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Characterization of Two Chitinase Genes and One Chitosanase Gene Encoded by Chlorella Virus PBCV-1

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Chlorella virus PBCV-1 encodes two putative chitinase genes, *a181/182r* and *a260r,* and one chitosanase gene, *a292l.* The three genes were cloned and expressed in *Escherichia coli.* The recombinant A181/182R protein has endochitinase activity, recombinant A260R has both endochitinase and exochitinase activities, and recombinant A292L has chitosanase activity. Transcription of *a181/182r, a260r,* and *a292l* genes begins at 30, 60, and 60 min p.i., respectively; transcription of all three genes continues until the cells lyse. A181/182R, A260R, and A292L proteins are first detected by Western blots at 60, 90, and 120 min p.i., respectively. Therefore, *a181/182r* is an early gene and *a260r* and *a292l* are late genes. All three genes are widespread in chlorella viruses. Phylogenetic analyses indicate that the ancestral condition of the *a181/182r* gene arose from the most recent common ancestor of a gene found in tobacco, whereas the genealogical position of the *a260r* gene could not be unambiguously resolved.

INTRODUCTION

Chitin, an insoluble linear homopolymer of β-1,4-linked *N*-acetylglucosamine (GlcNAc) residues, is one of the most abundant biopolymers in nature (Cabib, 1987). It is a common component of insect exoskeletons, shells of crustaceans, and fungal cell walls (Gooday *et al.,* 1986). Chitin is specifically degraded by chitinases (exochitinases and endochitinases) and β-*N*-acetylglucosaminidases. Endochitinases randomly cleave the chitin polymer, whereas exochitinases release chitobiose from the nonreducing end of the chitin polymer. Typically β-*N*-acetylglucosaminidases hydrolyze chitobiose to GlcNAc, although some β-*N*-acetylglucosaminidases can also cleave terminal GlcNAc units from the polymer. Chitinases are produced by many organisms in addition to ones that contain chitin, including bacteria and higher plants (Flach *et al.,* 1992). Chitinases from all organisms fall into two phylogenetically distinct glycosyl hydrolase families (Henrissat, 1991; Henrissat and Bairoch, 1993; Henrissat and Davies, 1997; Gooday, 1997). Many organisms contain members of glycosyl hydrolase Family 18, suggesting that genes encoding these enzymes have an ancient origin. In contrast, members of glycosyl hydrolase Family 19 primarily exist in plants.

Chitosan, a β-1, 4-linked polymer of D-glucosamine, is a deacetylated derivative of chitin. Chitin and chitosan are distinguished by the amount of acetylation of the D-glucosamine residues. Polymers containing more than 60% acetylation are considered chitin; those with less than 40% acetylation are called chitosan (Ando *et al.,* 1992). Chitosan, a major structural component of many fungal cell walls (Bartnicki-Garcia, 1968; Datema *et al.,* 1977; Davis and Bartnicki-Garcia, 1984), is cleaved by chitosanases. Chitosanases, which are assigned to glycosyl hydrolase Family 46 (Henrissat and Bairoch, 1996), have no amino acid sequence similarities to those of Family 18 chitinases.

PBCV-1 is a large, polyhedral, plaque-forming, dsDNA-containing virus that infects a unicellular, eukaryotic green alga, *Chlorella* NC64A (Van Etten *et al.,* 1983). The 330-kb PBCV-1 genome has been sequenced and it contains 377 ORFs predicted to encode proteins (Lu *et al.,* 1995, 1996; Li *et al.,* 1995, 1997; Kutish *et al.,* 1996). Three ORFs, A181R, A182R, and A260R, resemble microbial chitinases and one ORF, A292L, resembles bacterial chitosanases (Lu *et al.,* 1996). We were surprised that PBCV-1 encoded four putative enzymes that degrade chitin or chitin-related materials because chitin is rare in algae (Herth *et al.,* 1986).

In this study, we show that: (i) due to two DNA sequencing errors, PBCV-1 ORFs A181R and A182R encode a single 91-kDa chitinase, named A181/182R, (ii) expression of the two PBCV-1 chitinase genes and one chitosanase gene in *Escherichia coli* yield recombinant proteins

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TABLE 1

Plasmids Constructed for This Study

Plasmid	Vector	Insertion site	Insertion size (bp)	Expression product
pP22	pBluescript (KS^{II-})	*Pst*I	3730	A181/182R
pGEX2T-A181R	pGEX-2T	*Eco*RI	1425	GST-A181R
pGEX2T-A182R	pGEX-2T	*Bam*HI	690	GST-A182R
pGEX2T-A260R	pGEX-2T	*Bam*HI	1452	GST-A260R
pGEX2T-A292L	pGEX-2T	*Bam*HI/*Eco*RI	984	GST-A292L
pET11d-A181/182R	pET-11d	*Nco*I	2490	A181/182R

with the expected enzyme activities, (iii) these three genes are transcribed and translated in PBCV-1-infected cells, and (iv) these three genes are widely distributed among chlorella viruses.

RESULTS

PBCV-1 encodes two chitinase genes

We previously reported that PBCV-1 encoded three putative chitinase genes, *a181r, a182r,* and *a260r* (Lu *et al.,* 1996). However, the following observations led us to suspect that the adjacent ORFs A181R and A182R might be a single ORF. (i) Hybridization of *a181r* and *a182r* gene probes to RNA isolated at various times after PBCV-1 infection produced identical Northern blots, hybridizing to a 2.7-kb transcript at 30 min p.i. (ii) Antiserum prepared against the recombinant A181R protein reacted with a 91-kDa protein from PBCV-1-infected cells. A 91-kDa protein is larger than the predicted size of either A181R or A182R proteins, but is the expected size if *a181r* and *a182r* comprise a single gene. (iii) *E. coli* cells containing plasmid pP22 (Table 1), which has a 3.7-kb PBCV-1 DNA insert encompassing genes *a181r* and *a182r,* expressed a 91- kDa protein that reacted with the A181R antiserum. Therefore, we resequenced the *a181r* and *a182r* genes and their flanking regions and found that a G near the 3′ end of ORF A181R had been overlooked as well as a 5′-CCCCT sequence instead of the reported 5′ CCTG sequence in the intergenic region between *a181r* and *a182r.* Correcting these two sequencing errors revealed a single 830-amino-acid ORF with a predicted mass of 90.7 kDa; the ORF was named A181/182R (Fig. 1).

Comparison of the A181/182R ORF to proteins in the databases with FASTA and BLAST programs revealed that A181/182R has three distinct domains (Table 2). Ninety amino acids near the N-terminus encompass a cellulose-binding domain. Chitinases often contain such domains because of structural similarities between cellulose and chitin (Tomme *et al.,* 1995; Warren, 1996). The cellulose-binding domain is followed by two regions that resemble catalytic domains of bacterial and fungal chitinases. Amino acid residues 104 to 411 comprise the first chitinase domain and residues 553 to 825 encompass the second. These two chitinase domains, which have 36% amino acid identity, are separated by a 140-amino-acid, Pro-rich region beginning at residue 412; this region probably serves as a linker. Before realizing that A181/182R is a single ORF, we had individually expressed A181R and A182R in *E. coli.* The recombinant A181R protein, but not the A182R protein, had detectable chitinase activity (results not shown), suggesting that the chitinase domains can function independently.

