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Characterization of a β-1,3-Glucanase Encoded by Chlorella Virus PBCV-1[1]

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Sequence analysis of the 330-kb chlorella virus PBCV-1 genome revealed an open-reading frame, A94L, that encodes a protein with significant amino acid identity to Glycoside Hydrolase Family 16 β-1,3-glucanases. The *a94l* gene was cloned and the protein was expressed as a GST-A94L fusion protein in *Escherichia coli.* The recombinant A94L protein hydrolyzed the β-1,3-glucose polymer laminarin and had slightly less hydrolytic activity on β-1,3-1,4-glucose polymers, lichenan and barley β-glucan. The recombinant enzyme had the highest activity at 65°C and pH 8. We predicted that the *a94l*-encoded β-1,3-glucanase is involved in degrading the host cell wall either during virus release and/or is packaged in the virion particle and involved in virus entry. Therefore, we expected *a94l* to be expressed late in virus infection. However, contrary to expectations, both the *a94l* mRNA and the A94L protein appeared 15 min after PBCV-1 infection and disappeared 60- and 120-min p.i. postinfection, respectively, indicating that *a94l* is an early gene. Twenty-seven of 42 chlorella viruses contained the *a94l* gene. To our knowledge, this is the first report of a virus-encoded β-1,3-glucanase.

INTRODUCTION

Microorganisms and higher plants often either contain or encounter polysaccharides classified as β-1,3-glucans. For example, β-1,3-glucans serve as structural components of fungal cell walls, as cytoplasmic and vacuolar reserve materials, and as extracellular substrates (Bielecki and Galas, 1991). Consequently, enzymes that degrade β-1,3-glucans are widely distributed among bacteria, fungi, and plants where they participate in many physiological functions, including β-glucan mobilization in fungi (Bielecki and Galas, 1991) and fungal pathogen-plant interactions (de la Cruz *et al.,* 1995).

Genes encoding β-1,3-glucanases and β-1,3-1,4-glucanases have been characterized from many bacteria including *Bacillus* species (Murphy *et al.,* 1984; Hofemeister *et al.,* 1986; Borriss *et al.,* 1990; Yahata, *et al.,* 1990; Fiske *et al.,* 1990), *Fibrobacter succinogenes* (Erfle *et al.,* 1988; Irvin and Teather, 1988), *Cellvibrio mixtus* (Sakellaris *et al.,* 1993), *Thermotoga neapolitana* (Dakhova *et al.,* 1993), *Ruminococcus flavefaciens* (Flint *et al.,* 1989, 1993), *Oerskovia xanthineolytica* (Ferrer *et al.,* 1996), *Clostridium thermocellum* (Schimming *et al.,* 1991, 1992), and *Rhodothermus marinus* (Spilliaert *et al.,* 1994). Bacterial *endo*-β-1,3-glucanases (also called laminarinases) and *endo*-β-1,3-1,4-glucanases (also called lichenases) have common amino acid sequences. Together with a few other glycosidases, these enzymes compose the Glycoside Hydrolase Family 16 (Henrissat, 1991; Henrissat and Bairoch, 1993, 1996). In contrast, most eukaryotic *endo*-β-1,3-glucanases and *endo*-β-1,3-1,4-glucanases are found in Glycoside Hydrolase Family 17. However, exceptions to this bacterial-eukaryotic paradigm are being discovered. For example, *endo*-β-1,3-glucanases from the Archaeon *Pyrococcus furiosus* (Gueguen *et al.,* 1997), sea urchin eggs (Bachman and McClay, 1996), and the fungus *Cochliobolus carbonum* (Gorlach *et al.,* 1998) resemble Family 16 glycoside hydrolases.

While sequencing the 330-kb dsDNA genome of chlorella virus PBCV-1, the prototype virus of the genus *Chlorovirus* (family Phycodnaviridae) (Van Etten and Meints, 1999), we discovered a gene, *a94l,* encoding a putative β-1,3-glucanase (Li *et al.,* 1995). In this manuscript, we show that: (i) *a94l* encodes a functional β-1,3-glucanase, (ii) both transcription of the *a94l* gene and translation of the A94L protein occur early after PBCV-1 infection, and (iii) the *a94l* gene is common, but not universal in chlorella viruses.

RESULTS

Sequence analyses

The deduced 364 amino acid protein encoded by the PBCV-1 *a94l* gene (Fig. 1A) has 26 to 30% amino acid identity with Family 16 *endo*-β-1,3-glucanases from several bacteria including *Bacillus circulans* strains WL-12 (Yahata *et al.,* 1990) and IAM1165 (Yamamoto *et al.,* 1993),

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A

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  1  MSQVDTVVDS VVDVENHQPT HIDTFPYNKR VIESKPKKNM IVRGVVICMA
 51  ILIFGGAIAT AIVVSSDNSS DQAPAPAPGP ALIYKGAYID EPPPFEPKAG
101  FEAMWWDEFD GEEIDRTKWY IQPDIVDYYT GNRQIQHYID SPSTIEVSND
151  TLHIIANNPG EVQYNETSSN YDQTYYTSAR INTKTTGGHW YPGMEVNGTT
201  WNTIRVEARL KAPRGPGVVG AFWMLPIDNS CFPEIDIFET PYCERASMGT
251  WYVNKDVPRG ISKHGTTITE SYDKFCDEYV TYAVEWNADY IAFYAGDAET
301  PVFVTGKEIW AGKCDANDTD APYNRPFYII LNTSIGSAWG GIPLNDIFPA
351  VLDVDYVRVS GIRD*
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B

