBCV-1-encoded endonuclease V homolog can initiate pyrimidine photodimer repair. Furthermore, chlorella host cells can photoreactivate UV-damaged PBCV-1 DNA. Since chlorella viruses are exposed to constant solar radiation, two separate DNA repair systems, one that functions in the dark and one that functions in the light, should significantly enhance their survival.

MATERIALS AND METHODS

Chlorella, viruses, and plasmids. The hosts for the chlorella viruses, *Chlorella* strain NC64A and *Chlorella* strain Pbi, were grown on MBBM medium (40) and FES medium (25), respectively. Procedures for producing, purifying, and plaquing virus PBCV-1 and the other chlorella viruses and the isolation of host and virus DNAs have been described previously (38, 40, 42). Three PBCV-1 DNA restriction fragments containing ORF A50L were subcloned from cosmid cYL5 (16) into the polylinker region of pBluescript KS(+) (Stratagene) as indicated below (see Fig. 1B). The resultant plasmids were transformed into *E. coli* AB2480 (*uvrA recA*), kindly provided by Mary Berlyn of the *E. coli* Genetic Stock Center at Yale University, and grown in LB medium (17) at 37°C. The PBCV-1 *A50L* gene probe used in hybridization experiments was amplified from cosmid cYL5 by PCR (21). Oligonucleotide primers complementary to the 5′ and 3′ ends of the gene (accession no. U42580) were designed to introduce *Bam*HI restriction sites at the translation initiation codon and immediately 3′ of the translation stop codon. The PCR product was cleaved with *Bam*HI and cloned into the pUC19 *Bam*HI site.

UV radiation source and measurement. Samples were irradiated with a 15-W General Electric G15T8 germicidal lamp, and doses were quantified with a UVX radiometer and a UV-25 probe (Ultraviolet Products). This lamp was operated for 100 h prior to conducting experiments to stabilize the UV output. The lamp was illuminated for 10 to 15 min before exposing samples. Lamp output was determined for each experiment and was typically 0.1 J m^{-2} s^{-1}. Dose was varied by changing the exposure time.

UV protection assays. (i) Complementation of *E. coli* AB2480 by the PBCV-1 *A50L* gene. The protocol was a modification of the methods used by Kokjohn and Miller (13) to quantify CFU. Cells were grown to the mid-log phase in LB medium at 37°C (50 Klett units at 660 nm), harvested by centrifugation, suspended in an equal volume of 0.85% saline, and irradiated for various time

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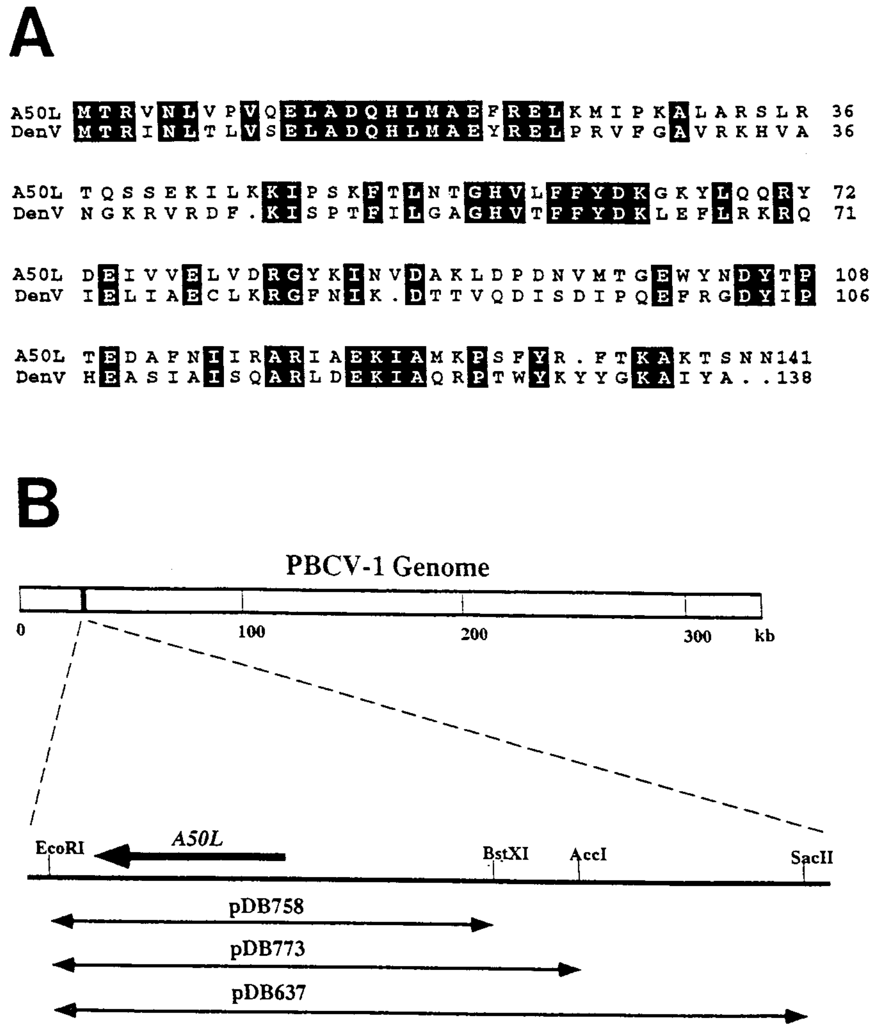