The deduced 484-codon A260R ORF produced FASTA scores of 526 to 567 and had 32 to 36% amino acid identity to bacterial and fungal chitinases. However, the microbial chitinases that resembled A260R differed from those that resembled A181/182R (Table 3); these differences reflect the lack of similarity between A260R and A181/182R. Despite the differences between A181/182R

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  1  MATVPSTKLE LTVSKTSDWN TGYDGQFKLE NKNDYDILQW GMTFDFPESF
 51  NFTWFSEGDL VRKGNKVTMI PKDWNMSIPA GTTKIIPFGG VKALPGNLKY
101  NQILPLVGKD PSLAKRGKWS SKAVAPYVDA CAFPTPDLPA ISKASGLKFF
151  TLAFITADSN NKASWAGTIP LSSQHLLSQV RQIRSSGGDI SISFGGANGI
                                  *
201  ELADAIKDVD ALVAEYSRVI DLYSLTRIDF DIEGGAVADT EEVDRRNKAI
251  NILNKKYPNL QITYCLPVLP TGLALAGELL VRNARVNNAI IHSFNGMSMD
301  FGDSAAPDPE GRMGDYVIMS CQNLRTQVLS AGYDSPNIGT IPMIGVNDVE
351  SEVFRISDAK KVYDFFQSIP WMTYVGFWST NRDNAGQGQG ANPFNSGIKQ
401  NPYDFSKTFL Gkkvleldps prpnpphipp pggdpnplpp vgpvdpspkp
451  ptpkpptpnp ptnpekpqkp vqkpnvnadw cnvslefvrr crdgeapdav
501  ikdlqtrysg lgpenqkalk klldpskpvd pkpvdpkpvd pkpvdpkppv
551  ksNRFFTPYT ESWQYWSGWN NAKTLEQIPT KNVTLAFVLY ADGVPKFDGT
601  MDANIYVDQA KIVQTKGGIV RISFGGATGT ELALGIKDVN KLAAAYESVI
                          *
651  KMYNTRNIDM DIEGGPASDM DSITRRNKAL VILQKKYPDL KVDYTLAVMQ
701  TGLSTQGLDI LKDAKKQGLK VHAVNIMAMD YGTNEKQMGK AAISAATATK
751  KPCDDLGLVY EGVGITPMIG LNDTSPETFT IDNAKEVVDF AKKTSWVNFL
801  GFGATGRDNA KDTKVKQVMW EFTNIFNTFA
```

FIG. 1. The predicted amino acid sequence of the PBCV-1-encoded chitinase A181/182R. The protein has three domains, a cellulose-binding domain from amino acids 6 to 101 (dotted underline), a chitinase catalytic domain from amino acids 104 to 411 (single underline), a Pro-rich linker region from amino acids 412 to 552 (lowercase), and a second chitinase catalytic domain from amino acids 553 to 825 (double underline). The asterisks indicate the expected catalytic Glu residue in each chitinase domain.

TABLE 2

Regions of PBCV-1 ORF A181/182R That Resemble Other Proteins in the Databases

From AA	To AA	AA identity	Description
17	90	32%/74	*Cellulomonas fimi* cellulose-binding domain (P07986)
12	81	30%/70	*Mycobacterium tuberculosis* cellulose-binding domain (P11220)
6	101	27%/97	*Streptomyces lividans* cellulose-binding domain (P36909)
10	90	27%/81	*Microbispora bispora* cellulose-binding domain (P26414)
16	90	26%/75	*Thermomonospora fusca* endoglucanase (P26221)
104	409	30%/326	*Streptomyces coelicolor* probable hydrolase (AL009199)
119	409	31%/310	*Escherichia coli yheB* gene product (U18997)
119	383	31%/280	*Metarhizium anisopliae* chitinase (AF036320)
134	410	26%/286	*Ewingella americana* chitinase (X90562)
228	388	36%/168	*Aeromonas* sp. chitinase II (D31818)
136	411	27%/281	*Saccharopolyspora erythraea* chitinase (P14529)
553	829	33%/311	*S. coelicolor* probable hydrolase (AL009199)
584	808	31%/249	*E. coli yheB* gene product (U18997)
584	808	30%/246	*Metarhizium anisopliae* chitinase (AF036320)
620	809	35%/201	*E. americana* chitinase (X90562)
558	810	30%/268	*Aeromonas* sp. chitinase II (D31818)
566	825	28%/278	*S. erythraea* chitinase (P14529)

and A260R proteins, antisera to A181R and A260R cross-react (see below) and both proteins belong to glycosyl hydrolase Family 18 (Henrissat, 1991; Henrissat and Bairoch, 1993; Henrissat and Davies, 1997).

The deduced amino acid sequence of A292L ORF has 97% identity with a chitosanase from another chlorella virus, CVK2 (Yamada *et al.,* 1997), and 28 to 29% identity with bacterial chitosanases (Table 3). The A292L protein belongs to glycosyl hydrolase Family 46 (Henrissat and Bairoch, 1996).

Cloning and expression of putative chitinase and chitosanase genes

The putative chitinase and chitosanase genes were amplified from PBCV-1 genomic DNA by the polymerase chain reaction (PCR) using the primers listed in Table 4, cloned into vectors pET-11d or pGEX-2T, and expressed in *E. coli* BL21 (DE3). *E. coli* containing plasmids pET11d-A181/182R, pGEX2T-A260R, and pGEX2T-A292L (Table 1) produced the expected 91-, 83-, and 66-kDa recombinant proteins, respectively (Fig. 2). The recombinant A181/182R and glutathione S-transferase (GST)-A260R fusion proteins were primarily in the soluble fraction of the *E. coli* extracts, whereas only about 20% of the GST-A292L protein was soluble. *E. coli* strains containing the *a181/182r* gene grow slowly and plasmids containing *a181/182r* are easily lost.

Recombinant chitinases and chitosanase are enzymatically active

Chitinase activity was assayed either qualitatively with colloidal chitin as a substrate or quantitatively with one of four 4-methylumbelliferyl glycosides of *N*-acetylglucosamine oligosaccharides [4MU-$(GlcNAc)_{1-4}$], referred

TABLE 3

Similarity of PBCV-1-Encoded Chitinases and a Chitosanase to Those from Other Organisms

PBCV-1 protein	Description	AA identity	FASTA scores
Chitinase A181/182R	*E. coli* hypothetical protein	34%/310	425
	Saccharopolyspora chitinase	30%/296	380
	Ewingella chitinase	39%/198	366
	Streptomyces putative chitinase	35%/323	332
Chitinase A260R	*Aphanocladium* chitinase	36%/407	567
	Trichoderma chitinase	36%/286	533
	Coccidiomyces endochitinase	34%/386	531
	Streptomyces chitinase C	32%/380	526
Chitosanase A292L	Chlorella virus CVK2 chitosanase	97%/328	2188
	Bacillus chitosanase	29%/252	295
	Nocardioides chitosanase	28%/240	267
	Streptomyces chitosanase	29%/228	244

TABLE 4

PCR Primers Used in This Study

Primer	Primer sequence	RE* site	Gene
a181r 5′	5′-AAGGAATTCATATGGCGACCGTACCAAGCAC	*Eco*RI	*a181r*
a181r 3′	5′-AGGGAATTCTCACATTCGGTTTCTGAACT	*Eco*RI	*a181r*
a182r 5′	5′-AAGGGATCCCCGATGGACGCGAATATTTATGT	*Bam*HI	*a182r*
a182r 3′	5′-AGGGGATCCTTACGCAAATGTGTTGAATA	*Bam*HI	*a182r*
a260r 5′	5′-AAGGGATCCCCGATGGCCCTTGCGAAACCTGCT	*Bam*HI	*a260r*
a260r 3′	5′-AGGGAATTCTCATTTTAATACCACCATTAT	*Eco*RI	*a260r*
a2921 5′	5′-ATCGGATCCATGTCTCAAGTAGACACCG	*Bam*HI	*a2921*
a2921 3′	5′-TCCGAATTCATGAAATAACTATGTTTTTA	*Eco*RI	*a2921*
a181/182r 5′	5′-AAGCCATGGCGACCGTACCAAGCAC	*Nco*I	*a181/182r*
a181/182r 3′	5′-AGGCCATGGTCACATTCGGTTTCTGAACT	*Nco*I	*a181/182r*