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                                          *     *
Rhodothermus marinus (O52754)          145--DNGEIDIMEHVGFNPDVVHGT--[276]
Bacillus circulans (P23903)            549--ASGEIDVMEARGRLPGSVSGT--[682]
Bacillus circulans (Q45095)            552--SSGEIDVMEAKGRLPGSTSGA--[877]
Thermotoga neapolitana (Q60039)        335--TCGEIDIMEMLGHDTRTVLRT--[646]
Oerskovia xanthineolytica (Q51333)     179--TSGEIDIMENVGNAPHEVHGT--[306]
Oerskovia xanthineolytica (O68641)     168--SSGEIDIMENVGFEPHRVHGT--[435]
Pyrococcus furiosus (O73951)           167--NCGEIDIMEFLGHEPRTIHGT--[297]
Strongylocentrotus purpuratus (Q26660) 325--ASGEIDLVESRGNADIKDADG--[499]

PBCV-1 (Q84415)                        231--CFPEIDIFETPYCERASM-GT--[364]

Bacillus amyloliquefaciens (P07980)    127--PWDEIDI-EFLGKDTTKVQFN--[239]
Bacillus brevis (P37073)               139--PWDEIDI-EFLGKDTTRIQFN--[259]
Bacillus licheniformis (P27051)        131--PWDEIDI-EFLGKDTTKVQFN--[243]
Bacillus macerans (P23904)             125--QWDEIDI-EFLGKDTTKVQFN--[237]
Bacillus polymyxa (P45797)             126--QWDEIDI-EFLGKDTTKVQFN--[238]
Bacillus sp. (A00896)                  141--PWDEIDI-EFLGKDTTKIQFN--[276]
Bacillus subtilis (Q45691)             127--PWDEIDI-EFLGKDTTKVQFN--[239]
Bacillus subtilis (P04957)             130--PWDEIDI-EFLGKDTTKVQFN--[242]
Clostridium thermocellum (P29716)      133--PWDEIDI-EFLGKDTTKVQFN--[334]
Fibrobacter succinogenes (P17989)       76--PWVEVDI-EVLGKNPGSFQSN--[349]
Ruminococcus flavefaciens (Q53317)     687--PWDEIDI-EILGKNTTQVQFN--[802]
Streptococcus bovis (O07856)           124--KWDEIDI-EFLGKDTTKVQFN--[237]
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FIG. 1. (A) The deduced amino acid sequence of the PBCV-1-encoded glucanase. The catalytic domain for Glycoside Hydrolase Family 16 glucanases (Prosite-PS01034) is underlined. (B) Alignment of the active site domain of the PBCV-1-encoded glucanase with β-1,3-glucanases (above the PBCV-1 sequence) and β-1,3-1,4-glucanases (below the PBCV-1 sequence) from other organisms. The two amino acids Glu^{234} and Glu^{239} (asterisks) critical for glucanase activity are indicated (Hoj *et al.,* 1992; Juncosa *et al.,* 1994; Hahn *et al.,* 1995).

O. xanthineolytica (Ferrer *et al.,* 1996), *R. marinus* (Borriss and Krah, Accession No. O52754), and the Archeon *P. furiosus* (Gueguen *et al.,* 1997). These proteins range in size from 276 to 877 amino acid residues. The catalytic site of Family 16 *endo*-β-1,3- and *endo*-β-1,3-1,4-glucanases contains the sequence E(LIV)D(LIV)$x_{0,1}$Ex_2(GQ)(KRNF)x(PSTA) (Prosite PS01034). Crystal structures, as well as inhibitor and site-directed mutation studies of *Bacillus* β-1,3-1,4-glucanases have established that the two Glu and probably the Asp residues in this sequence are essential for catalysis (Ay *et al.,* 1998; Hahn *et al.,* 1995, 1995a; Hoj *et al.,* 1992; Juncosa *et al.,* 1994; Keitel *et al.,* 1993). Two amino acids in this catalytic region typically distinguish β-1,3-glucanases from β-1,3-1,4-glucanases (Fig. 1B). The first Glu in β-1,3-glucanases is usually preceded by a Gly rather than an Asp residue and β-1,3-glucanases have an extra amino acid, typically a Met, preceding the second Glu. The PBCV-1 A94L ORF contains a EIDIFETPycEr sequence, including the three critical amino acids Glu^{234}, Asp^{236}, and Glu^{239}. The PBCV-1 ORF is clearly more similar to β-1,3-glucanases than to β-1,3-1,4-glucanases. However, the putative active site of A94L differs slightly from other β-1,3-glucanases; the most significant difference is a Tyr at amino acid residue 242 rather than the consensus Gly (Fig. 1B). The predicted molecular weight of the A94L protein is 40,734 and the predicted isoelectric point is 4.4.

