Isolation and characterization of a new type of chlorovirus that infects an endosymbiotic Chlorella strain of the heliozoon Acanthocystis turfacea

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A novel virus, named Acanthocystis turfacea Chlorella virus (ATCV), that infects endosymbiotic Chlorella algae of the heliozoon Acanthocystis turfacea was isolated from freshwater samples. Electron microscopic analysis of ATCV revealed that the viral capsid has a distinct icosahedral shape with a diameter of 140–190 nm. Filamentous structures extending from some of the virus vertices, which may aid attachment of the virus to host cells, were also observed. The capsid is made up of one major coat protein of about 50 kDa and contains a large dsDNA genome. ATCV is a member of the genus Chlorovirus, which belongs to the family Phycodnaviridae, a group of large, icosahedral, dsDNA-containing viruses that infect algae and are ubiquitous in natural environments. However, ATCV is clearly distinct from the prototype Chlorovirus, Paramecium bursaria Chlorella virus (PBCV-1), in some aspects of its genome structure and gene content and therefore must be regarded as a member of a new group of Chlorella viruses.

INTRODUCTION

Chlorella viruses are large, icosahedral, dsDNA viruses that belong to the genus Chlorovirus in the family Phycodnaviridae (Wilson et al., 2005). They are ubiquitous in fresh water and infect endosymbiotic strains of the unicellular green alga Chlorella (for review see Van Etten, 2003). The viruses form plaques on lawns of Chlorella algae (Van Etten et al., 1983). When grown in liquid cultures, virus particles can be obtained in large quantities from lysed algal cells (Van Etten et al., 1983). The complex virions have diameters of 140–190 nm and are composed of a multilaminate shell surrounding an icosahedral capsid. The virus particles contain proteins, dsDNA and lipids. Of the virions’ more than 50 structural proteins, several are glycosylated and located in the outer layer of the capsid (Yan et al., 2000). Likewise, the lipid component is located inside the outer capsid shell and is required for virus infectivity. The surface structure of the virions is dominated by the major capsid protein, a glycoprotein accounting for approximately 40% of the total virion protein (Wang et al., 1993). One of the remarkable features of Chlorella viruses is their large linear dsDNA genome of 330–390 kbp. The linear genome is non-permutated and has covalently closed hairpin ends (Rohozinski et al., 1989; Yamada & Higahiyama, 1993; Zhang et al., 1994). It has been established that the genomes of Chlorella viruses represent a mosaic of prokaryotic and eukaryotic genes, some of which are of considerable economic interest, e.g. DNA methyltransferases and DNA site-specific restriction endonucleases (Van Etten & Meints, 1999). Chlorella virus isolates show strict specificity towards certain host strains and have been grouped accordingly. NC64A viruses infect Chlorella strain NC64A isolated from Paramecium bursaria collected in the USA, Pbi viruses infect Chlorella strain Pbi isolated from P. bursaria collected in Goettingen, Germany, and Hydra viridis Chlorella virus (HVCV) infects Chlorella strain N1A isolated from Hydra viridis. The viruses cannot infect host strains of the other groups because they do not attach to the algal cell walls (Reisser et al., 1988). This feature may be related to cell wall characteristics of the respective host strain, but probably also depends on variations in virion proteins.

Endosymbiotic Chlorella algae have been found in association with many organisms from different classes of the animal kingdom like Rhizopoda, Ciliata, Hydrozoa and Turbellaria. However, so far, viruses have only been isolated from symbiotic Chlorella strains of P. bursaria (Ciliata) and H. viridis (Hydrozoa). Some viruses infecting P. bursaria Chlorella strains have been characterized in detail. Paramecium bursaria Chlorella virus (PBCV-1) is the prototype of this virus group and the complete nucleotide sequence of the viral genome is available.