to as substrates 1 to 4. *N*-Acetylglucosaminidase releases 4-MU from substrate 1; exochitinases release 4-MU from substrate 2 more rapidly than from substrate 3, while endochitinases release 4-MU from substrate 3 more rapidly than from substrate 2 (Robbins *et al.,* 1988).

Both recombinant chitinases, A181/182R and GST-A260R, produced clear zones on agar plates containing 0.2% colloidal chitin, indicating chitinase activity (results not shown). Recombinant A181/182R protein released 4-MU more rapidly from substrate 3 than from the other substrates (Figs. 3A and 3D), whereas GST-A260R protein released 4-MU more rapidly from substrate 2 than from the other three substrates (Figs. 3B and 3E). However, A260R also releases significant 4-MU from substrates 3 and 4. Accordingly, A181/182R is classified as

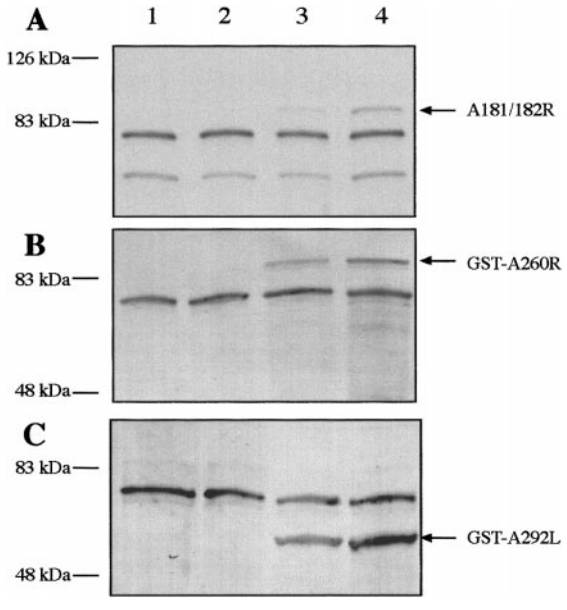

FIG. 2. Western blots of PBCV-1 encoded chitinases A181/182R and A260R and chitosanase A292L expressed in *Escherichia coli.* BL21(DE3). (A) Cells containing plasmid pET11d-A181/182R and probed with antiserum against A181R. (B) Cells containing plasmid pGEX2T-A260R and probed with antiserum against GST. (C) Cells containing plasmid pGEX2T-A292L and probed with antiserum against GST. In each panel, lanes 1 and 2 are from control *E. coli* before and after IPTG induction, and lanes 3 and 4 are from plasmid containing *E. coli* before and after IPTG induction. *Note.* The A181R antibody cross-reacts with two *E. coli* proteins (A) and the GST antibody cross-reacts with one *E. coli* protein (B and C).

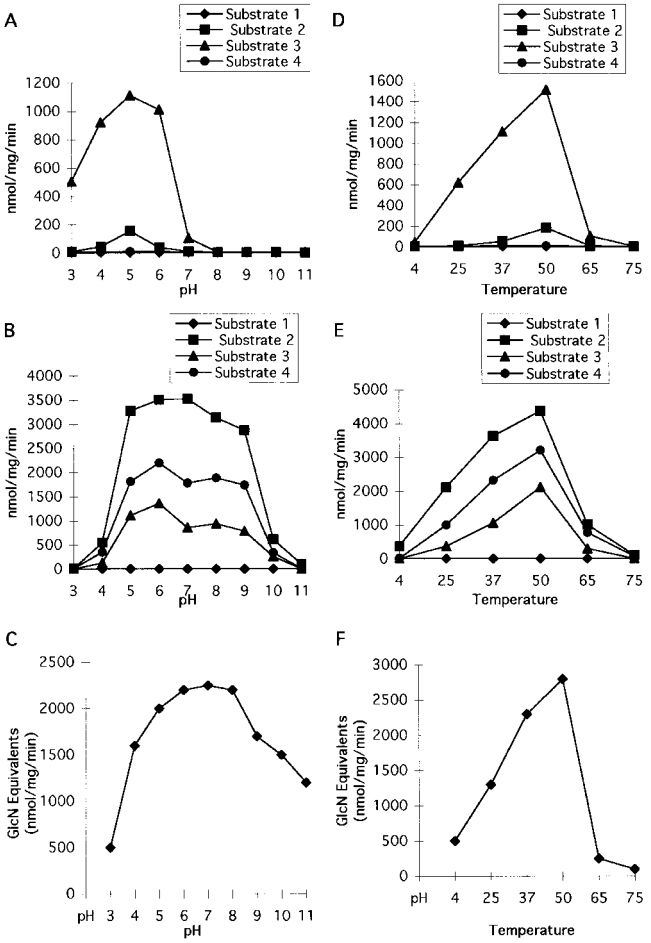

FIG. 3. Effect of pH on the activity of chitinases A181/181R (A) and A260R (B) and chitosanase A292L (C). Effect of temperature on the activity of chitinases A181/182R (D) and A260R (E) and chitosanase A292L (F). The two chitinases were assayed with four 4-MU-GlcNAc oligosaccharides as substrates.

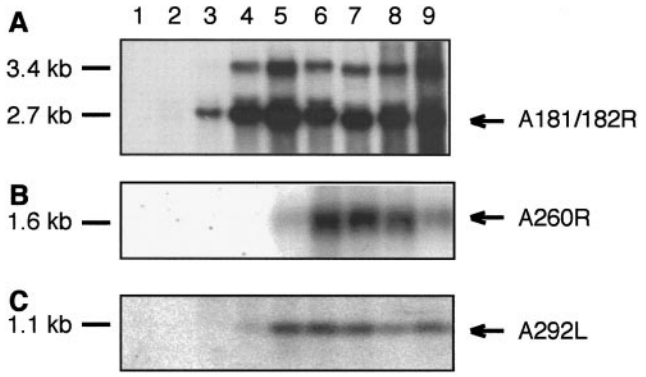

FIG. 4. Transcription of PBCV-1-encoded chitinases A181/182R and A260R and chitosanase A292L. Total RNAs were isolated from uninfected (lane 1) and PBCV-1-infected cells at 15, 30, 45, 60, 90, 120, 240, and 360 min p.i. (lanes 2–9) and hybridized with ^{32}P-labeled *a181/182r* (A), *a260r* (B), and *a292l* (C) gene probes.

an endochitinase and A260R has both endochitinase and exochitinase activities. A181/182R has a pH optimum of 4 to 6 (Fig. 3A) and is most active at 50°C (Fig. 3D). The GST-A260R enzyme is active in a pH range of 5 to 9 (Fig. 3B) and is also most active at 50°C (Fig. 3E).

Chitosanase activity was measured by monitoring formation of either glucosamine (GlcN) or oligomers of GlcN from chitosan. Recombinant chitosanase GST-A292L released GlcN from chitosanase at a rate of about 2000 nmol/mg protein/min (results not shown). The enzyme has a pH optimum of 5 to 8 (Fig. 3C) and is also most active at 50°C (Fig. 3F).

No chitinase or chitosanase activities were detected in enzyme extracts from *E. coli* cells that contained the vector plasmid.

Transcription and expression of PBCV-1 chitinases and chitosanase genes

PBCV-1 DNA replication begins about 1 h p.i. (Van Etten *et al.,* 1984). Genes transcribed before 1 h p.i. are defined as early genes, while genes transcribed after 1 h p.i. are considered late genes (Schuster *et al.,* 1986). To determine when the chitinase and chitosanase genes initiate transcription, RNA was extracted from cells at various times after infection and hybridized with appropriate gene probes. We also assayed protein extracts from these samples by Western blotting.

The *a181/182r* gene hybridized to a 2.7-kb RNA that appeared at 30 min p.i. and to a lesser extent with a 3.4-kb RNA that appeared at 60 min p.i.; both transcripts were maintained throughout the replication cycle (Fig. 4A). The 2.7-kb band was about the size expected for the 2490-bp *a181/182r* coding region. The source of the larger 3.4-kb transcript is unknown. Consistent with the transcription pattern, a 91-kDa A181/182R protein was detected at 60 min p.i. and was maintained throughout the replication cycle. The antiserum, originally made against A181R, also reacted with a 60-kDa protein, beginning at 90 min p.i. (Fig. 5A). The 60-kDa reacting protein is probably the product of the *a260r* gene.

The *a260r* gene hybridized to a 1.6-kb RNA beginning at 60 min p.i. and continuing until the cells lysed (Fig. 4B). The 1.6-kb RNA is the expected size for the 1452-bp coding region of the *a260r* gene. The highest *a260r* mRNA level occurred at 90 and 120 min p.i. Antiserum to the recombinant A260R protein reacted with a 60-kDa protein beginning at 90 min p.i. (Fig. 5B). The native A260R protein is slightly larger than its predicted size of 54 kDa as well as the recombinant A260R protein (Fig. 5D), suggesting that the native A260R protein could be posttranslationally modified. The A260R antiserum also cross-reacted with the A181/182R protein (Fig. 5B).

The chitosanase *a292l* mRNA first appeared at 60 min p.i. and was maintained throughout the replication cycle (Fig. 4C). Like the *a260r* gene, the highest level of *a292l* mRNA occurred at 90 and 120 min p.i. The size of the *a292l* transcript was 1.1 kb, as expected for a 984-bp coding region. Western analysis detected the A292R 37-kDa protein between 120 and 240 min p.i. Therefore, *a260r* and *a292l* are defined as late genes, whereas *a181/182r* is an early gene. The A292L antiserum also cross-reacted with two host proteins of about 60 and 70 kDa.

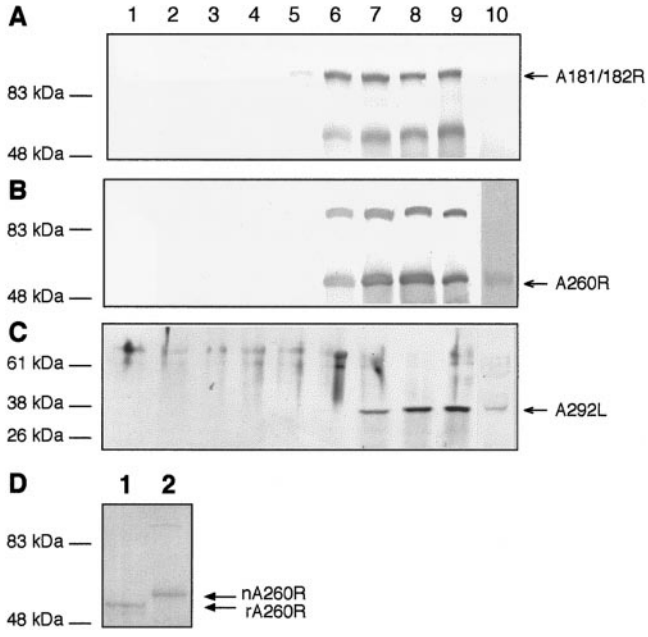

FIG. 5. Appearance of PBCV-1-encoded chitinases A181/182R and A260R and chitosanase A292L proteins. Protein extracts were isolated from uninfected cells (lane 1), PBCV-1-infected cells at 15, 30 45, 60, 90, 120, 240, and 360 min p.i. (lanes 2–9), and PBCV-1 virions (lane 10). The proteins were probed with antiserum against A181R (A), A260R (B, D), and A292L (C). (D) Migration of recombinant A260R protein (lane 1) and native A260R protein (lane 2). Note that the recombinant protein electrophoreses faster than the native protein.

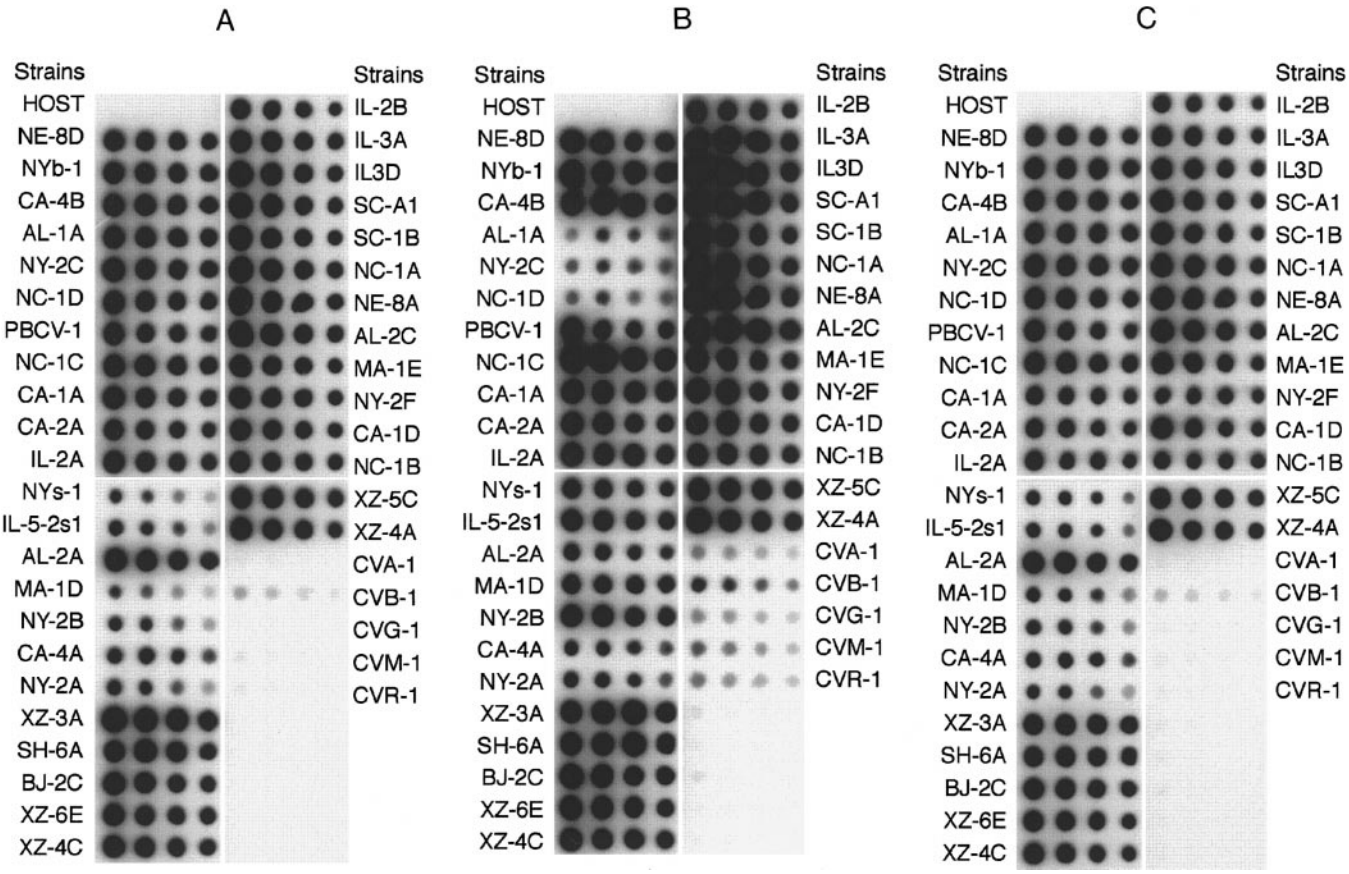