The coding region of the PBCV-1 *a94l* gene is 45% G + C which is slightly higher than the 40% G + C content of the PBCV-1 genome. The 49 to 90 nucleotide region preceding the *a94l* ATG translation start codon contains

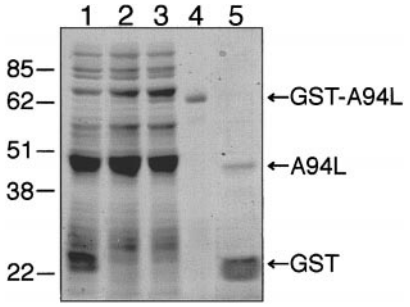

FIG. 2. SDS-PAGE analysis of protein from *E. coli* BL21 (DE3) expressing the PBCV-1 A94L fusion protein. Arrows indicate the position of the recombinant A94L-GST-fusion protein, and the thrombin cleaved A94L and GST peptides. Soluble extracts from cells containing plasmid pGEX-2T (lane 1) and recombinant plasmid pGEX-2T-A94L, before (lane 2) and 3 h after induction (lane 3). Protein after chromatography over a GST-binding column before (lane 4) and after thrombin cleavage (lane 5).

six direct repeats consisting of the seven nucleotide sequence ATGACAA. The first 6 nucleotides of this sequence resemble the TTGACA sequence characteristic of the -35 portion of bacterial -35 and -10 promoters. The ATGACAA sequence closest to the ATG start codon is 15 nucleotides from a TAAAAT sequence, which resembles the consensus bacterial -10 TATAAT sequence, and it has the proper spacing of a bacterial -35 and -10 promoter sequence.

Expression of the *a94l* gene in *E. coli*

The *a94l* gene was amplified from PBCV-1 DNA by the polymerase chain reaction (PCR), cloned into vector pGET-2T, and expressed in *E. coli* BL21 (DE3) as a glutathione *S*-transferase (GST)-A94L-fusion protein. Cells containing plasmid pGEX-2T-A94L produced the expected 68-kDa recombinant fusion protein (Fig. 2) after induction with isopropyl-β-D-thiogalactopyranoside (IPTG). About 20% of the GST-A94L-fusion protein was in the soluble fraction of the bacterial extract. Purification of the fusion protein over a glutathione-Sepharose 4B column and cleavage with thrombin produced proteins of the expected size (Fig. 2, lanes 4 and 5).

ORF A94L encodes a functional glucanase

The purified recombinant GST-A94L-fusion protein hydrolyzed laminarin (a β-1,3-glucan) in a linear fashion for 10 min (Fig. 3A) and the reaction depended on protein concentration (Fig. 3B). The recombinant protein was active from pH 4 to 10, with an optimum at about 8 (Fig. 3C). At pH 8, glucanase activity increased slightly as the incubation temperature increased from 25 to 65°C (Fig. 3D). Glucanase activity was inhibited significantly by 1 mM silver, cobalt, copper, or manganese ions (Table 1).

Substrate specificity of the PBCV-1 glucanase

The ability of the GST-A94L protein to release glucose from polysaccharides is summarized in Table 2. The

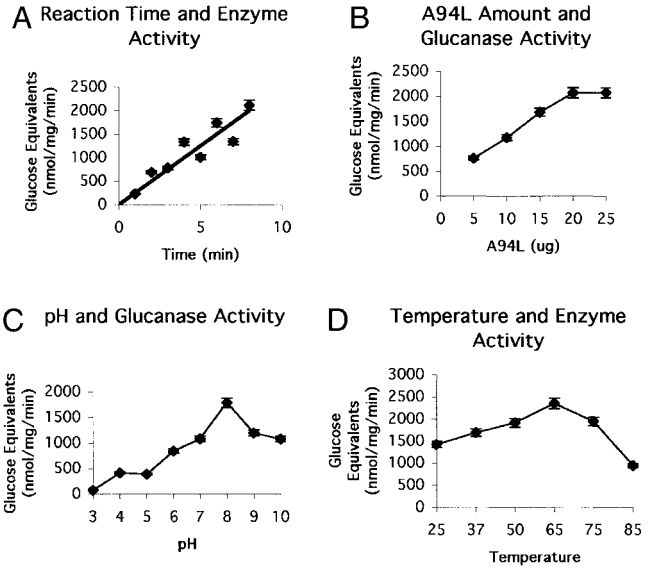

FIG. 3. Effect of time (A), protein concentration (B), pH (C), and temperature (D) on PBCV-1 recombinant glucanase activity.

protein was most active with laminarin as a substrate and had slightly less activity with the two β-1,3-1,4-glucans, barley β-glucan and lichenan. The protein did not hydrolyze CM cellulose (β-1,4-glucan), glycogen (α-1,4:1,6-branched glucan), dextran (α-1,6-glucan), and salicin (β-glucoside). Cell extracts from *E. coli* containing the vector alone were inactive with all substrates. These experiments establish that A94L encodes a functional glucanase that can hydrolyze both β-1,3- and β-1,3-1,4-glucans.

Transcription and translation of PBCV-1 *a94l*

RNA was extracted from *Chlorella* NC64A cells at various times after virus infection and hybridized to an *a94l*-specific probe in order to determine when the gene

TABLE 1

Effect of Metal Ions on PBCV-1-Encoded Glucanase Activity

Salt (1 mM)	β-1,3-Glucanase activity[a] (as % of control)
None	100%
$AgNO_3$	35%
$CaCl_2$	126%
$CoCl_2$	56%
$CuCl_2$	66%
$FeCl_3$	101%
$HgCl_2$	101%
$MgCl_2$	81%
$MnCl_2$	38%
$NiNO_3$	124%
$ZnCl_2$	83%

[a] The recombinant enzyme was incubated with the salt for 1 h at 25°C prior to addition of the substrate.

