Digestion of Chlorella Cells by Chlorovirus-encoded Polysaccharide Degrading Enzymes

Nitit ChuChird¹, Shingo HiRAMATSU¹, Ichiro SUgimoto¹, Makoto FUjie¹, Shoji UsAMI¹ and Takashi YAMADA¹*

¹ Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1–3–1 Kagamiyama, Higashi-Hiroshima 739–8530, Japan

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Using a halo-forming assay with polysaccharide-degrading enzymes encoded by Chlorella virus (chlorovirus) CVK2, several free-living Chlorella strains, especially those belonging to C. vulgaris, were found to be sensitive to the viral enzymes. Among these sensitive strains, a taxonomically established strain C. prototechoides 211-6 served as a new laboratory host for chloroviruses (NC64A-viruses). Many zoochlorella strains isolated from Paramecium were totally resistant to the enzymes. Attachment of CVK2 to the cells of both Chlorella strain NC64A and C. prototechoides 211-6 was markedly blocked by treatment of the cells with a mixture of the viral enzymes. The treatment with vAL-1 may destroy the virus receptor molecules.

Key words: chlorovirus, new laboratory host, halo-assay, zoochlorella

Large icosahedral, dsDNA-containing, plaque-forming viruses that infect certain strains of the unicellular green alga Chlorella (chlorovirus, Phycodnaviridae) are widespread in natural aquatic environments. These viruses were first found in Chlorella-like algae (zooclorella) that are endosymbiotic with Paramecium bursaria. Lytic viruses appear in zooclorella after these algae are released from Paramecium cells. No virus particles were detected in the endosymbionts growing inside the paramecium. Such lytic viruses were isolated from zooclorella of Hydra viridis and P. bursaria. Van Etten et al. established a plaque assay for these viruses with an exsymbiotic Chlorella strain NC64A as an experimental or laboratory host, so the viruses can be easily detected in natural environments by plaque formation and produced in large quantities in laboratories. Plaque-forming viruses on Chlorella strain NC64A (NC64A-viruses) are common in continental American, Japanese, and Chinese, but not European, fresh water. Viruses that infect other exsymbiotic Chlorella strains SAG-241-80 and Pbi as laboratory hosts (Pbi-viruses) were also discovered in then-Soviet and European fresh water. Interestingly, NC64A-viruses do not infect strains SAG-241-80 and Pbi as hosts, and the strain NC64A does not serve as a host for the Pbi viruses.

These observations lead to the following questions: (i) What is the mechanism by which the viruses distinguish these Chlorella strains? (ii) Are these zooclorellae the exclusive viral hosts in native waters? (iii) What are the factors influencing the geographical distribution of NC64A and Pbi viruses in the natural environment?

To answer these questions, we have extensively examined the sensitivity of a variety of Chlorella strains to cell-wall-degrading enzymes encoded by CVK2, a chlorovirus isolated in Japan. The enzymes tested include chitosanase, chitinase, vAL-1, and vAL-2. We have found that C. prototechoides 211-6 can serve as a laboratory host strain for NC64A-viruses and that the treatment of the host cells with these viral enzymes blocked the viral attachment.
Materials and Methods

Cells and viruses

Cells of Chlorella strain NC64A were cultured in modified Bold’s basal medium (MBBM) as described previously. Chlorella virus CVK2 and other viruses were isolated from natural waters in Japan by plaque-forming assays. SAGV was isolated from a water sample collected in Galway, Ireland, with Chlorella strain SAG-241-80 as an experimental host. The production and purification of Chlorella viruses were performed as described previously. For halo-forming assay, C. vulgaris and C. ellipsoidea were obtained from the algal culture collection of the Institute of Molecular and Cellular Biosciences, The University of Tokyo. C. vulgaris 211-1e and 211-11b, C. saccharophila C-211, C. ellipsoidea C-87 (211-1a), C. prototichaoides C-206, and Chlorella sp. C-201 were obtained from the algal culture collection, Plant Physiology Institute, University of Goettingen. Paramecium bursaria zooclorella strains PB-3, PB-4, and PB-5 were provided by H. Hosoya, Hiroshima University; and strains MitB, Nn7, SO5, CT39, and T316 were from H. Takeda, Niigata University. All of these strains were cultured in MBBM at 25°C in light except for strains 211-6 and SAG-241-80. Strain 211-6 was cultured in a medium containing NH4Cl as the sole nitrogen source and thiamine according to Douglas and Huss. The strain SAG-241-80 was grown in jaworski’s medium supplemented with 0.2% Lab-lemco powder (Oxoid, Hampshire, UK).