FIG. 6. Hybridization of the PBCV-1 chitinase genes *a181/182r* and *a260r* and chitosanase gene *a292l* to DNA isolated from *Chlorella* NC64A and from 37 NC64A viruses and five Pbi viruses (CVA-1, CVB-1, CVG-1, CVM-1, and CVR-1). The DNAs were hybridized with ^{32}P-labeled *a181/182r* (A), *a260r* (B), and *a292l* (C) gene probes. The blots contain 1, 0.5, 0.25, and 0.12 μg DNA, left to right, respectively.

Presence of chitinase and chitosanase proteins in the PBCV-1 virion

Purified PBCV-1 virions were also examined for the presence of chitinase and chitosanase proteins by Western analyses. The A260R (Fig. 5B, lane 10) and A292L (Fig. 5C, lane 10) proteins, but not the A181/182R protein (Fig. 5A, lane 10), were detected in 1 mg of PBCV-1 virion protein.

Occurrence of chitinase and chitosanase genes in chlorella viruses

To determine whether the chitinase and chitosanase genes are common among the chlorella viruses, DNA from 42 viruses and the host alga were hybridized to *a181/182r, a260r,* and *a292l* gene probes. None of the probes hybridized to *Chlorella* NC64A DNA. DNA from all viruses infecting *Chlorella* NC64A hybridized to various degrees with all three probes (Fig. 6). The *a181/182r* and *a292l* probes produced similar patterns. All three probes hybridized weakly with DNA from one or more viruses that infect a related alga, *Chlorella* Pbi, i.e., viruses CVA-1, CVB-1, CVG-1, CVM-1, and CVR-1 (Reisser *et al.,* 1988). Therefore, all three genes are widespread among chlorella viruses.

Phylogenetic analyses of PBCV-1 chitinase and chitosanase genes

The G+C content of the coding regions of the *a181/182r, a260r,* and *a292l* genes are 45, 47, and 44%, respectively. These values are higher than the 40% G+C content of the entire PBCV-1 genome, suggesting that these three genes may have been acquired relatively recently by the chlorella viruses. Consequently, phylogenetic analyses were performed on the two PBCV-1 chitinase genes and the chitosanase gene to determine their possible origins. The two A181/182R chitinase domains were also analyzed separately because we suspected that one domain may have originated from the other.

Due to differences among tree search algorithms (quartet puzzling for PUZZLE; heuristic search with branchswapping in Protpars) the maximum likelihood tree for the chitinases was less resolved than the weighted parsimony tree, but among resolved relationships there was complete congruence. The weighted parsimony tree, arbitrarily rooted using the bacterial endosymbiont of *Glossina morsitans,* is shown in Fig. 7A. Though there was little resolution among the major clades basally, there was generally high bootstrap support among less inclusive nodes.

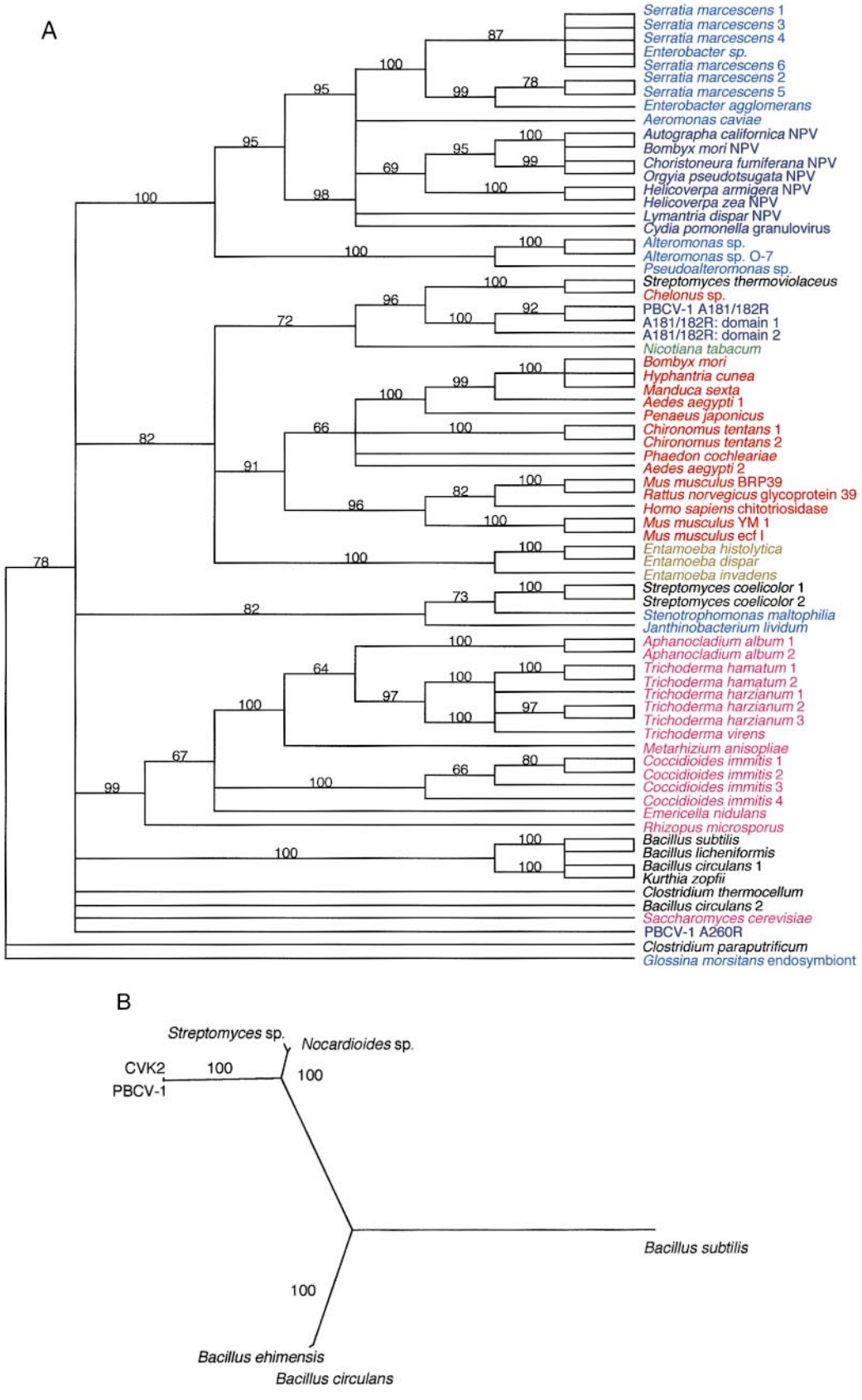

FIG. 7. (A) Chitinase gene genealogy reconstructed from amino acid sequences using weighted parsimony and maximum likelihood. Values preceeding resolved nodes indicate the percentage of bootstrap support (weighted parsimony, 100 replicates). Key: Firmicutes in black; Proteobacteria in turquoise; Fungi in mangenta; Entamoeba in olive green; Metazoa in red; Plants in green; dsDNA viruses in indigo. (B) Relationships among chitosanase genes for members of glycosyl hydrolase Family 46. The tree was reconstructed using weighted parsimony and maximum likelihood

The chitinase gene genealogy (with a few notable exceptions) is only slightly discordant with current hypotheses of organismal phylogeny. For example, mammalian and fungal chitinase genes (with the exception of *Saccharomyces,* whose relationship to the rest of the fungi is unresolved) comprise monophyletic groups, as do the Entamoebidae. Metazoan chitinase genes also form a monophyletic group, but with the curious inclusion of chitinase genes from a Firmicute bacterium (*Streptomyces thermoviolaceus*), a green plant (*Nicotiana tabacum*), and the PBCV-1 *a181/182l* gene as well as both of its chitinase domains. The inclusion of plant and bacterial chitinase genes within the metazoa suggests dramatic convergent evolution or horizontal gene transfer. The distribution of chitinase genes among the Firmicutes and Proteobacteria appears somewhat promiscuous, with at least two paraphyletic events (*Streptomyces coelicolor* genes within a clade of Proteobacteria and a *Streptomyces thermoviolaceus* gene within the Metazoa). The PBCV-1 *a181/182l* chitinase gene shares a most recent common ancestor with a Firmicute bacterium (*S. thermoviolaceus*) and the chitinase gene of a metazoan (*Chelonus* sp.). The ancestral condition of the PBCV-1 *a181/182l* gene (if the tree is rooted appropriately) is the gene found in the green plant *N. tabacum.* The phylogenetic position of the *a260r* gene could not be unambiguously resolved.

The chitosanase gene genealogy recovered by maximum likelihood and weighted parsimony trees were congruent and well supported (Fig. 7B). Although the tree cannot be unambiguously rooted, the two chlorella virus chitosanase genes appear to be more similar to the *Streptomyces* and *Nocardioides* genes than to the three *Bacillus* genes.

DISCUSSION

Like many bacteriophages, virus PBCV-1 infects its host *Chlorella* NC64A by attaching to the cell wall, degrading the wall at the point of attachment, and releasing viral DNA into the cell (Meints *et al.,* 1984; Van Etten *et al.,* 1991). Following a 6- to 8-h replication phase, nascent PBCV-1 particles are released from the host cell by localized lysis of the cell wall (Meints *et al.,* 1986; Van Etten *et al.,* 1991). Because digestion of host cell walls is important in the PBCV-1 replication cycle, one would predict that the virus encodes one or more cell wall degrading enzymes. Results presented in this paper establish that PBCV-1 encodes at least three proteins that could be involved in this lytic process: A181/182R, an endochitinase; A260R, with both exochitinase and endochitinase activities; and A292L, a chitosanase. The A181/182R protein is interesting because it has two separate chitinase catalytic domains. The two domains have 36% amino acid identity, suggesting that one domain originated as the result of a duplication of the other and that they have been modified subsequently. The phylogenetic analysis is consistent with this hypothesis.