FIG. 1. (A) Sequence alignment of PBCV-1 ORF A50L with bacteriophage T4 endonuclease V (T4endoV) was performed. (B) The PBCV-1 ORF A50L was subcloned from cosmid cYL5 by using DNA restriction endonuclease *Sac*II, *Acc*I, or *Bst*XI to cleave 1,168 bp (pDB637), 667 bp (pDB773), or 475 bp (pDB758), respectively, upstream of the translational start codon of the gene and *Eco*RI to cleave 105 bp after the translational stop codon of the gene. Each fragment was inserted into the polylinker region of pBluescript KS(+).

periods. All manipulations after irradiation were conducted in the dark. Irradiated cells were diluted in saline, and duplicate samples were plated on LB agar. Plates were incubated in the dark at 37°C, and colonies were counted after 16 h.

(ii) UV resistance of PBCV-1 wild type and deletion mutants. The UV sensitivity of PBCV-1 and two deletion mutants of PBCV-1 lacking the *A50L* gene (viruses named P1050 and P1210 [14]) was measured by procedures similar to those used by Simonson et al. (31) for determining host cell reactivation of UV-irradiated bacteriophages. Virus lysates were diluted in 50 mM Tris-HCl (pH 7.8) and exposed to increasing doses of UV radiation. UV sensitivity was quantified by plaque assay. The titering plates were incubated either in the dark (dark repair conditions) or in the light (photoreactivation conditions) for 3 to 5 days.

Pyrimidine dimer-specific nicking activity assays. PBCV-1 virions were disrupted by sonication, and chlorella cells were disrupted in a French press in buffer containing 25 mM NaH_2PO_4 (pH 6.8), 100 mM KCl, 10 mM EDTA, and 100 μg of bovine serum albumin ml^{-1}. Cell extracts were normalized by chlorophyll content. The extracts and 0.4 ng of a γ-^{32}P-labelled, 5′-end-labelled duplex 49-mer oligonucleotide containing a centrally located cyclobutane pyrimidine dimer (gift of J. S. Taylor, Washington University, St. Louis, Mo.) were incubated in cell disruption buffer at 37°C for 30 min. The reactions were stopped by adding buffer containing 95% formamide, 20 mM EDTA, 0.02% (wt/vol) bromophenol blue, and 0.02% (wt/vol) xylene cyanol. The samples were heated to 90°C for 5 min prior to electrophoresis on a 15% polyacrylamide–urea gel.

Other procedures. Radioactive DNA probes were prepared by nick translation with a kit from Bethesda Research Laboratories. For dot blots, virus DNAs were denatured and applied to nitrocellulose membranes as described previously (27). Total RNA was isolated and analyzed from PBCV-1-infected cells as described previously (29).