Halo-forming assay with polysaccharide-degrading enzymes encoded by CVK2

Two-hundred μl of the host Chlorella cells, at a cell density of 2×10^6 cells/ml, were poured with 2.5 ml of 0.75% soft-agar MBBM onto a 1.5% MBBM agar plate. Sample discs (5 mm in diameter) of 3 MM filter paper were put on the plate. To each sample disc was applied 5 μl of enzyme solution containing approximately 1 μg of protein of purified vChta-1 chitosanase, vCht-1 chitinase, or vAL-1 lytic enzyme. Purification of the enzymes was as previously described: vChta-1 chitosanase, vCht-1 chitinase, and vAL-1 lytic enzyme. In the case of vAL-2, Escherichia coli lysate harboring a cosmid clone 1G1 containing the gene for vAL-2 was added to the disc. Bacterial lysate was prepared as follows: cells were grown in 20 ml of 2×YT medium for 12 h at 37°C, harvested and washed in phosphate-buffered saline (PBS) by centrifugation at 5,000×g, and sonicated in an As-trason Ultrasonic Processor (Heat System-Ultrasonics, Inc., NY) at output control 4.5, for 30 sec 10 times on ice. The lysate was centrifuged at 1,700×g for 10 min at 4°C, and the supernatant containing 20 mg protein/ml was used as a sample for the halo assay. The halo-plate was incubated in light at 25°C for 2 days. A clear zone (halo) around the disc (expanding with time) showed lysis of the cells.

Virus adsorption assay

Cells of Chlorella NC64A, C. prototichaoides 211-6, and C. ellipsoidea C-87 (control) growing exponentially were harvested and pretreated with the viral enzymes: 500 μl of cell suspension (10^7 cells/ml) was added with 150 μl of enzyme solution containing 10 μg of purified vChta-1, vCht-1, vAL-1, or all three and incubated with shaking for 3 h at 25°C. The adsorption of CVK2 to the enzyme-treated Chlorella cells was assayed by addition of 50 μl of virus preparation containing 500 pfu of CVK2 to the cell suspension. The samples were incubated for 30 min at 25°C and the reaction was stopped by centrifugation at 5,000 g for 10 min at 4°C. Aliquots of 140 μl (equivalent to 100 pfu of CVK2 per plate) were titered by plaque assay with strain NC64A as a host. The data are expressed as the percentage of unadsorbed virus.

Effects of vAL-1-digested cell wall material on CVK2 infection

Cell wall materials were prepared from Chlorella strain NC64A as previously described. After treatment of the cell wall materials (1 mg) with purified vAL-1 enzyme (20 μg protein) for 24 h at 37°C, the degradation products were separated by centrifugation and checked by thin-layer chromatography (TLC) as before. Various amounts of the degradation products were added to CVK2 preparations before plaque assay with strain NC64A as a host. For comparison, same amounts of untreated NC64A cell wall, glycol chitin (Seikagaku Corp.), and glycol chitosan (Sigma) that were mechanically grinded were added in the same way.