TABLE 2

Ability of the PBCV-1-Encoded Glucanase to Cleave Various Polysaccharides

Substrate	Structure	% Glucose released[a]
Laminaran	β-1,3-glucan	100
Barley β-glucan	β-1,3-1,4-glucan	87
Lichenan	β-1,3-1,4-glucan	75
CM cellulose	β-1,4-glucan	nd[b]
Glycogen	α-1,4:1,6-branched glucan	nd
Dextran	α-1,6-glucan	nd
Salicin	β-glucoside	nd

[a] Relative to the glucose released from laminaran.
[b] nd is none detected.

is transcribed during PBCV-1 replication. The *a94l* probe hybridized to a diffuse 1.1-kb RNA band present in cells at 15, 30, 45, and 60 min postinfection (p.i.) (Fig. 4A). The intensity of the RNA band, which is the expected size for the *a94l* gene, is strongest at 15 min p.i. and disappears by 60 min p.i. Since PBCV-1 DNA synthesis begins at 60 to 90 min p.i. (Van Etten *et al.,* 1984), *a94l* is an early gene. The diffuse nature of the *a94r* transcript suggests that the RNA might have been isolated under less than ideal conditions. However, repeated RNA isolations and *a94l* hybridization experiments always produced the same diffuse pattern, whereas, well-defined RNA bands were obtained on sister blots hybridized with other PBCV-1 gene probes. It has been our experience that certain PBCV-1 genes produce diffuse hybridization signals, whereas others do not. Possible explanations are that the *a94l* gene lacks a well-defined transcriptional stop signal or that the *a94l* mRNA is rapidly degraded.

Western blotting detected the A94L protein in cell extracts prepared at various times after PBCV-1 infection. Antibody to the A94L-GST-fusion protein reacted with two protein bands, 45 and 41 kDa, expressed at 30, 45, 60, 90, and 120 min p.i. (Fig. 4B). The 45-kDa band electrophoreses at the same position as recombinant A94L protein minus the GST tag, even though the predicted size of the A94L protein is 41 kDa. Therefore, we suspect that the 41-kDa migrating protein band is either a degradation product or a posttranslational modified product of A94L. Interestingly, both of these proteins disappear in virus infected cells by 120 min p.i. This disappearance indicates that the A94L protein is either degraded during late stages of virus replication or that it is released into the medium. Although the A94L protein lacks an obvious N-terminal export signal sequence, we looked for the protein in the growth medium. Cells were removed from 20 ml of growth medium at 150 and 300 min p.i. by centrifugation and extracellular proteins were precipitated from the supernatant with 5 vol of acetone, electrophoresed on a SDS-polyacrylamide gel (SDS-PAGE), and probed with A94L antiserum. No A94L antiserum-reacting protein was detected. As a control, A94L protein was detected when 2 μg of recombinant protein was added to the medium and carried through the same process.

PBCV-1 glucanase does not degrade the host cell wall

Initially it was hypothesized that the glucanase might help degrade the host cell wall during virus release and/or the enzyme is packaged in the virion particle and digests the cell wall during virus entry. In either scenario, *a94l* should be expressed late in virus infection. However, Northern and Western analyses indicate that *a94l* is transcribed and translated early in virus replication; consequently the glucanase is probably not involved in host cell wall degradation. Four additional experiments support this conclusion. (i) No A94L protein was detected in the PBCV-1 virion by Western analyses. (ii) Incubating *Chlorella* NC64A ghost cells (Meints *et al.,* 1988) overnight with recombinant glucanase did not prevent subsequent PBCV-1 attachment, indicating the enzyme does not cleave the host virus receptor (Table 3). As a control, the ghost cells were treated with equivalent protein concentrations of "lysin," a crude enzyme extract prepared from PBCV-1 lysates that degrades the chlorella cell wall and destroys the virus receptor (Table 3) (Meints *et al.,* 1988). (iii) Addition of A94L antibody to the lysin preparation did not inhibit its activity (Table 3). (iv) The A94L protein was not detected in the lysin preparation by Western analyses.

Occurrence of *a94l* gene in other chlorella viruses

To determine the distribution of the *a94l* gene among chlorella viruses, the *a94l* probe was hybridized to DNA from 41 virus isolates from diverse geographical regions

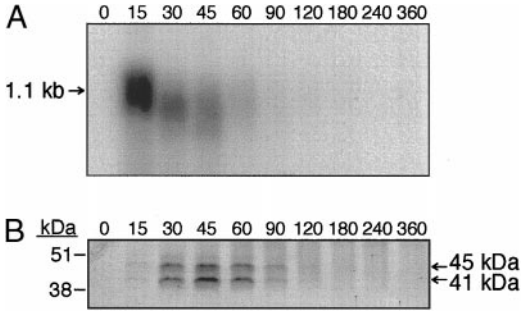

FIG. 4. (A) Transcription of PBCV-1-encoded glucanase gene *a94l.* Total RNAs were isolated from uninfected (lane 1) and PBCV-1-infected cells at the indicated times and hybridized with a ^{32}P-labeled *a94l* probe. (B) Appearance of PBCV-1-encoded glucanase. Total proteins were isolated from uninfected (lane 1) and PBCV-1-infected cells at the indicated times. The proteins were probed with antiserum against A94L-GST. Note: the thrombin-treated recombinant A94L band (Fig. 2, lane 5) migrates at the same rate as the larger (45 kDa) of the two reacting bands.