RESULTS

Complementation of *E. coli* by the PBCV-1 *A50L* gene. Figure 1A shows the predicted amino acid alignment of PBCV-1 ORF A50L with bacteriophage T4 endonuclease V. The two ORFs are about the same size and have 41% amino acid identity. Critical amino acids required for T4 endonuclease V structure and function are known from crystal (20) and cocrystal structures (44) and from site-directed mutations (3, 4, 11, 18, 28). These critical amino acids include the αNH_2 group of Thr-2 which serves as a nucleophile (3, 28) and Glu-23 (4, 11, 18). The predicted amino acid sequence of ORF A50L has these two key amino acids.

To determine if the *A50L* gene product was active, three DNA constructs containing the *A50L* gene (Fig. 1B) were transformed into UV repair-deficient *E. coli* AB2480 (*uvrA recA*) (12) and tested for repair of UV damage to the bacterial genome. All three transformants survived UV radiation better

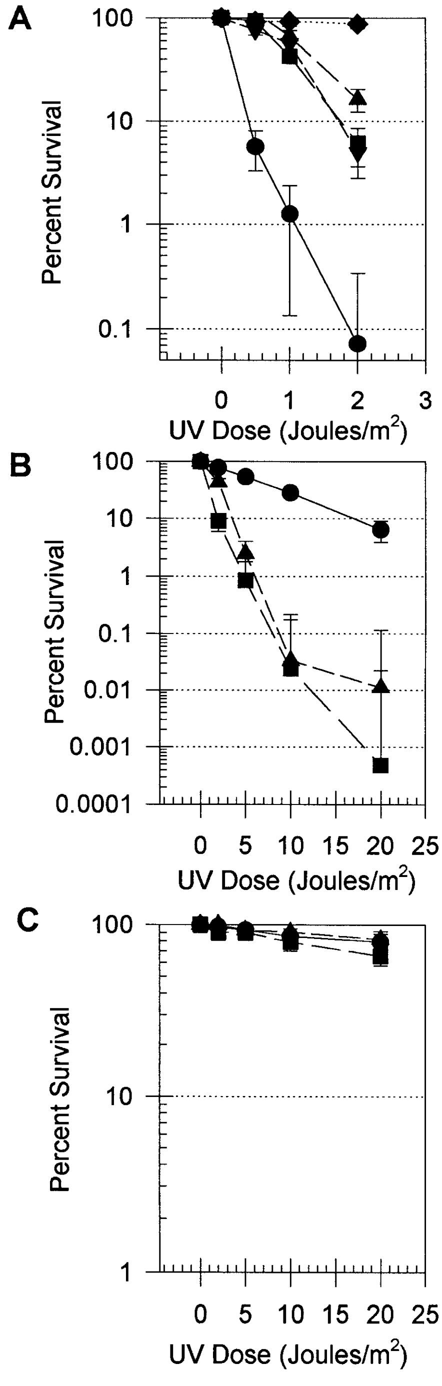

FIG. 2. (A) UV sensitivity of *E. coli* AB2480 (*uvrA recA*) with and without plasmids containing the PBCV-1 *A50L* gene. *E. coli* AB2480 contained pBluescript KS(+) (circles), pDB637 (squares), pDB773 (inverted triangles), or pDB758 (triangles). *E. coli* AB1157 (*uvrA*$^+$ *recA*$^+$) served as the control (diamonds). Means and standard errors are illustrated. (B and C) UV inactivation of PBCV-1 virus and two deletion mutants lacking the *A50L* gene under dark repair (B) and light repair conditions (C). PBCV-1 wild type (circles) and deletion mutants P1050 (squares) and P1210 (triangles) are shown. Means and standard errors are illustrated.

than a transformant with the plasmid vector alone (Fig. 2A). Thus, the PBCV-1 *A50L* gene, like the *denV* gene (15, 36), complements the UV repair deficiencies of this *E. coli* strain. Furthermore, like many chlorella virus genes (e.g., see reference 19), *A50L* has an upstream region that functions as a promoter in *E. coli.*