Initially, we were surprised to discover chitinase and chitosanase genes in the PBCV-1 genome because chitin and chitin-like compounds are rare in algal cell walls (Herth *et al.,* 1986). However, Kapaun and Reisser (1995) reported that cell walls of *Chlorella* Pbi (the host for the Pbi viruses) have a chitin-like glycan. A similar polymer(s) may exist in *Chlorella* NC64A walls. Three additional observations support this hypothesis: (i) glucosamine comprises 7 to 17% of the total sugars in cell walls of endosymbiotic *Chlorella* (Meints *et al.,* 1988; Kapaun *et al.,* 1992; Takeda, 1995), (ii) the three recombinant enzymes characterized in this report partially digest *Chlorella* NC64A walls (Liangwu Sun and James Van Etten, unpublished results), and (iii) preliminary experiments indicate that the spent culture medium from healthy *Chlorella* NC64A cells contains exochitinase and β-*N*-acetylglucosaminidase activities (Liangwu Sun and James Van Etten, unpublished results). This last observation suggests that *Chlorella* NC64A encodes one or

models of sequence evolution. Values at nodes indicate the percentage of bootstrap support (weighted parsimony, 100 replicates). Database Accession numbers of sequences used in these alignments: (A) *Serratia marcescens* 1 gi[999631], *S. marcescens* 2 gi[152818], *S. marcescens* 3 gi[2126178], *S. marcescens* 4 gi[3308994], *S. marcescens* 5 gi[96882], *S. marcescens* 6 gi[729133], *Enterobacter* sp gi[2811113], *E. agglomerans* gi[1899048], *Aeromonas caviae* gi[483820], *Autographa californica* nucleopolyhedrovirus (NPV) gi[1168936], *Bombyx mori* NPV gi[3745945], *Choristoneura fumiferana* NPV gi[2351556], *Orgyia pseudotsugata* NPV gi[2493674], *Helicoverpa armigera* NPV gi[4559269], *H. zea* NPV gi[2078320], *Lymantria dispar* NPV gi[3822305], *Cydia pomonella granulovirus* gi[3273311], *Alteromonas* sp gi[423799], *Alteromonas* sp. O-7 gi[416794], *Pseudoalteromonas* sp. S9 gi[3928777], *Streptomyces thermoviolaceus* gi[625938], *S. coelicolor* 1 gi[4519549], *S. coelicolor* 2 gi[4519551], *Stenotrophomonas maltophilia* gi[2429326], *Janthinobacterium lividum* gi[458468], *Aphanocladium album* 1 gi[484528], *A. album* 2 gi[1345774], *Trichoderma hamatum* 1 gi[1419324], *T. hamatum* 2 gi[2738109], *T. harzianum* 1 gi[630419], *T. harzianum* 2 gi[1345775], *T. harzianum* 3 gi[2133296], *T. virens* gi[2967701], *Metarhizium anisopliae* gi[2565425], *Coccidioides immitis* 1 gi[1255728], *C. immitis* 2 gi[1457963], *C. immitis* 3 gi[2118035], *C. immitis* 4 gi[1705804], *Emericella nidulans* gi[4038642], *Rhizopus microsporus* var. *oligosporus* gi[1565203], *Bacillus subtilis* gi[3193265], *B. licheniformis* gi[1845337], *B. circulans* 1 gi[116300], *B. circulans* 2 gi[1711282], *Kurthia zopfii* gi[927653], *Clostridium thermocellum* gi[1418680], *C. paraputrificum* gi[2696017], *Bombyx mori* gi[1841851], *Hyphantria cunea* gi[1841853], *Manduca sexta* gi[544013], *Aedes aegypti* gi[2564719], *A. aegypti* 2 gi[2564721],*Penaeus japonicus* gi[1256180], *Chironomus tentans* 1 gi[2113832], *C. tentans* 2 gi[2113834], *Mus musculus* BRP39 protein gi[2137174], *Rattus norvegicus* glycoprotein 39-precursor gi[4558458], *Homo sapiens* chitotriosidase precursor gi[1050958], *Mus musculus* YM 1-secretory protein precursor gi[285015], *Mus musculus* ecf 1-precursor gi[1545819], *Entamoeba histolytica* gi[1685362], *E. dispar* gi[1685360], *E. invadens* gi[1685364], *Phaedon cochleariae* gi[4210812], *Chelonus* sp gi[1079185], *Nicotiana tabacum* gi[505267], *Saccharomyces cerevisiae* gi[2132509], *Glossina morsitans* endosymbiont gi[2842409]. (B) *Streptomyces* sp. gi[153214], *Nocardioides* sp. gi[703203], Chlorella virus CVK2 gi[2160011], *Bacillus subtilis* gi[1934630], *Bacillus ehimensis* gi[2626878], *Bacillus circulans* gi[216297].

more chitinases or chitosanases which participate in normal algal growth. Chlorella cells replicate by increasing in size and dividing into two, four, eight, or more progeny, which are released by digestion of the parental (chitin-containing?) cell wall. However, the host chitinases or chitosanases must differ from the three PBCV-1 genes because: (i) the virus genes do not hybridize with host DNA even at low stringency, (ii) primers to the virus genes do not prime PCR synthesis with host DNA, and (iii) with the exception of A292L, no host proteins react with antisera to the virus enzymes.

Assuming that the two PBCV-1-encoded chitinases and the chitosanase facilitate either virus infection and/or release of nascent viruses from the infected cells, one predicts that the three genes would be expressed late in virus replication. Otherwise, the infected cell would lyse prematurely and release incomplete, noninfectious virus particles. Two of the virus genes, *a260r* and *a292l,* fulfill this prediction. However, *a181/182r* transcription and translation products appear as early as 30 to 60 min p.i. This finding suggests that the A181/182R chitinase might serve another function in the virus life cycle. The presence of A260R and A292L proteins, but not A181/182R protein, in PBCV-1 virions likewise suggests functional differences between the enzymes.

A chitosanase gene has been characterized from another *Chlorella* NC64A virus, CVK2 (Yamada *et al.,* 1997). The CVK2 gene encodes two transcripts, a 1.0-kb mRNA and a 2.0-kb mRNA, and two proteins, a 37-kDA protein, which is the expected size from the DNA sequence, and a 65-kDa protein. Nascent virions contain the larger protein, but the smaller protein is found only in infected cells. Interestingly, PBCV-1 encodes the same chitosanase gene (*a292l* has 95% nucleotide identity with the CVK2 gene) which is flanked by two ORFs, A295L and A289L. Homologs of these two ORFs also flank the CVK2 gene except that the CVK2 genome has an "extra" 245-codon ORF inserted immediately downstream of its chitosanase gene (between the chitosanase and an *a289l* homolog) and which accounts for the bigger 65-kDa protein, presumably as a readthrough product. In contrast, the PBCV-1 chitosanase gene produces only one mRNA and antiserum to the chitosanase protein only reacts specifically with a 37-kDa protein in PBCV-1-infected cells and isolated virions. However, two host proteins of about 65 and 70 kDa cross-react with the A292L antiserum.