Pulsed-field gel electrophoresis

Chromosomal DNA molecules of Chlorella cells were separated by pulsed-field gel electrophoresis as described previously. Contour-clamped homogeneous electric field (CHEF) gel electrophoresis was carried out in a 1% agarose gel in 0.25×TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA at pH 8.3) with 5 min and 6 min switching intervals for 37 h and 50 h, respectively, at 2 V/cm using a CHEF-DRII system (Bio-Rad, Hercules, CA).
**Results**

**Halo-forming assay for Chlorella strains with virus-encoded lytic enzymes**

Previously, a total of 13 *Chlorella* strains (C. vulgaris 211-1e, 211-11b, C-135, and C-150; C. saccharophila 211-9a and C-211; C. ellipsoidea C-87; C. prototechoides 211-7a, 211-10d, and C-206; Chlorella. sp. C-201 and SAG-241-80) were tested for halo-formation with an algal-lytic enzyme, vAL-1 encoded by chlorovirus CVK2. In that study, we found that all four *C. vulgaris* strains and *Chlorella* strain SAG-241-80 are sensitive to this degradation activity. Nevertheless, none of these sensitive strains serve as a host for CVK2 infection. This raises the question of how viral-encoded lytic enzymes contribute to determining the host specificity. Apparently, the vAL-1 activity by itself is not the initial determinant of host recognition by the virus.

In addition to vAL-1, one chitosanase and two chitinase activities are known to be encoded by CVK2 and by PBCV-1 (*Paramecium bursaria* chlorella-like algal virus). Another lytic enzyme vAL-2 was also detected by Sugimoto et al. To clarify the roles of these enzymes in the viral life cycle, we have further examined, using an enzyme-halo assay, 21 *Chlorella* strains, many of which are of symbiotic origin. The sensitivity of each of these strains to chloroviruses was also examined. When strain NC64A was subjected to the assay, each CVK2 chitosanase, chitinase, and vAL-2 gave a clear lytic zone on the plate (Fig. 1), indicating the contribution of each in algal lysis as suggested previously. The results summarized in Table 1 distinguish four groups of *Chlorella* strains on the basis of sensitivity to the three different CVK2 enzymes as well as chloroviruses: (i) *C. prototechoides* C211-6 was newly found to serve as an experimental host of CVK2 as well as PBCV-1. Like NC64A, this strain was also sensitive to all three enzyme activities. (ii) All five strains of *C. vulgaris* and *Chlorella* sp. SAG-241-80 were sensitive to three CVK2 enzymes but resistant to CVK2/PBCV-1 infection. (iii) Three strains of PB3-5 isolated from *P. bursaria* in Hiroshima (H. Hosoya, personal communication) were sensitive to vAL-1 and vAL-2, marginal to chitosanase and chitinase, and resistant to viral infection. (iv) Six exsymbiotic strains (MitB, Nn7, SO5, CT39, T316, and C-201) isolated from *Paramecium* were all resistant to the enzyme activities. Similarly, three strains of *C. prototechoides* (211-7a, 211-10d, and C-206) did not form a halo.

Therefore, it is apparent that some free-living *Chlorella* strains, especially those belonging to *C. vulgaris*, are sensitive to the viral enzymes, even if they cannot experimentally serve as viral hosts. Some symbiotic strains isolated from *Paramecium* were totally resistant to the viral enzymes.

**Infection of CVK2 in *C. prototechoides* 211-6**

As described above, *C. prototechoides* 211-6 was found to serve as a new laboratory host for CVK2. PBCV-1 and all viruses isolated in Japan also infected strain 211-6 with an efficiency almost equal to that of strain NC64A. The viral life cycle, burst size, and plaque morphology were indistinguishable when either strain was the host (data not shown). CVK2 progeny grown in strain NC64A efficiently infected strain 211-6 and gave a burst size of ca. 300 on either strain, indicating no difference in the host selectivity between the two strains. No apparent differences were discerned in the restriction fragmentation patterns of the genomic DNA between the CVK2 progeny grown on NC64A and those grown on 211-6. Viral structural proteins separated by SDS-PAGE showed exactly the same patterns in NC64A-grown and 211-6-grown CVK2 progenies. Therefore, no host-dependent modifications or mutations in the viral genome were detected.