TABLE 3

Ability of PBCV-1 to Adsorb to *Chlorella* NC64A Ghosts after Treatment with Lysin or PBCV-1-Encoded Glucanase

Sample[a]	PBCV-1 titer ($\times 10^7$ PFU/ml)	% unbound
Control (minus ghosts)	150	100
Ghosts	14	9
Ghosts incubated with 10 μg lysin for 20 h	139	93
Ghosts incubated with 10 μg lysin and 1 μl of A94L-GST antiserum for 20 h[b]	123	82
Ghosts incubated with 10 μg lysin and 5 μl A94L-GST antiserum for 20 h[b]	112	75
Ghosts incubated with 10 μg A94L-GST for 20 h	19	13
Ghosts incubated with 40 μg A94L-GST for 20 h	20	13

[a] *Chlorella* NC64A ghosts (1.0×10^8; cell walls) were incubated for 20 h with either lysin, lysin plus A94L-GST antiserum, or A94L-GST enzyme for 16 h at 25°C. After this treatment, 1.0×10^9 PBCV-1 were added to each reaction and incubated for 1 h at 25°C, and the samples were plaqued for virus. The numbers reflect PBCV-1 attachment to the host receptor.

[b] The lysin was preincubated with A941-GST antiserum for 5 h at 4°C prior to the 20-h incubation with the ghosts.

(Fig. 5). These viruses infect either *Chlorella* NC64A or *Chlorella* Pbi. This experiment produced the following results. (i) The PBCV-1 *a94l* probe did not hybridize to host *Chlorella* NC64A DNA. (ii) The *a94l* probe hybridized to DNAs from 28 of the 37 viruses (including PBCV-1) that infect *Chlorella* NC64A (NC64A viruses). (iii) The *a94l* probe did not hybridize to DNAs from the five viruses, CVA-1, CVB-1, CVG-1, CVM-1, and CVR-1, that infect *Chlorella* Pbi (Pbi viruses) (Reisser *et al.,* 1988).

Clearly *a94l* expression is not essential for chlorella virus growth since 10 NC64A viruses lack the gene. Interestingly, the hybridization pattern of *a94l* to the 37 NC64A viruses is identical to the hybridization pattern of another PBCV-1 virus gene, *a98r* (Graves *et al.,* 1999). The *a98r* gene, which is adjacent to *a94l,* encodes a glycosyltransferase enzyme, hyaluronan synthase. Assuming that these two genes are colinear in the NC64A viruses, the apparent absence of both genes in certain viruses suggests that these genes are acquired or deleted as a unit. The assumption of colinearity among chlorella viruses is supported by the report that the overall gene order of another NC64A virus, CVK2, resembles that of PBCV-1 (Nishida *et al.,* 1999).

Phylogenetic analyses

The complete multiple sequence alignment of 43 glucanase genes with amino acid sequence composition similar to PBCV-1 A94L was 1660 residues in length. Of these, only 684 aligned positions inferred confident homology statements. Maximum-likelihood and parsimony-jackknife trees differed only slightly in terms of resolution of clades and support indices. Both solutions depict a poorly resolved tree with a large polytomy that includes the PBCV-1 *a94l* gene (Fig. 6). The high degree of sequence dissimilarity and difficulty in inferring homology prevents a reliable reconstruction of its evolutionary history despite the presence of ample phylogenetic signal ($P < 0.001$; Lyons-Weiler *et al.,* 1996).

DNA sequencing errors

As individual PBCV-1 genes are characterized, we resequence them and their flanking regions to check for errors in the original DNA sequence. This DNA sequencing effort revealed two mistakes in the 3′ flanking regions of the *a94l* gene, an A after position 48,912 and a G after position 49,011 were missed. Insertion of these two nucleotides creates a single 359 codon ORF called A92/93L rather than two separate ORFs, A92L and A93L. The deduced protein translation product of A92/93L did not resemble any proteins in the databases except PBCV-1 ORFs A278L, A282L, and A676R. Thus these four ORFs represent a PBCV-1 gene family. With these two nucleotide additions, the PBCV-1 genome consists of