Baculoviruses are the only other viruses known to encode a chitinase (*chiA*). The *chiA* gene from *A. californica* nuclear polyhedrosis virus (AcMNPV) has been extensively characterized (Hawtin *et al.,* 1995, 1997; Thomas *et al.,* 1998). The AcMNPV gene is expressed late in infection and the resultant chitinase, which has both endo- and exo-activity, aids in liquifying the virus-infected insect larvae, releasing virus from insect cadavers.

The baculovirus chitinase genes are nested within a larger, exclusive Proteobacteria clade (Fig. 7A); this clade includes the enteric insect-pathogenic bacterium *Serratia marcescens.* The *S. marcescens* chitinase (Jones *et al.,* 1986) has 61% amino acid identity with the AcMNPV chitinase, suggesting that AcMNPV *chiA* was transferred horizontally from a bacterial source (Hawtin *et al.,* 1995). However, the baculovirus and bacterial chitinases represent distinct lineages if more baculovirus and bacterial chitinases are included in the analyses (Kang *et al.,* 1998). The PBCV-1 chitinase A260R has 28% amino acid identity with the AcMNPV chitinase and about 30% amino acid identity with several bacterial chitinases. However, the AcMNPV enzyme does not resemble the PBCV-1 A181/182R chitinase.

MATERIALS AND METHODS

Viruses, vectors, and host strains

The growth of PBCV-1 host *Chlorella* NC64A on MBBM medium, the production and purification of the viruses, and the isolation of virus DNAs have been described (Van Etten *et al.,* 1981, 1983). GST fusion proteins were produced in plasmid pGEX-2T (Smith and Johnson, 1988). Plasmid pET-11d (Studier and Moffatt, 1986), obtained from Novagene, Inc. (Madison, Wisconsin), also served as an expression vector. Cloning and recombinant gene expression were in *E. coli* strains XL-1-Blue (Stratagene, LaJolla, CA) and BL21 (DE3) (Novagen).

Cloning and expression of the chitinase and chitosanase genes

The chitinase and chitosanase genes were amplified from PBCV-1 DNA by PCR with oligonucleotide primers complementary to the 5′ and 3′ ends of the genes (Table 4). The PCR products were digested with appropriate restriction enzyme(s), separated on agarose gels, and ligated into either pGEX-2T or pET-11d vectors that were previously digested with the same enzyme(s). Overnight cultures of *E. coli* strains containing plasmids pET11d-A181/182R, pGEX2T-A260R, or pGEX2T-A292L were diluted 10-fold with fresh LB medium and incubated for 1 h at 37°C. Recombinant protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM, and cultures were incubated an additional 3 h.

Purification of GST fusion proteins and making antisera

The IPTG induced *E. coli* cells were collected by centrifuging at 5000 rpm for 5 min and cells were disrupted by sonication in phosphate-buffered saline, pH 7.3 (1× PBS), containing 1% Triton X-100. Fusion proteins were purified by affinity chromatography on a glutathione–Sepharose 4B column (Pharmacia Biotech. Inc., Uppsala,

Sweden) and used to produce polyclonal antibodies in rabbits or mice as described previously (Van Etten *et al.,* 1982).

Assay of recombinant chitinase and chitosanase activities

Chitinase activity was assayed qualitatively by monitoring clearing zones on agar plates containing 0.2% colloidal chitin, which were prepared as described (Hirano and Nagao, 1988). Chitinase activity was also assayed by measuring the release of fluorescence from one of four 4-methylumbelliferyl glycosides of 4MU-$(GlcNAc)_{1-4}$ (Robbins *et al.,* 1988; Hawtin *et al.,* 1995). A typical reaction contained 40 μg of protein and 5 μM 4MU-$(GlcNAc)_{1-4}$ in 100 μl of 0.1 M $NaPO_4$, pH 6.0, and was incubated for 60 min at 37°C. Reactions were terminated by diluting to 2 ml with 0.1 M glycine, pH 10.4. Fluorescence was excited at 350_{nm} and monitored at 440_{nm}. Buffers for pH measurements were 0.1 M sodium citrate (pH 3–5), 0.1 M $NaPO_4$ (pH 6–8), 0.1 M glycine (pH 9–10), and 0.1 M 3-(cyclohexylamino)-1-propanesulfonic acid (pH 11).

Chitosanase activity was assayed by following the release of GlcN from chitosan (Sigma, St. Louis, MO). GlcN levels were measured with fluorescamine (Sigma), which produces a fluorescent product with the amino sugar (Osswald *et al.,* 1992). Chitosanase activity was calculated on the basis of GlcN equivalents using a GlcN standard curve.

RNA isolation and Northern blot analysis

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 under denaturing conditions on 1.5% agarose/formaldehyde denaturing gels, stained with ethidium bromide, and transferred to nylon membranes (Micron Separations Inc., Westborough, MA). Membranes were subsequently photographed under UV illumination to visualize transfered RNA. The RNA was hybridized with *a181/182r, a260r,* or *a292l* probes; the probes were labeled with ^{32}P using a random-primed DNA labeling kit (GIBCO BRL), at 65°C in 50 mM $NaPO_4$, 1% bovine serum albumin, and 2% SDS.

After hybridization, radioactivity bound to the membranes was detected and quantified 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 stained membranes to digital images using a Hewlett–Packard ScanJet 4C scanner and analyzing the images with ImageQuant software.

SDS–polyacrylamide gel electrophorsis (PAGE) and Western blot analysis

Chlorella cells, prepared as described above, were combined with 200 μl 1× PBS and 200 mg 0.25 to 0.30-mm glass beads and vortexed at high speed for 5 min. Proteins (about 20 μg per sample) were electrophoresed in 7.5 or 15% SDS–PAGE at 100 V for 1 h (Laemmli, 1970). Protein gels were stained with Coomassie brilliant blue R or transferred to nylon membranes as described and analyzed by Western blotting (Wang *et al.,* 1993).

Purified PBCV-1 virions, equivalent to 1 mg of protein, were incubated in 4 M urea for 1 h at 4°C and then centrifuged for 5 min at 5000 rpm. The precipitate was dissolved in 300 μl 1× PBS, sonicated for 30 s, and again centrifuged for 5 min at 5000 rpm. Protein was precipitated from the supernatant by adding 6 vol of acetone and then stored at −80°C for 1 h. Protein was recovered by centrifugation, washed with 70% ethanol, and dissolved in 1× protein loading buffer.