In this study, a new Chlorovirus from an endosymbiotic Chlorella strain of the heliozoon Acanthocystis turfacea (Rhizopoda), designated Acanthocystis turfacea Chlorella virus (ATCV), was identified. It was isolated from a freshwater pond in Stuttgart, Germany. The virus was...
purified and characterized with respect to its structural, physical and biochemical properties. In addition, a conserved region of the DNA polymerase gene and random parts of the DNA genome of ATCV were sequenced. Data were compared to the sequence of PBCV-1, the prototype of the genus Chlorovirus, and to other DNA viruses.

**METHODS**

**Algal strains and viruses.** Chlorella virus PBCV-1 was obtained from J. Van Etten, University of Nebraska, Lincoln, USA. Chlorella strains SAG 211-6 (endosymbiont of P. bursaria, host for PBCV-1), SAG 241-80 (endosymbiont of P. bursaria, host for Phi viruses), SAG 3.83 (endosymbiont of A. turfarceae), SAG 242-80 (endosymbiont of H. viridis) and SAG 15.93 (free-living Chlorella species) were obtained from the algal culture collection (SAG) at the Institute of Plant Physiology, University of Goettingen, Goettingen, Germany. All Chlorella strains were cultivated in modified Bold’s basal medium (MBBM) as described previously (Van Etten et al., 1983).

**Isolation of virus particles.** Water samples were collected from freshwater ponds around the University of Hohenheim, Stuttgart, Germany. Water samples were filtered through a nitrocellulose membrane filter (0–45 μm pore size) and 1 ml of each water sample was used to infect an exponentially growing culture of Chlorella SAG 3.83. After 2 weeks cultivation, algae were pelleted by centrifugation at 5000 g for 10 min. The culture supernatants were used for virus plaque assays. To this end, 1·2×10^6 Chlorella cells were mixed with 100 μl samples of each culture supernatant and 2·5 ml soft agar and the mixtures were plated on MBBM. Plates were incubated at 25°C in continuous light. After 3–5 days, plaque formation could be observed.

For large-scale virus purification, a single plaque was picked from the algal lawn and transferred to an exponentially growing culture of Chlorella SAG 3.83 (2×10^6 cells ml^-1) in 800 ml MBBM in a 2 litre Erlenmeyer flask. The culture was propagated for 5 days at 25°C in continuous light on a rotating shaker at 200 r.p.m. until cell lysis was observed. The lysate was centrifuged at 5000 g for 5 min to remove cell debris. Triton X-100 was added to the supernatant to a final concentration of 1% and virus particles were precipitated by centrifugation for 3 h at 8,000 g. Purified virus particles formed a major band in the middle of the sucrose gradient. The virus band was removed from the gradient with a sterile needle and diluted to 30 ml with 50 mM Tris/HCl, pH 7·8, and were further purified on a 10–40% sucrose gradient by centrifugation for 20 min at 72,000 g. Purified virus particles formed a major band in the middle of the sucrose gradient. The virus band was removed from the gradient with a sterile needle and diluted to 30 ml with 50 mM Tris/HCl, pH 7·8. The particles were pelleted by centrifugation for 3 h at 75,000 g and resuspended in 8 ml 50 mM Tris/HCl, pH 7·8. The concentration of the purified virus particles (p.f.u. ml^-1) was determined on a lawn of Chlorella SAG 3.83.

**Electron microscopy.** For electron microscopic studies, purified virus particles were treated with 2% phosphotungstic acid and analyzed using an LEO EM 912 AB transmission electron microscope (LEO Oberkochen).

**Analysis of viral proteins.** Protein analysis of ATCV-1, ATCV-2 and PBCV-1 was carried out using 15% SDS-PAGE with 2 μl (4·0×10^6 p.f.u. ml^-1) purified virus particles as described previously (Laemmli, 1970). Proteins were visualized by Coomassie blue staining (Ausubel et al., 1987).