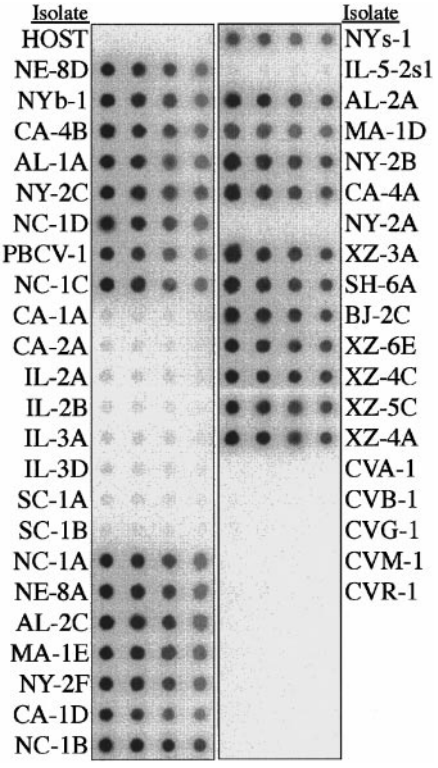

FIG. 5. Hybridization of the PBCV-1 *a94l* probe to DNA isolated from *Chlorella* NC64A and from 37 NC64A viruses and 5 Pbi viruses (CVA-1, CVB-1, CVG-1, CVM-1, and CVR-1). The DNAs were hybridized with a ^{32}P-labeled *a94l* probe. The blots contain 1, 0.5, 0.25, and 0.12 μg DNA, left to right, respectively.

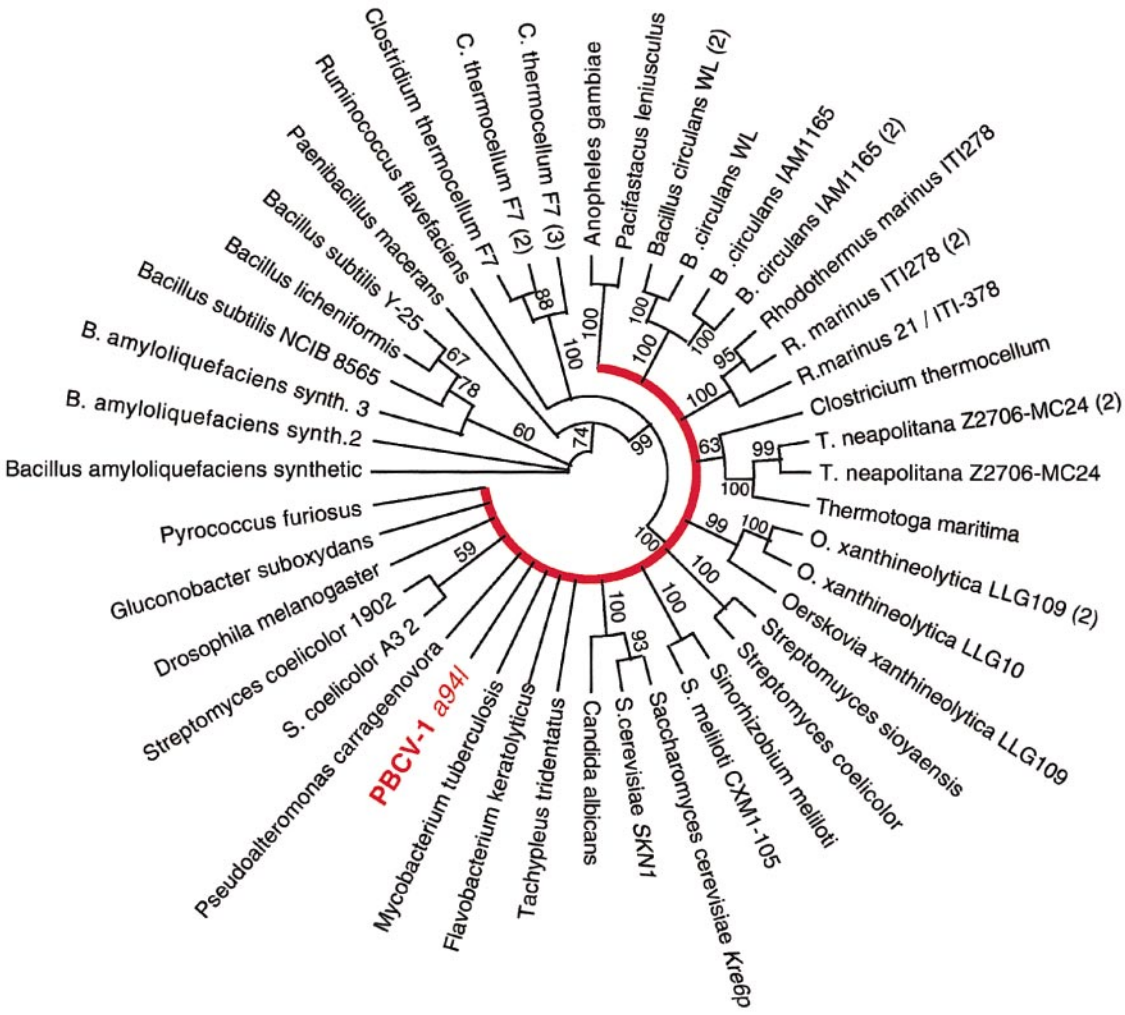

FIG. 6. Parsimony-jackknife tree (10,000 replicates, 37% deletion with fast-stepwise addition, emulating jaq resampling). PBCV-1 gene *a94l* and its membership in a large polytomous clade are highlighted in red.

330, 744 bp and is predicted to contain 375 protein encoding genes.