These results suggest that the two strains, *Chlorella* sp. NC64A and *C. prototechoides* 211-6, share common features that are specifically recognized and utilized by chloroviruses for their replication. To elucidate the host factors involved in chlorovirus replication, a detailed comparison of the two strains would be interesting.
Comparison of *Chlorella* sp. NC64A and *C. proto- techoides* 211-6

Taxonomical characteristics of strains NC64A and 211-6 were described by Douglas and Huss[1]. According to the growth and several physiological characteristics, strain NC64A was assigned to *C. vulgaris*. This was also supported by the DNA base composition and DNA-hybridization experiments. On the other hand, strain 211-6 is *C. proto- techoides* as confirmed by DNA-hybridization, and differs from *C. vulgaris* in its poor growth on nitrate as a nitrogen source, thiamine requirement, and cell unstainability with ruthenium red.

To clarify the differences between the two strains, we compared the chromosome patterns obtained by pulsed-field gel electrophoresis (electrophoretic karyotypes). As shown in Fig. 2, the karyotypes were very different between the two strains: under electrophoretic conditions to separate chromosomal DNAs smaller than 3 Mbp, strain NC64A gave seven bands ranging in size from 1 Mbp to 2.8 Mbp (lane 2). In contrast, only four chromosome bands (1 Mbp–2.6 Mbp) were separated for strain 211-6 (lane 3), whose pattern was apparently different from that of strain NC64A. From these results, the phylogenetic difference between the two strains was confirmed.

Since chloroviruses specifically attach to the host cell wall and digest it at the point of attachment in the initial stage of infection, cell walls of host *Chlorella* strains may share characteristic properties. Table 2 compares the sugar composition of the cell walls of strains NC64A, 211-6, and Pbi. The presence of glucosamine and relatively high contents of glucose and rhamnose are characteristics common to the three strains. It may be worth noting that both strains NC64A and 211-6, which serve as hosts for NC64A viruses, contain little fucose, in contrast with SAG-241-80, which is a host of Pbi viruses. The treatment of the cell wall materials from strain 211-6 with vAL-1 enzyme produced degradation products whose separation pattern on TLC was very similar to that of the NC64A cell wall[16] (data not shown). These properties may be involved in the specific host-virus interaction.

As shown in Table 1, these strains are all sensitive to CVK2-encoded lytic enzymes, so that some structural com-
ponents common to their cell walls may be specifically degraded by the enzymes. Such components may include virus-receptors. To test this possibility, we examined the attachment of CVK2 particles to host cells pretreated with lytic enzymes. Cells of strain NC64A and 211-6 (2 x 10^7 cells/10^9 l) were treated with 1 x 10^9 g of purified vChta-1 chitosanase, vChti-1 chitinase, and vAL-1 lytic enzyme, respectively, at 25°C for 6 h. The washed cells were added with CVK2 (2 x 10^7 pfu), stained with DAPI, and observed with a fluorescence microscope. The attachment of CVK2 particles to host cells was not markedly affected by the treatment with these enzymes, except for when NC64A and 211-6 cells were treated with vAL-1. This treatment reproducibly reduced the number of CVK2 particles attached to the cell wall (approximately 80% compared to the untreated control). These results were confirmed by a more quantitative method, a virus adsorption assay. When cells of strains NC64A and 211-6 were treated with vChta-1 chitosanase, 5.7% and 3.4% of CVK2 particles were unattached to the cells, respectively (Table 3). Similarly, 3.4% and 9.1% of viruses were detected unadsorbed to NC64A and 211-6 cells, respectively, following treatment with vChti-1 chitinase. Treatment with vAL-1 had a more marked inhibitory effect on the attachment: 13.6% and 17.0% of viruses were unattached to enzyme-treated NC64A and 211-6 cells, respectively. When the cells were treated with a mixture of the three enzymes, 55–60% of the virus particles could not attach to the cells of either strain (Table 3). These results indicate that some components of the cell wall of both strains were specifically removed or degraded by these enzymes and vAL-1-sensitive molecules may be involved in the virus-host interaction.