**Preparation of viral DNAs and construction of a genomic library.** DNA was isolated from purified ATCV-1, ATCV-2 and PBCV-1 by phenol extraction after treatment of virus isolates with proteinase K (0·1 mg ml^-1) for 8 h at 55°C. Viral DNAs were digested with EcoRI, BamHI or HindIII and samples were separated overnight on a 1% agarose gel. DNA was visualized by ethidium bromide staining. For construction of a genomic library, 15 μg extracted viral DNA was digested overnight with BamHI. Reaction products were extracted with phenol, precipitated with ethanol, resuspended in TE (10 mM Tris/HCl, pH 8·0, 1 mM EDTA) and ligated into the BamHI site of pUC18. The ligation was transformed into Escherichia coli DH5α competent cells and plasmid DNA from minipreparations was used for sequencing viral fragments (Sambrook et al., 1989; Sanger et al., 1977).

**PCR amplification of DNA polymerase sequences.** For amplification of a conserved region of the DNA polymerase gene of ATCV, degenerate primers were designed according to Chen & Suttle (1995). Using the upstream primer pol1 5‘-GA(A/G)GGGCCACIGGT(T/C)TIGA(T/C)GC-3’ and the downstream primer pol2 5‘-(G/C)(A/T)(A/G)TCIGT(A/G)TCICC(A/G)TA-3’, a 440 bp fragment could be amplified from 100 ng ATCV-1 or ATCV-2 DNA under the conditions given by Chen & Suttle (1995). PBCV-1 DNA was used as a positive control. The amplified DNA polymerase fragments were inserted into pUC18 by TA cloning and sequenced.

**RESULTS**

**Virus isolation and plaque assay for ATCV-1 and ATCV-2**

So far, viruses infecting symbiotic Chlorella algae have only been described for symbionts of P. bursaria and H. viridis. Since symbiotic algae are found in quite different organisms from the animal kingdom, we set out to investigate whether viruses also exist for symbiotic Chlorella strains other than those that infect P. bursaria and H. viridis. To this end, the endosymbiotic Chlorella strain SAG 3.83, which was originally isolated from the heliozoon A. turfarceae (Fig. 1), was infected with freshwater samples collected from a pond in the botanical garden at the University of Hohenheim,
Stuttgart, Germany. In the initial plating experiment, no plaques were detected. After additional amplification of virus in an exponentially growing algae culture, however, plaques formed on a lawn of Chlorella SAG 3.83 after 5 days incubation. Two different types of plaques were obtained, which differed in size. Viruses from both plaque phenotypes were picked, propagated and purified on sucrose gradients. The isolated viruses were named Acanthocystis turfacea Chlorella virus (ATCV). Upon plating, virus isolated from small plaques yielded uniformly small plaques on Chlorella SAG 3.83 (Fig. 2a). This virus strain was designated ATCV-1. Likewise, virus purified from large plaques, designated ATCV-2, formed uniformly large plaques upon replating (Fig. 2b). The concentration of purified virus particles was calculated for each virus strain; the titre of purified ATCV-1 was $1 \times 10^{11}$ p.f.u. ml$^{-1}$ and the titre for ATCV-2 was $2 \times 10^{8}$ p.f.u. ml$^{-1}$.

**Host specificity of ATCV**

The host specificity of Chlorella viruses is generally limited to one or only a few algal strains. To investigate whether this is also the case for ATCV, different Chlorella strains were infected with PBCV-1, ATCV-1 or ATCV-2. PBCV-1 produced plaques exclusively on SAG 211-6, whereas infection by ATCV-1 or ATCV-2 could only be observed with Chlorella strain SAG 3.83 (Fig. 2). Chlorella strains SAG 211-6 (endosymbiont of P. bursaria, host for PBCV-1), SAG 241-80 (endosymbiont of P. bursaria, host for Pbi viruses), SAG 242-80 (endosymbiont of H. viridis) and SAG 15.93 (free-living Chlorella species) were not infected by ATCV-1 or ATCV-2 (data not shown).

**Morphology of ATCV**

Chlorella viruses are known to be large icosahedral particles. Depending on the microscopic technique used, diameters of 140–190 nm have been reported for these viruses (Van Etten et al., 1991). Electron microscopic analysis of ATCV-1 and ATCV-2 with negative staining revealed that their capsids also have a distinct icosahedral shape with a diameter of 160–190 nm (Fig. 3). There are no apparent structural dissimilarities between ATCV-1 and ATCV-2 (data not shown). The particles of both viruses have filamentous structures extending from the vertices (Fig. 3b). Earlier investigations on the structure of PBCV-1 had demonstrated similar extensions and it has been suggested that the filamentous structures may be involved in attachment of the virus to the host cell wall (Van Etten et al., 1991).