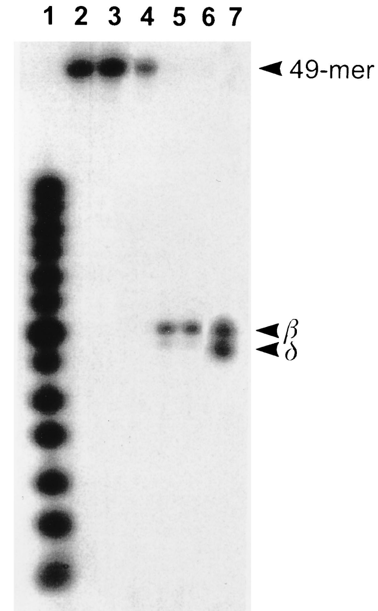

FIG. 3. Pyrimidine dimer-specific nicking activity isolated from PBCV-1-infected chlorella cells. Lanes: 1, oligonucleotide size markers (8 to 32 bases); 2 and 3, 5′-end-labelled duplex 49-mer oligonucleotide containing a centrally located cyclobutane pyrimidine dimer incubated alone (lane 2) or with extracts from purified virions (lane 3); 4, uninfected chlorella; 5, chlorella cells at 90 min after PBCV-1 infection; 6, chlorella cells 180 min after PBCV-1 infection; 7, T4 endonuclease V enzyme.

PBCV-1 mutants that lack the *A50L* gene. We recently isolated four PBCV-1 mutants with 27- to 37-kb deletions in the left end of the 330-kb genome (14). These mutants, which lack the *A50L* gene, replicate like the parent virus except for slightly reduced burst sizes. We compared the UV sensitivity of wild-type PBCV-1 and two deletion mutants designated P1050 and P1210 to determine if the *A50L* gene product functions in vivo. When infected cells were incubated in the dark (host cells grow heterotrophically in the dark and support virus replication), both deletion mutants were roughly 1,000-fold more sensitive to UV radiation than the wild-type virus was (Fig. 2B). In contrast, when infected cells were incubated in the light, which permits host photoreactivation of DNA damage, the deletion mutants were as UV resistant as the wild-type PBCV-1 virus (Fig. 2C).

Appearance of *A50L* gene product during virus replication. In their native environment, chlorella viruses are constantly exposed to solar radiation. Therefore, it seemed likely that the A50L enzyme might be packaged in the virions to initiate DNA repair immediately upon entry into the host cell. However, two experiments indicate that the enzyme is not packaged in the virion. (i) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of total proteins from PBCV-1 and PBCV-1 mutants lacking the *A50L* gene did not reveal a protein band of the expected size in wild-type virions that was absent in the deletion mutants, even after overloading the gel and silver staining (results not shown). (ii) Purified virions were disrupted by sonication, and these viral extracts were assayed for pyrimidine dimer-specific nicking activity with a ^{32}P-labelled oligonucleotide that contains a site-specific cyclobutane pyrimidine dimer. There was no difference between the untreated oligonucleotide (Fig. 3, lane 2) and the oligonucleotide treated with the

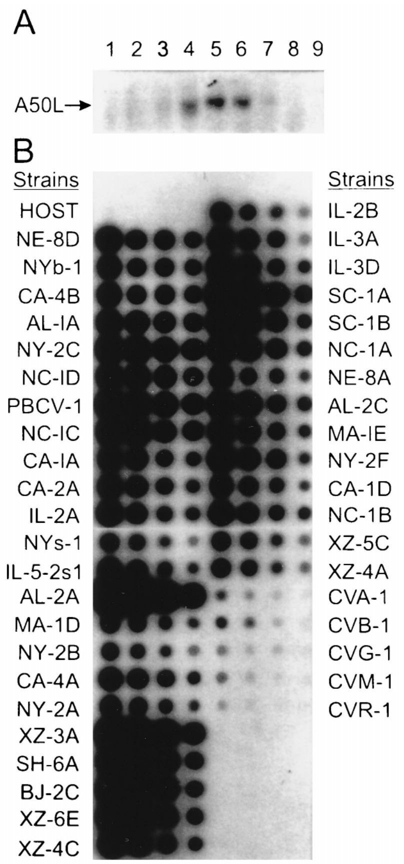

FIG. 4. (A) Northern blot analysis of RNAs isolated from uninfected and from PBCV-1-infected chlorella cells at 15, 30, 45, 60, 90, 120, 240, and 360 min after infection (lanes 1 to 9, respectively). The blot was hybridized with the *A50L* gene. (B) Hybridization of the PBCV-1 *A50L* gene to DNA isolated from the host *Chlorella* strain NC64A and from 37 chlorella viruses that infect *Chlorella* strain NC64A and 5 viruses (CVA-1, CVB-1, CVG-1, CVM-1, and CVR-1) that infect *Chlorella* strain Pbi. The blots contain 1, 0.5, 0.25, and 0.12 μg of DNA, left to right, respectively. Note that the *A50L* gene did not hybridize to the host chlorella DNA even after prolonged exposure.

virion extract (Fig. 3, lane 3), whereas T4 endonuclease V (Fig. 3, lane 7) generated the two expected β and δ elimination products.