Phylogenetic analyses

Algorithms for weighted parsimony (Protpars program in PHYLIP 3.573c for UNIX; Felsenstein, 1993) and maximum likelihood (PUZZLE version 4.0.2 for UNIX; Strimmer and Von Haeseler, 1996) were used to recover genealogical relationships among the two PBCV-1 chitinase genes and other chitinase genes available in GenBank. Amino acid sequences with high BLAST score similarity to the two PBCV-1 chitinase genes were aligned using CLUSTAL-X, version 1.64b, for PPC (Thompson *et al.,* 1997). In addition, the two chitinase domains of the *a181/182r* gene were also included in the analyses and aligned as separate entities. Although widely divergent, some regions of the aligned genes were conserved such that confident statements of homology could be inferred. For parsimony analyses, regions of the alignment that were highly variable or of dubious homology were removed such that only regions of high similarity and phylogenetic utility among the majority of taxa were included for analysis (455 residues). The data matrix was randomly resampled with replacement 100 times using Seqboot (Felsenstein, 1993). Most parsimonius trees for each of the 100 data sets were found using Protpars with randomized taxon addition (jumbled five times) and global rearrangements. Bootstrap support for each of the nodes was calculated by creating a majority rule consensus tree in PAUP* (kindly supplied by David Swofford) (Fig. 7A).

Because maximum likelihood models of evolution require contiguous residues (as opposed to the removal of parsimony-uninformative characters), a stretch of 613 conserved amino acids was selected for analysis using PUZZLE (version 4.0.2 for UNIX). The amino acid substitution model employed was that of Jones *et al.* (1992), with the assumption of uniform rate heterogeneity and

25,000 puzzling steps. The large number of genes included in this analysis prohibited the exploration of more elaborate models of sequence evolution.

The PBCV-1 chitosanase gene *a292l* was aligned with other known members of the glycosyl hydrolase Family 46. Amino acid sequences were aligned using CLUSTAL-X. Genealogy was reconstructed using maximum likelihood (model of sequence evolution after Jones *et al.,* 1992) and assuming a gamma distribution of rate heterogeneity (alpha estimated from the data set) and eight categories of rate variation. Chitinase genes were also analyzed by weighted parsimony (above).

Other procedures

Chlorella virus DNAs for dot blots were denatured and applied to nylon membranes (Micron Separations Inc.), fixed by UV cross-linking, and hybridized with the same ^{32}P-labeled probes used in the Northern analyses. DNA fragments were sequenced from both strands at either Geneseek (Lincoln, NE) or the University of Nebraska-Lincoln 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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Note added in proof. After this manuscript was accepted for publication, Hiramatsu *et al.* (1999) published an article on a PBCV-1 A181/182R homolog from chlorella virus CVK2. Their results were similar to ours except that the PBCV-1 chitinase gene was expressed early and the CVK2 gene was expressed as a late gene.

REFERENCES

Ando, A., Noguchi, K., Yanagi, M., Shinoyama, H., Kagawa, Y., Hirata, H., Yabuka, M., and Fujii, T. (1992). Primary structure of chitosanase produced by *Bacillus circulans* MH-K1. *J. Gen. Appl. Microbiol.* **38,** 135–144.

Bartnicki-Garcia, S. (1968). Cell wall chemistry, morphogenesis, and taxonomy of fungi. *Annu. Rev. Microbiol.* **22,** 87–108.

Cabib, E. (1987). The synthesis and degradation of chitin. *Adv. Enzymol.* **59,** 59–101.

Datema, R., Van Den Ende, H., and Wessels, J. G. H. (1977). The hyphal wall of *Mucor mucedo* 2. Hexosamine-containing polymers. *Eur. J. Biochem.* **80,** 621–626.

Davis, L. L., and Bartnicki-Garcia, S. (1984). The co-ordination of chitosan and chitin synthesis in *Mucor rouxii. J. Gen. Microbiol.* **130,** 2095–2102.

Felsenstein, J. (1993). PHYLIP (Phylogeny Inference Package) Version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle.

Flach, J., Pilet, P. E., and Jollès, P. (1992). What's new in chitinase research? *Experientia* **48,** 701–716.

Genetics Computer Group. (1997). Wisconsin Package Version 9.1, Madison, WI.

Gooday, G. W. (1997). The many uses of chitinases in nature. *Chitin Chitosan Res.* **3,** 233–243.

Gooday, G. W., Humphreys, A. M., and McIntosh, W. H. (1986). Role of chitinases in fungal growth. *In* "Chitin in Nature and Technology" (R. Muzzarelli, C. Jeuniaux, and G. W. Gooday, Eds.), pp. 83–91. Plenum, New York.

Hawtin, R. E., Arnold, K., Ayres, M. D., Zanotto, P. M., Howard, S. C., Gooday, G. W., Chappell, L. H., Kitts, P. A., King, L. A., and Possee, R. D. (1995). Identification and preliminary characterization of a chitinase gene in the *Autographa californica* nuclear polyhedrosis virus genome. *Virology* **212,** 673–685.

Hawtin, R. E., Zarlowska, T., Arnold, K., Thomas, C. J., Gooday, G. W., King, L. A., Kuzio, J. A., and Possee, R. D. (1997). Liquefaction of *Autographa californica* nucleopolyhedrovirus-infected insects is dependent on the integrity of virus-encoded chitinase and cathepsin genes. *Virology* **238,** 243–253.

Henrissat, B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* **280,** 309–316.

Henrissat, B., and Bairoch, A. (1993). New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* **293,** 781–788.

Henrissat, B., and Bairoch, A. (1996). Updating the sequence-based classification of glycosyl hydrolases. *Biochem. J.* **316,** 695–696.

Henrissat, B., and Davies, G. (1997). Structural and sequence-based classification of glycoside hydrolases. *Curr. Opin. Struct. Biol.* **7,** 637–644.

Herth, W., Mulisch, M., and Zugenmaier, P. (1986). Comparison of chitin fibril structure and assembly in three unicellular organisms. *In* "Chitin in Nature and Technology" (R. Muzzarelli, C. Jeuniaux, and G. W. Gooday, Eds.), pp. 107–120. Plenum, New York.