**Analysis of the major capsid protein of ATCV**

Chlorella virus particles are complex. PBCV-1, for example, contains at least 50 different proteins (Van Etten, 2003). The capsid of PBCV-1 is composed of 5040 copies of the 54 kDa major capsid protein VP54. A three-dimensional image reconstruction of PBCV-1 indicates that VP54 is arranged in homotrimeric capsomers that cover the surface of the virus. The most abundant capsid protein of the Pbi virus Chlorella
virus G-1 (CVG-1) is VP49. VP49 is dimeric and identical to the PBCV-1 major coat protein VP54 in the N-terminal amino acid sequence (Plugge et al., 1999). To determine whether the ATCV capsid is constructed similarly, the protein composition of ATCV-1 and ATCV-2 was analysed by SDS-PAGE and compared to that of PBCV-1. Like PBCV-1, the ATCV-1 and ATCV-2 capsids are composed of only one major coat protein. The size of the ATCV coat protein is about 50 kDa, which is smaller than PBCV-1 coat protein VP54, but similar in size to the corresponding protein of Pbi viruses (Fig. 4).

**Physical stability of ATCV**

Another significant distinction between ATCV and PBCV-1 is the stability of the virus particles. PBCV-1 can only be stored at 4 °C and is completely inactivated by freezing. In contrast, virus particles of ATCV-1 and ATCV-2 can be frozen and stored at −70 °C without any loss of infectivity.

**Analysis of ATCV genomic DNA**

To analyse the relatedness between ATCV and PBCV-1, and, in particular, between ATCV-1 and ATCV-2 at the genomic level, purified viral DNAs were incubated with the restriction endonucleases EcoRI, BamHI and HindIII. Digested DNAs were separated on a 0·8 % agarose gel and the DNA patterns were compared to each other (Fig. 5). Whereas the restriction enzyme fragmentation patterns of ATCV-1 and ATCV-2 DNAs were identical with the three endonucleases, the restriction pattern of PBCV-1 DNA was completely different from the ATCV pattern. This result may suggest a rather low overall similarity between ATCV and PBCV-1 at the DNA level. On the other hand, the number and size of the restriction fragments produced by different restriction enzymes indicate that ATCV has a large DNA genome of about 350 kb, like many phycodnaviruses, including PBCV-1.

To further clarify the relationship between ATCV-1 and ATCV-2 and that between ATCV and PBCV-1, genomic DNAs from ATCV-1 and ATCV-2 were cut with BamHI and the fragments were ligated into BamHI-digested pUC18. Random clones from ATCV-1 and ATCV-2 DNA, which appeared identical in size, were chosen and sequenced from both ends. Three clones from corresponding genes of ATCV-1 and ATCV-2 were analysed. The analysed sequences encompassed a total of 5 kbp from six different protein-coding regions. The encoded proteins exhibited similarity to a cellulose synthase from *Acetobacter*, a human DNA-binding protein, a GDP-mannose dehydratase from *Yersinia enterocolitica*, a mouse procollagen, a cellulose synthase from *Neurospora crassa* and a ribonucleotide reductase from *Roseophage SIO1*, respectively (Table 1). The nucleotide sequences obtained were identical for both viruses (data not shown), indicating that ATCV-1 and ATCV-2 can be regarded as two strains of the same virus. However, comparison of the sequences with those in databases revealed that there is no identity at the nucleotide level between ATCV and PBCV-1 in the regions analysed. This result is consistent with the high diversity in restriction enzyme patterns observed previously with ATCV and PBCV-1 genomic DNAs.