DISCUSSION

This paper establishes that the chlorella virus PBCV-1 *a94l* gene encodes a β-1,3-glucanase. PBCV-1 is the first virus known to encode a β-1,3-glucanase. The amino acid sequence of the virus-encoded enzyme, especially in the catalytic region, and its ability to release glucose from β-1,3-glucans (laminarin) as well as mixed β-1,3-1,4-glucans (barley β-glucan and lichenan) places the enzyme in Glycosyl Hydrolase Family 16 (Henrissat, 1991; Henrissat and Bairoch, 1993, 1996). Like other β-1,3-glucanases, the PBCV-1 enzyme did not degrade CM cellulose (β-1,4-glucan), glycogen (α-1,4:1,6-branched glucan), dextran (α-1,6-glucan), or salicin (β-glucoside).

Bacterial β-1,3- and β-1,3-1,4-glucanases are usually transported out of the cell and hydrolyze extracellular polysaccharides. Consequently, bacterial enzymes have N-terminal 20 to 30 amino acid signal peptides. The PBCV-1 enzyme contains no obvious N-terminal signal. Furthermore, no extracellular A94L protein was detected in the culture medium of virus-infected cells.

Like many bacteriophages, PBCV-1 infects its host *Chlorella* NC64A by attaching to the cell wall, degrading the wall with a virus-packaged enzyme(s), and then releasing viral DNA into the cell (Meints *et al.,* 1984; Van Etten *et al.,* 1991). After a 6- to 8-h replication phase, localized lysis of the host cell wall releases nascent PBCV-1 particles (Meints *et al.,* 1986; Van Etten *et al.,* 1991). Thus, digestion of host chlorella cell walls is critical at two stages of the PBCV-1 life cycle. Consequently, one predicts that the virus encodes one or more cell wall degrading enzymes, including a possible β-1,3-glucanase. If this prediction(s) is correct, *a94l* should be expressed late and the enzyme might be packaged in the virions. However, contrary to these expectations, *a94l* is expressed early, the A94L protein is not packaged in the

virion, and prior incubation of host walls with the recombinant enzyme does not alter virus attachment. An even bigger surprise is that the A94L protein is the first PBCV-1-encoded protein we have observed to disappear during late stages of virus replication.

If the PBCV-1 β-1,3-glucanase is not involved in degrading the host cell wall, then it must serve another function. One possibility is degradation of host storage polysaccharides (i.e., β-glucans) to produce glucose as an energy source for virus replication. In this regard, laminarin (a β-1,3-glucan) is a common reserve polysaccharide in many algae, especially brown algae (Craigie, 1974). However, there is no evidence *Chlorella* NC64A contains laminarin. In fact, starch (α-1,4-glucans) is usually the primary storage polysaccharide of green algae (Van den Hoek *et al.,* 1995).

Complicating the biological significance of the PBCV-1-encoded β-1,3-glucanase is the finding that about 25% of chorella viruses lack the *a94l* gene. It may be significant that all the viruses that lack the *a94l* gene also lack the *a98r* gene, which is adjacent to the *a94l* gene in PBCV-1 (Graves *et al.,* 1999). The *a98r* gene encodes hyaluronan synthase, a plasma membrane-associated glycosyltransferase (DeAngelis *et al.,* 1997). Hyaluronan is a linear polysaccharide chain composed of alternating β-1,4-glucuronic acid and β-1,3-*N*-acetylglucosamine residues. Interestingly, both of these sugars are synthesized from glucose, the product of the β-1,3-glucanase enzyme.

Regardless of the biological function of the PBCV-1-encoded β-1,3-glucanase, like the hyaluronan synthase enzyme, it is not essential for survival of the viruses in nature because all of these viruses have been isolated from native sources within the past 20 years.

MATERIALS AND METHODS

Strains and culture conditions

Growth of PBCV-1 host *Chlorella* NC64A on MBBM medium, production and purification of PBCV-1, and isolation of PBCV-1 DNA have been described (Van Etten *et al.,* 1981, 1983). The gene was cloned and expressed in *E. coli* strains XL1-Blue (Stratagene, LaJolla, CA) and BL21 (DE3) (Novagen, Madison, WI), respectively. Plasmid pGEX-2T (Smith and Johnson, 1988) was used to create GST-fusion proteins.

Cloning and expression of the *a94l* gene

The *a94l* gene was amplified from PBCV-1 DNA with Vent DNA polymerase (New England BioLabs, Beverly, MA) by PCR. The 5′ primer (5′-ATCGGATCCATGTCTCAA GTAGACACCG) and the 3′ primer (5′-ACGGAATTCAATCGCGAATGCCTGAAACC) used to amplify the *a94l* gene contained *Bam*HI and *Eco*RI sites (underlined), respectively. The 1.1-kb PCR product was digested with *Bam*HI and *Eco*RI and cloned into the equivalent sites of pGEX-2T. The resultant plasmid, pGEX-2T-A94L, was transformed into *E. coli* XL1-Blue or BL21 (DE3) for expression.

Overnight cultures of *E. coli* BL21 (DE3) containing pGEX-2T-A94L were diluted 10-fold with fresh LB medium and incubated for 1 h at room temperature. Expression of the recombinant fusion protein was induced by adding IPTG to a final concentration of 0.1 mM and cultures were incubated for an additional 3 h.