To determine if the virus receptor is solubilized and re-

Table 2. Comparison of sugar composition of Chlorella cell walls.

<table>
<thead>
<tr>
<th>Sugars</th>
<th>Percentage of total sugars</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strains NC64A^a</td>
</tr>
<tr>
<td>Arabinose</td>
<td>5.3</td>
</tr>
<tr>
<td>Fucose</td>
<td>0.7</td>
</tr>
<tr>
<td>Galactose</td>
<td>9.1</td>
</tr>
<tr>
<td>Glucose</td>
<td>51.3</td>
</tr>
<tr>
<td>Mannose</td>
<td>5.3</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>16.0</td>
</tr>
<tr>
<td>Xylose</td>
<td>5.5</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>6.9</td>
</tr>
<tr>
<td>Unknown</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
</tr>
</tbody>
</table>

^a Data are from Meints et al.11).
^b Data are from Takeda18).
^c Data are from Kapaun et al.6).
^d Exact amount is unknown.

cells, respectively (Table 3). Similarly, 3.4% and 9.1% of viruses were detected unadsorbed to NC64A and 211-6 cells, respectively, following treatment with vChti-1 chitinase. Treatment with vAL-1 had a more marked inhibitory effect on the attachment: 13.6% and 17.0% of viruses were unattached to enzyme-treated NC64A and 211-6 cells, respectively. When the cells were treated with a mixture of the three enzymes, 55–60% of the virus particles could not attach to the cells of either strain (Table 3). These results indicate that some components of the cell wall of both strains were specifically removed or degraded by these enzymes and vAL-1-sensitive molecules may be involved in the virus-host interaction.

To determine if the virus receptor is solubilized and re-

Table 3. Adsorption of CVK2 to Chlorella cells treated with the enzymes.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Percentage of unbound CVK2^a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strains NC64A</td>
</tr>
<tr>
<td>No enzyme</td>
<td>0</td>
</tr>
<tr>
<td>vChta-1</td>
<td>5.7</td>
</tr>
<tr>
<td>vChti-1</td>
<td>3.4</td>
</tr>
<tr>
<td>vAL-1</td>
<td>13.6</td>
</tr>
<tr>
<td>Mixture</td>
<td>60.0</td>
</tr>
</tbody>
</table>

^a Data are means of three different experiments with standard deviations of less than 2%.
^b The number of CVK2 plaques that appeared when a control strain (C. ellipsoidea C-87) without enzyme treatment was used for adsorption was 100%.
leased intact by vAL-1 treatment, *Chlorella* NC64A cell wall preparations were treated with purified vAL-1 for 24 h as before\(^7\) and the soluble fraction was tested for effects on CVK2 attachment. The soluble fraction had no effect on CVK2 infection (data not shown), indicating that the treatment with vAL-1 destroyed the virus receptor. The CVK2 infection was also unaffected by the soluble fraction of the cell wall treated with a mixture of vChta-1, vChti-1, and vAL-1.