**DISCUSSION**

**Similarity of proteins encoded by ATCV genes to proteins from PBCV-1 and other species**

Although the similarity between ATCV and PBCV-1 is very low at the DNA level, there is considerable similarity...
between proteins encoded by genes from ATCV, PBCV-1 and other species at the amino acid level. Several interesting conclusions can be drawn from the data shown in Table 1. Of the six ATCV putative protein-coding genes analysed, proteins encoded by three of them exhibit highest identity to corresponding proteins from prokaryotes or phages (ORF1, ORF3 and ORF6), whereas the other three are most similar to proteins from eukaryotes (ORF2, ORF4 and ORF5). In general, the genome of ATCV seems to be a mosaic of genes of prokaryotic and eukaryotic origin, as has been described previously for PBCV-1. For all ATCV protein-coding genes analysed, homologues were found in the PBCV-1 genome. The degree of identity between the homologous ATCV and PBCV-1 proteins is, however, remarkably variable, ranging from 0 to 88%. Furthermore, the relative positions of some of the homologous genes within the genomes of ATCV and PBCV-1 are different. The genes for GDP-mannose dehydratase (ORF3) and the Pro-rich protein (ORF4), for example, which are located on a 5 kbp BamHI fragment in the ATCV genome, are 39 kbp apart in the PBCV-1 genome.

Even more interestingly, some ATCV proteins are more similar to proteins from distant species than to the homologous PBCV-1 proteins (Table 1). The GDP-mannose dehydratase of ATCV (ORF3) exhibits 66% identity to the GDP-mannose dehydratase of Y. enterocolitica, whereas identity to the PBCV-1 protein A118R is only 42%. ORF6 of ATCV shows 27% identity to the prokaryotic-type RB ribonucleotide reductase of Roseophage SIO1, a T7-like virus infecting marine prokaryotic host organisms. At the same genome position, PBCV-1 contains a gene for the small subunit of ribonucleotide reductase, A476R. The PBCV-1 gene exhibits no similarity to the ribonucleotide reductase of ATCV. On the other hand, A476R displays a high identity (58%) to ribonucleotide reductase from tobacco and thus is of eukaryotic origin. Together, these data suggest that ATCV and PBCV-1 contain genes with corresponding functions that have been obtained from different sources. Since ATCV and PBCV-1 are clearly structurally related, they most likely developed from a common ancestor virus. During evolution, however, the two viruses appear to have acquired genes with equivalent functions from quite diverse sources.

### Table 1. Amino acid identity of proteins encoded by ATCV genes to proteins from PBCV-1 and other species

<table>
<thead>
<tr>
<th>ATCV gene</th>
<th>Homologous protein (% identity)</th>
<th>Homologous PBCV-1 protein (% identity)</th>
<th>Distance in ATCV (kb)</th>
<th>Distance in PBCV-1 (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORF1</td>
<td>Cellulose synthase of Acetobacter (25)</td>
<td>A222/226R (88)</td>
<td>~3.5</td>
<td>250-0</td>
</tr>
<tr>
<td>ORF2</td>
<td>Human DNA-binding protein (44)*</td>
<td>A540L (32)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORF3</td>
<td>GDP-mannose-4,6-dehydratase of Yersinia enterocolitica (66)</td>
<td>A118R (42)</td>
<td>5.0</td>
<td>39-0</td>
</tr>
<tr>
<td>ORF4</td>
<td>Procollagen of Mus musculus (37)*</td>
<td>A189R (51)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORF5</td>
<td>Cellulose synthase of Neurospora crassa (51)*</td>
<td>A473L (58)*</td>
<td>5.0</td>
<td>2.6</td>
</tr>
<tr>
<td>ORF6</td>
<td>RB ribonucleotide reductase of Roseophage SIO1 (27)</td>
<td>A476R (−)*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Genes of eukaryotic origin.