Hiramatsu, S., Ishihara, M., Fujie, M., Usami, S., and Yamada, T. (1999). Expression of a chitinase gene and lysis of the host cell wall during chlorella virus CVK2 infection. *Virology* **260,** 308–315.

Hirano, S., and Nagao, N. (1988). An improved method for the preparation of colloidal chitin by using methanesulfonic acid. *Agric. Biol. Chem.* **52,** 2111–2112.

Jones, J. D. G., Grady K. L., Suslow, T. V., and Bedbrook, J. R. (1986). Isolation and characterization of genes encoding two chitinase enzymes from *Serratia marcescens. EMBO J.* **5,** 467–473.

Jones, D. T., Taylor, W. R., and Thornton, J. M. (1992). The rapid generation of mutation data matrices from protein sequences. *CABIOS* **8,** 275–282.

Kang, W., Tristem, M., Maeda, S., Crook, N. E., and O'Reilly, D. R. (1998). Identification and characterization of the *Cydia pomonella* granulovirus cathepsin and chitinase genes. *J. Gen. Virol.* **79,** 2283–2292.

Kapaun, E., Loos, E., and Reisser, W. (1992). Cell wall composition of virus-sensitive symbiotic chlorella species. *Phytochemistry* **31,** 3103–3104.

Kapaun, E., and Reisser, W. (1995). A chitin-like glycan in the cell wall of a *Chlorella* sp. (Chlorococcales, Chlorophyceae). *Planta* **197,** 577–582.

Kutish, G. F., Li, Y., Lu, Z., Furuta, M., Rock, D. L., and Van Etten, J. L. (1996). Analysis of 76 kb of the chlorella virus PBCV-1 330-kb genome: Map positions 182 to 258. *Virology* **223,** 303–317.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Li, Y., Lu, Z., Burbank, D. E., Kutish, G. F., Rock, D. L., and Van Etten, J. L. (1995). Analysis of 43 kb of the chlorella virus PBCV-1 330-kb genome: Map positions 45 to 88. *Virology* **212,** 134–150.

Li, Y., Lu, Z., Sun, L., Ropp, S., Kutish, G. F., Rock, D. L., and Van Etten,

J. L. (1997). Analysis of 74 kb of DNA located at the right end of the 330-kb chlorella virus PBCV-1 genome. *Virology* **237,** 360–377.

Lu, Z., Li, Y., Que, Q., Kutish, G. F., Rock, D. L., and Van Etten, J. L. (1996). Analysis of 94 kb of the chlorella virus PBCV-1 330-kb genome: Map positions 88 to 182. *Virology* **216,** 102–123.

Lu, Z., Li, Y., Zhang, Y., Kutish, G. F., Rock, D. L., and Van Etten, J. L. (1995). Analysis of 45 kb of DNA located at the left end of the chlorella virus PBCV-1 genome. *Virology* **206,** 339–352.

Meints, R. H., Burbank, D. E., Van Etten, J. L., and Lamport, D. T. A. (1988). Properties of the chlorella receptor for the virus PBCV-1. *Virology* **164,** 15–21.

Meints, R. H., Lee, K., Burbank, D. E., and Van Etten, J. L. (1984). Infection of a chlorella-like alga with the virus, PBCV-1: Ultrastructural studies. *Virology* **138,** 341–346.

Meints, R. H., Lee, K., and Van Etten, J. L. (1986). Assembly site of the virus PBCV-1 in a chlorella-like green alga: Ultrastructural studies. *Virology* **154,** 240–245.

Osswald, W. F., McDonald, R. E., Niedz, R. P., Shapiro, J. P., and Mayer, R. T. (1992). Quantitative fluorometric analysis of plant and microbial chitosanases. *Anal. Biochem.* **204,** 40–46.

Reisser, W., Burbank, D. E., Meints, S. M., Meints, R. H., Becker, B., and Van Etten, J. L. (1988). A comparison of viruses infecting two different chlorella-like green algae. *Virology* **167,** 143–149.

Robbins, P. W., Albright, C., and Benfield, B. (1988). Cloning and expression of a *Streptomyces plicatus* chitinase (chitinase-63) in *Escherichia coli*. *J. Biol. Chem.* **263,** 443–447.

Schuster, A. M., Girton, L., Burbank, D. E., and Van Etten, J. L. (1986). Infection of a chlorella-like alga with the virus PBCV-1: Transcriptional studies. *Virology* **148,** 181–189.

Smith, D. B., and Johnson, K. S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* **67,** 31–40.

Strimmer, K., and Von Haeseler, A. (1996). Quartet puzzling: A quartet maximum likelihood method for reconstructing tree topologies. *Mol. Biol. Evol.* **13,** 964–969.

Studier, F. W., and Moffatt, B. A. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189,** 113–130.

Takeda, H. (1995). Cell wall composition and taxonomy of symbiotic chlorella from *Paramecium* and *Acanthocystis*. *Phytochemistry* **40,** 457–459.

Thomas, C. J., Brown, H. L., Hawes, C. R., Lee, B. Y., Min, M. K., King, L. A., and Possee, R. D. (1998). Localization of a baculovirus-induced chitinase in the insect cell endoplasmic reticulum. *J. Virol.* **72,** 10207–10212.

Tomme, P., Warren, R. A. J., and Gilkes, N. R. (1995). Cellulose hydrolysis by bacteria and fungi. *Adv. Microbiol. Physiol.* **37,** 1–81.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997). The CLUSTAL-X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25,** 4876–4882.

Van Etten, J. L., Burbank, D. E., Joshi, J., and Meints, R. H. (1984). DNA synthesis in a chlorella-like alga following infection with the virus PBCV-1. *Virology* **134,** 443–449.

Van Etten, J. L., Burbank, D. E., Xia, Y., and Meints, R. H. (1983). Growth cycle of a virus, PBCV-1, that infects chlorella-like algae. *Virology* **126,** 117–125.

Van Etten, J. L., Lane, L. C., and Meints, R. H. (1991). Viruses and virus-like particles of eukaryotic algae. *Microbiol. Rev.* **55,** 586–620.

Van Etten, J. L., Meints, R. H., Burbank, D. E., Kuczmarski, D., Cuppels, D. A., and Lane, L. C. (1981). Isolation and characterization of a virus from the intracellular green alga symbiotic with *Hydra viridis*. *Virology* **113,** 704–711.

Van Etten, J. L., Meints, R. H., Kuczmarski, D., Burbank, D. E., and Lee, K. (1982). Viruses of symbiotic chlorella-like algae isolated from *Paramecium bursaria* and *Hydra viridis*. *Proc. Natl. Acad. Sci. USA* **79,** 3867–3871.

Wang, I. N., Li, Y., Que, Q., Bhattacharya, M., Lane, L. C., Chaney, W. G., and Van Etten, J. L. (1993). Evidence for virus-encoded glycosylation specificity. *Proc. Natl. Acad. Sci. USA* **90,** 3840–3844.

Warren, R. A. J. (1996). Microbial hydrolysis of polysaccharides. *Annu. Rev. Microbiol.* **50,** 183–212.

Yamada, T., Hiramatsu, S., Songsri, P., and Fujie, M. (1997). Alternative expression of a chitosanase gene produces two different proteins in cells infected with chlorella virus CVK2. *Virology* **230,** 361–368.