**Discussion**

In this study, we have demonstrated that CVK2-encoded lytic enzymes actually function on host *Chlorella* cells (Fig. 1). The activities of these enzymes apparently contribute to host-selection by viruses, because a good correlation was seen between the virus-sensitivity and the lytic enzyme-sensitivity of *Chlorella* strains, except for several strains belonging to *C. vulgaris* and a few zoochlorellae (Table 1). In addition to the strains listed in Table 1, many strains of *C. fusca*, *C. saccharophila*, *C. ellipsoidea*, and *C. luteoviridis* were also resistant to these enzymes (data not shown). The authentic host, *Chlorella* strain NC64A, was previously assigned to *C. vulgaris* based on several taxonomic characteristics\(^1\). The data obtained in the present study (Table 1) support an affinity of strain NC64A to *C. vulgaris*. The finding that CVK2 lytic enzymes specifically function on the laboratory strains of *C. vulgaris* means that certain carbohydrate polymers which are sensitive to the enzymes are also common to the cell wall of these strains but that the virus receptor molecules are not present. Presumably the cell wall of these strains, like other *Chlorella* strains, is a composite of several polymers. As shown in this study (Table 3), at least part of the receptor may be destroyed or modified by treatment with vAL-1. Sugimoto et al.\(^{16}\) revealed that vAL-1 degrades NC64A cell wall materials and releases smaller molecules that can be separated on TLC. The released degradation products were demonstrated to have inhibitory effects on the attachment of CVK2 to host cells (Table 3). At present, the nature of these molecules is unknown, but they most likely contain acidic sugar components such as uronic acids since only TLC under conditions to separate acidic sugars could detect these molecules\(^{16}\). Our results are consistent with the report of Meints et al.\(^{11}\) that the virus receptor is a carbohydrate which is unaffected by treatment with cellulase and pectinase but is destroyed by an enzyme preparation from PBCV-1 lyase.

In the course of this study, we found *C. protothecoides* 211-6 to serve as a host for chloroviruses. This strain was originally isolated from *P. bursaria* in the USA by Loefer\(^{10}\) and was formerly labeled as *C. paramecii* Loefer 211-6\(^{6}\) or *C. protothecoides* Kruger CCAP 211-6\(^{1,5}\). Based on taxonomical criteria, Douglas and Huss\(^1\) confirmed the assignment of this strain to *C. protothecoides*. The electrophoretic karyotypes determined in this study also could be used to distinguish between strains 211-6 and NC64A (Fig. 2). CVK2 as well as other NC64A viruses, including PBCV-1, infected strain 211-6 with the same efficiency as strain NC64A. No host-dependent changes in the viral genome or particles were observed between CVK2 progeny grown on strains NC64A and 211-6. Therefore, in spite of taxonomical differences, both strains must share common properties that are required for recognition and replication of chloroviruses. A detailed comparison of these strains will provide insight into the molecular mechanisms involved in host-virus interaction and cell wall digestion, and the penetration of host cells by chloroviruses.

*Chlorella* sp. SAG-241-80 is a laboratory host of European Pbi-viruses, and NC64A-viruses do not attach to this strain. However, as shown in Table 1, all four lytic enzymes encoded by CVK2 functioned on this strain, indicating that some components of its cell wall are similar to those of strains NC64A, 211-6, and *C. vulgaris*. From this result, it is predicted that Pbi-viruses also encode very similar lytic enzymes and utilize them in infection. Moreover, the host-selectivity of chloroviruses may depend solely on some structural differences of receptor molecules for viruses on the cell wall. The biochemical characterization of cell wall components of strains NC64A, 211-6, and SAG-241-80 should help to clarify this matter.

The only known hosts for chloroviruses are symbiotic zoochlorellae, some of which can be cultured under laboratory conditions. *Chlorella* strains NC64A, 211-6, and SAG-241-80 were all originally isolated from *P. bursaria*, and are zoochlorellae. Therefore, we are interested in testing zoochlorellae recently isolated from *P. bursaria* for their sensitivity to CVK2 lytic enzymes. As shown in Table 1, three zoochlorella strains isolated in Japan (PB3-5) were sensitive to the enzymes, although vChta-1 chitosanase and vChti-1 chitinase worked weakly. The other six strains isolated in various areas of Japan and China showed complete resistance to the enzymes. These results suggest that zoochlorellae are naturally heterogeneous in terms of their sensitivity to the viral enzymes, and that they are not always the only natural hosts of chloroviruses.
Acknowledgements

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References