In contrast, extracts from chlorella cells infected with PBCV-1 for 90 or 180 min had cyclobutane pyrimidine dimer-specific nicking activity (Fig. 3, lanes 5 and 6). Separate dilution experiments indicated that the cells infected for 180 min contained about 10 times more enzyme activity than the cells infected for 90 min. No nicking activity was detected in extracts from uninfected chlorella (Fig. 3, lane 4).

Northern (RNA) blot experiments revealed that a 450-nucleotide *A50L* transcript appeared about 30 min after PBCV-1 infection and disappeared about 120 min after virus infection (Fig. 4A). Thus, *A50L* is an early virus gene. Furthermore, since cells infected for 180 min contained high levels of dimer-specific nicking activity, the A50L enzyme is very stable.

T4 *denV* gene homologs are widespread in the chlorella viruses. As mentioned in the introduction, several microorganisms contain T4 endonuclease V-like activities. However, these other enzymes differ substantially from endonuclease V and the A50L enzyme in size and amino acid sequence. To determine if the *A50L* gene is widespread among the chlorella viruses, the *A50L* gene probe was hybridized to DNA isolated from 42 other chlorella viruses (Fig. 4B). The probe hybridized strongly to 37 chlorella viruses, each of which replicates in the same chlorella host that PBCV-1 does. The probe hybridized weakly to the other five virus DNAs, each of which replicates in another chlorella isolate, strain Pbi (25). Thus, the *A50L* gene is common in the chlorella viruses. The *A50L* probe did not hybridize to host chlorella DNA (Fig. 4B).

DISCUSSION

The following observations and experimental results support the hypothesis that the chlorella virus PBCV-1 *A50L* gene is a homolog of the *E. coli* bacteriophage T4 *denV* gene. (i) The predicted PBCV-1 A50L ORF has a Z-score of 34 and 41% amino acid sequence identity to endonuclease V, including critical amino acids known to be required for endonuclease V structure and function. (ii) Structural modelling studies between endonuclease V and the *A50L* gene product, with the X-ray crystal structure of endonuclease V as a guide, predict similar folding patterns between the two proteins (26a). (iii) Cloned DNA fragments containing the *A50L* gene complement the DNA damage repair defects of *E. coli* AB2480. (iv) Deletion mutants of virus PBCV-1 lacking the *A50L* gene are more susceptible to inactivation by UV irradiation. (v) Enzyme extracts from PBCV-1-infected cells, but not from uninfected cells, exhibit pyrimidine dimer-specific nicking activity. (vi) Repair activity of the A50L enzyme substantially overlaps that of the photoreactivation system, indicating that pyrimidine dimers are being removed. (vii) The *A50L* gene product is expressed as an early viral function. Taken together, these findings indicate that PBCV-1 encodes a homolog of the bacteriophage T4 *denV* gene and that this homolog is expressed and functions to eliminate UV-induced dimers in the viral genome during infection of the host alga. Thus, PBCV-1 is the first example of an algal virus encoding a DNA repair gene and only the second example of a virus of any type known to encode host-independent DNA UV repair functions.

Until the discovery of the PBCV-1 *A50L* gene, there were no known homologs of the T4 *denV* gene. Southern blot analysis of many independently isolated chlorella viruses (Fig. 4B) indicate that T4 *denV* gene homologs are common in this group of viruses. While the effects of convergent evolution in producing the apparent relationship between the T4 *denV* and PBCV-1 *A50L* genes cannot be discounted, the discovery of DNA repair gene homologs in unrelated viruses parasitizing hosts from different kingdoms suggests that these genes are ancient and widely disseminated in the biosphere. Indeed, Bernstein and Bernstein (1) have suggested that enzymes involved in DNA repair, recombination, and replication probably arose early and have been retained in biotic evolution. From an enzymological standpoint, the chlorella viruses are a new source of *denV*-like genes for genetic and structural studies.