Purification of recombinant A94L protein and antiserum production

IPTG-induced *E. coli* BL21 (DE3) cells were collected by centrifuging at 5000 rpm for 5 min, resuspended in 0.1 M sodium acetate (pH 5.5), and sonicated on ice for 30 s. The GST-fusion protein was purified by affinity chromatography on a glutathione-Sepharose 4B column (Pharmacia Biotech. Inc., Uppsala, Sweden) and used to produce polyclonal antibody in mice as described previously (Van Etten *et al.,* 1982).

Assay for recombinant glucanase activity

The standard assay for β-1,3-glucanase activity contained 100 μl of 0.5% laminarin (soluble β-1,3-glucan), 20 μl enzyme solution (15 μg of protein), and 180 μl of 0.1 M sodium acetate buffer (pH 5.5). The reaction was incubated at 37°C, for 10 min. Reducing sugars released from laminarin were measured by the method of Dygert *et al.* (1965). One unit of β-1,3-glucanase was defined as the amount of enzyme that released 1 μmol of glucose/min under these conditions.

The optimum pH for the reaction was determined by incubating the reaction at 37°C in either 0.1 M sodium citrate buffer (pH 3.0 to 5.0), 0.1 M $NaPO_4$ buffer (pH 6.0 to 8.0), or 0.1 M glycine buffer (pH 9.0 to 10.0). The purified recombinant GST-fusion protein was preincubated at 25°C for 60 min with the following salts (1 mM each) $AgNO_3$, $BaCl_2$, $CaCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_3$, $FeCl_2$, $HgCl_2$, $MgCl_2$, $MnCl_2$, or $ZnCl_2$; laminaran was added and enzyme activity was assayed under the standard conditions.

RNA analyses

Chlorella cells (1×10^9 cells/sample) were collected by centrifugation at various times after PBCV-1 infection, frozen in liquid nitrogen, and stored at −80°C. Total RNA was isolated using TRIzol reagent (GIBCO BRL, Gaithersburg, MD), electrophoresed on 1.5% agarose/formaldehyde denaturing gels, stained with ethidium bromide, and transferred to nylon membranes (Micro Separation Inc., Westborough, MA). Membranes were subsequently photographed under UV illumination to visualize transfered RNA. RNA was hybridized with a ^{32}P-labeled *a94l*

probe as described (Sun *et al.,* 1999). After hybridization, radioactivity bound to the membranes was visualized using a Storm 840 PhosphorImager and ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA). To monitor possible loading differences between samples, the relative amount of the 3.6-kb rRNA in each lane was determined by converting the photographs of the stained membranes to digital images using a Hewlett-Packard ScanJet 4C scanner and analyzing the images with ImageQuant software.

Protein analyses

Chlorella cells (1×10^9 cells/sample) were collected by centrifugation at various times after PBCV-1 infection and disrupted by vortexing with glass beads (0.25–0.30 mm in diameter) at high speed for 5 min. Purified PBCV-1 virions were disrupted by sonication (Furuta *et al.,* 1997). Proteins (20 μg/sample) were separated by SDS-PAGE (Laemmli, 1970). The protein gels were stained with Coomassie brilliant blue R or transferred to nylon membranes and reacted with antibody (Sun *et al.,* 1999). Protein concentrations were measured by the method of Bradford (1976) with bovine serum albumin as a standard.

Phylogenetic analyses

Sequences were retrieved by gapped pBLAST search (Altschul *et al.,* 1997) and from representative taxa on the CAZY database for Glycoside Hydrolase Family 16 enzymes (EC 3.2.1.39). Protein sequences were aligned by Clustal X (Thompson *et al.,* 1997) and edited manually to optimize the alignment.

From the optimized alignment, 976 ambiguously aligned amino acids were removed. Of the remaining 684, five hundred were informative for global parsimony analysis. The best estimate of gene genealogy was reconstructed via parsimony jackknifing (Farris *et al.,* 1996) (10,000 replicates) as employed in PAUP* version 4.0b4a (Swofford, 2000).

Because of extensive sequence divergence, and thus the possibility of long-branch attraction, a maximum-likelihood tree was also constructed (Felsenstein, 1978; Zharkikh and Li, 1993; but see also Steel *et al.,* 1993). The maximum-likelihood tree was estimated using PUZZLE version 3.1 (Strimmer and von Haeseler, 1996) and assumed the model of Jones *et al.* (1992) with amino acid frequencies estimated from the data set. We also assumed a gamma distributed rate heterogeneity with four rate categories, the gamma distribution being estimated empirically.

All trees were rooted arbitrarily with a synthetic sequence, a hybrid-*endo*-1,3-1,4-β-glucanase of *Bacillus amyloliquefaciens* (GenBank Accession No. CAA81093).

Other procedures

Chlorella virus DNAs used for dot blots were denatured and applied to nylon membranes (Micron Separation Inc.), fixed by UV cross-linking, and hybridized with the same ^{32}P-labeled probes used in the Northern analyses. Preparation of *Chlorella* NC64A ghost cells, the lysin used to degrade the algal cell walls, and virus attachment assays have been described (Meints *et al.,* 1988). DNA fragments were sequenced from both strands at the University of Nebraska Center for Biotechnology DNA sequencing core facility.

DNA sequences were analyzed with the University of Wisconsin Genetics Computer Group package of programs (Genetics Computer Group, 1997). The accession number for the PBCV-1 genome sequence is U42580.

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