### Evolutionary relationship of ATCV to Chlorella viruses and other DNA viruses

Phylogenetic trees based on the amino acid sequences of the main capsid proteins have demonstrated that the family Phycodnaviridae are related to the family Iridoviridae, members of which commonly infect insects and some vertebrates (Van Etten & Meints, 1999). Iridoviruses also share some other properties with phycodnaviruses, e.g. icosahedral capsid and a large dsDNA genome. In addition, cladistic analysis with genes shared by different virus groups supports the monophyly of poxviruses, African swine fever virus and phycodnaviruses (Iyer et al., 2001). Phylogenetic analysis based on sequences of the DNA polymerase conserved deoxynucleotide-binding domain from several algal viruses places the Pbi and NC64A Chlorella viruses in separate, albeit closely related groups (Chen & Suttle, 1996). Furthermore, these data suggest that phycodnaviruses are more closely related to herpesviruses than to other DNA viruses. To clarify the relatedness of ATCV to Chlorella viruses and to other viral groups, the conserved deoxynucleotide-binding domain of DNA polymerase from ATCV-1 and ATCV-2 was cloned. Degenerate primers were designed and a 440 bp fragment was amplified via PCR from the viral DNAs. Sequence analysis showed that there are no differences in the nucleotide sequence of this domain between ATCV-1 and ATCV-2 (data not shown), which supports our previous conclusion that ATCV-1 and ATCV-2 are two strains of the same virus.

The derived amino acid sequence was compared to the DNA polymerase domains from Pbi and NC64A viruses and, using CLUSTALW, to those from other DNA viruses. Fig. 6 shows an alignment of the amino acid sequence of the ATCV DNA polymerase domain with the corresponding sequences of the Pbi virus Chlorella virus A-1 (CVA-1) and the NC64A virus PBCV-1. The identity of the ATCV sequence to the CVA-1 sequence is 89%, whereas identity to the PBCV-1 sequence is only 82%. The main difference between the ATCV, CVA-1 and PBCV-1 sequences is a sequence of six additional amino acids in the PBCV-1 DNA polymerase domain. The significance of these additional amino acids in
the PBCV-1 DNA polymerase gene is not clear. However, the insertion might indicate the presence of a so far undetected intron in the PBCV-1 sequence. Introns have been found in other parts of the DNA polymerase gene of PBCV-1 and the positions and sequences of the introns are conserved in many NC64A viruses, but not in Pbi viruses (Zhang et al., 2001). The absence of the six amino acids in the DNA polymerase sequences of ATCV and CVA-1 indicates that ATCV is more closely related to Pbi viruses than to PBCV-1 and other NC64A viruses. This view is also supported by the phylogenetic tree generated from multiple alignment of the DNA polymerase deoxynucleotide-binding domain sequences from different DNA viruses by clustalw (Fig. 7). Although ATCV is closely related to

**Fig. 6.** Amino acid sequence alignment of the conserved DNA polymerase domains of ATCV-1, CVA-1 and PBCV-1. *Chlorella* virus CVA-1 is a member of the Pbi group and *Chlorella* virus PBCV-1 represents the NC64A viruses. Dashes were introduced to aid alignment of the sequences. Conservative amino acid exchanges in the CVA-1 and PBCV-1 sequences are indicated by +. The six amino acid insertion in the PBCV-1 sequence is shown in bold and underlined.

PBCV-1, the neighbour-joining algorithm puts ATCV on a separate branch together with the Pbi Chlorella viruses.

CONCLUSION

By isolation and characterization of the new phycodnavirus ATCV it has been demonstrated that viruses similar to PBCV-1 can also be found in symbiotic Chlorella strains from species not related to P. bursaria or H. viridis. However, although ATCV has a particle structure closely resembling that of PBCV-1, the similarity of ATCV to PBCV-1 at the genome level is rather low. Only highly conserved gene regions, such as the deoxynucleotide-binding domain of the DNA polymerase, showed considerable identity between ATCV and PBCV-1 as well as in other phycodnaviruses and animal herpesviruses. Based on the conserved DNA polymerase sequence and the size of the major capsid protein, ATCV is more closely related to Pbi viruses than to NC64A viruses like PBCV-1. Data strongly suggest that ATCV and PBCV-1, although descending from a common ancestor virus, have evolved independently from each other.

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