UV damage to the PBCV-1 genome can also be repaired by the host photoreactivation system. Thus, PBCV-1 has access to two independent repair systems to reverse DNA damage, i.e., photoreactivation using host-encoded gene products and a virus-encoded enzyme that initiates dark repair. The combined activities of these repair systems should enable PBCV-1 to effectively exploit their hosts under a range of environmental conditions and to retain infectivity in environments exposed to substantial solar radiation. Since the algal host for these viruses is a photosynthetic organism, the viruses are obviously exposed to solar radiation in their natural environment. Photoreactivation enables infecting damaged viruses to rapidly and continuously repair pyrimidine dimers, ensuring effective viral replication under conditions in which the host is exposed to sunlight. The presence of a separate light-independent repair system permits DNA repair in the absence of photoreactivation. Thus, PBCV-1 can replicate whenever suitable hosts are encountered, day or night. This finding helps explain the frequent occurrence and occasional high titer of these viruses in fresh water collected throughout the world (39, 43, 48, 50).

UV repair systems are widely distributed in aquatic microorganisms, and evidence suggests that some of their viruses might also encode DNA repair genes. The effects of virus-encoded DNA repair genes will need to be considered in developing models of aquatic ecosystems. Marine surface waters contain high concentrations of virus particles, 10^5 to 10^8 ml^{-1}, although the majority of these particles are probably not infectious at any one time (6, 34). Most of these particles consist of viruses that infect bacteria, cyanobacteria, and algae. Viruses exist in a dynamic state in these environments and influence the composition, activity, and genetic diversity of microbial communities (6). For example, solar UV radiation rapidly inactivates bacteriophage and cyanophage with loss rates as high as 0.4 to 0.8 h^{-1} in full sunlight (33, 35). Not surprisingly, infectivity disappears faster in the light than in the dark, leading to the suggestion that the concentration of infectious viruses in seawater may exhibit a strong diel signal (35). Suttle and Chen (35) have noted that sunlight inactivates bacteriophages at different rates, implying that bacteriophages differ in the ability to repair DNA damage. The capacity to repair UV-damaged DNA is clearly widespread among bacteriophages (10). Viruses may capitalize on available host-encoded repair systems, including photoreactivation, to eliminate UV damage (10, 47) or, as exemplified by bacteriophage T4 and virus PBCV-1, also encode novel, host-independent DNA repair functions. Presumably, viruses that can use two separate DNA repair systems have a better chance to survive in environments exposed to intense sunlight. Thus, it seems likely that many dsDNA-containing viruses found in environments subjected to solar radiation might encode DNA repair genes.

In summary, this study has three major implications. First, chlorella virus PBCV-1 and many, if not all, chlorella viruses encode a homolog of the bacteriophage T4 endonuclease V UV repair enzyme. Thus, the enzyme is not unique but a member of a widely distributed family. Second, the chlorella viruses will be a valuable source of endonuclease V homologs for structural and genetic studies. Third, the discovery of two independent repair systems, a host-encoded photorepair system and a combined virus- and host-encoded excision dark repair system, helps explain the ease with which chlorella viruses are recovered from surface waters throughout the world. Furthermore, similar mechanisms may help explain the surprisingly high concentrations of virus particles observed in marine surface waters.

ACKNOWLEDGMENTS

We thank Yoshikazu Tanaka for helpful discussions and an anonymous reviewer for making the manuscript better. *E. coli* AB2480 was kindly provided by Mary Berlyn of the *E. coli* Genetic Stock Center at Yale University.

This investigation was supported, in part, by Public Health Service grants GM-32441 to J.V.E. and ES04091 and ES06676 to R.S.L. and an EPA cooperative agreement (CR822163) to T.K.

ADDENDUM IN PROOF

After acceptance of this paper, S. Shiota and H. Nakayama (Proc. Natl. Acad. Sci. USA **94:**593–598, 1997) reported the characterization of a gene from *M. luteus* that also encodes a bacteriophage T4 endonuclease V-like enzyme. The PBCV-1-encoded enzyme is more similar to T4 endonuclease V than is the enzyme from *M. luteus.*

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