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Ornithine Decarboxylase Encoded by Chlorella Virus PBCV-1

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Abstract: Sequence analysis of the 330-kb genome of chlorella virus PBCV-1 revealed an open reading frame, A207R, which encodes a protein with 37–41% amino acid identity to ornithine decarboxylase (ODC) from many eukaryotic organisms. The *a207r* gene was cloned and the protein was expressed as a His-A207R fusion protein in *Escherichia coli.* The recombinant protein catalyzes pyridoxal 5'-phosphate-dependent decarboxylation of ornithine to putrescine, the first step in the polyamine biosynthetic pathway The enzyme has a pH optimum of 9.0 and a temperature optimum of 42°C, and it requires dithiothreitol for maximal activity. The enzyme has a K_m, for ornithine of 0.78 mM and a specific activity of 100 μmol/min/mg protein. PBCV-1 ODC is quite sensitive to the competitive inhibitor L-arginine and the irreversible inhibitor difluoromethylarginine but it is less sensitive to the irreversible inhibitor difluoromethylornithine. The *a207r* gene is expressed both early and late in PBCV-1 infection and is highly conserved among the chlorella viruses. The 42-kDa PBCV-1 ODC (372 amino acids) is the smallest ODC in the databases and, to our knowledge, is the first virus-encoded ODC.

Keywords: ornithine decarboxylase, polyamines, chlorella viruses, Phycodnaviridae

INTRODUCTION

Ornithine decarboxylase (ODC, EC 4.1.1.17) is the first enzyme and typically the rate-limiting enzyme in the polyamine biosynthetic pathway, catalyzing the formation of putrescine (1,4 diaminobutane) from ornithine (Fig. 1) (Davis *et al.,* 1992; Cohen, 1998). Putrescine is then modified by the addition of one and two propylamino groups, respectively, to form spermidine and spermine. The donor of the propylamino group is decarboxylated *S*-adenosylmethionine that is formed by the enzyme *S*-adenosyl-methionine decarboxylase.

Polyamines are multifunctional molecules that are involved in many cell activities. These activities include regulation of gene expression (Celano *et al.,* 1989), stabilization of chromatin (Snyder, 1989; Feuerstein *et al.,* 1990; Basu and Marton, 1995), protection of DNA from damaging agents (Khan *et al.,* 1992a,b; Tadolini, 1988; Dypbukt *et al.,* 1994; Ha *et al..* 1998), and the function of Kir type K^+ channels (Lopatin *et at.,* 1994; Ficker *et al.,* 1994; Lin *et al.,* 1997). In addition, spermidine is the source of the 4-aminobutyl moiety that is used in the posttranslational conversion of lysine to hypusine; hypusine serves a vital role in the function of the protein synthesis initiation factor eIF-5A (Park *et al.,* 1997). Because of the importance of polyamines, ODC has been characterized from a variety of organisms including *Escherichia coli* (Applebaum *et al.,* 1977), *Trypanosoma brucei (*Phillips *et al.,* 1987), *Neurospora crassa* (DiGangi *et al.,* 1987), *Saccharomyces cerevisiae* (Tyagi *et al.,* 1981), and tobacco (Heimer and Mizsrahi, 1982), as well as many mammals (e.g., Haddox and Russell, 1981; Seely *et al.,* 1982; Gupta and Coffino, 1985).

Enzymes that decarboxylate ornithine, lysine, or arginine require pyridoxal 5'-phosphate (PLP) as a cofactor and are classified into two families based on amino acid sequence similarities (Sandmeierefa/., 1994). Members in family 1 include ornithine and lysine decarboxylases from prokaryotic organisms and E *coli* biodegradative arginine decarboxylase; collectively, these proteins are referred to as group III decarboxylases (Sandmeier *et al.,* 1994). Members in family 2, referred to as group IV decarboxylases, include ornithine and diaminopimelic acid decarboxylases from eukaryotic organisms, plus bacterial and plant biosynthetic arginine decarboxylases (Sandmeier *et al.,* 1994). Family 2 ODCs function as homodimers; the subunits range in size from 44 kDa for *Drosophila melanogaster* (Rom and Kahana, 1993) and 46 kDa for *Datura starmonium* (jimsonweed) (Michael *et al.,* 1996) to 53 kDa for *N. crassa* (DiGangi *et al.,* 1987).

Unexpectedly, computer analysis of the 330-kb dsDNA genome of chlorella virus PBCV-1, the prototype virus

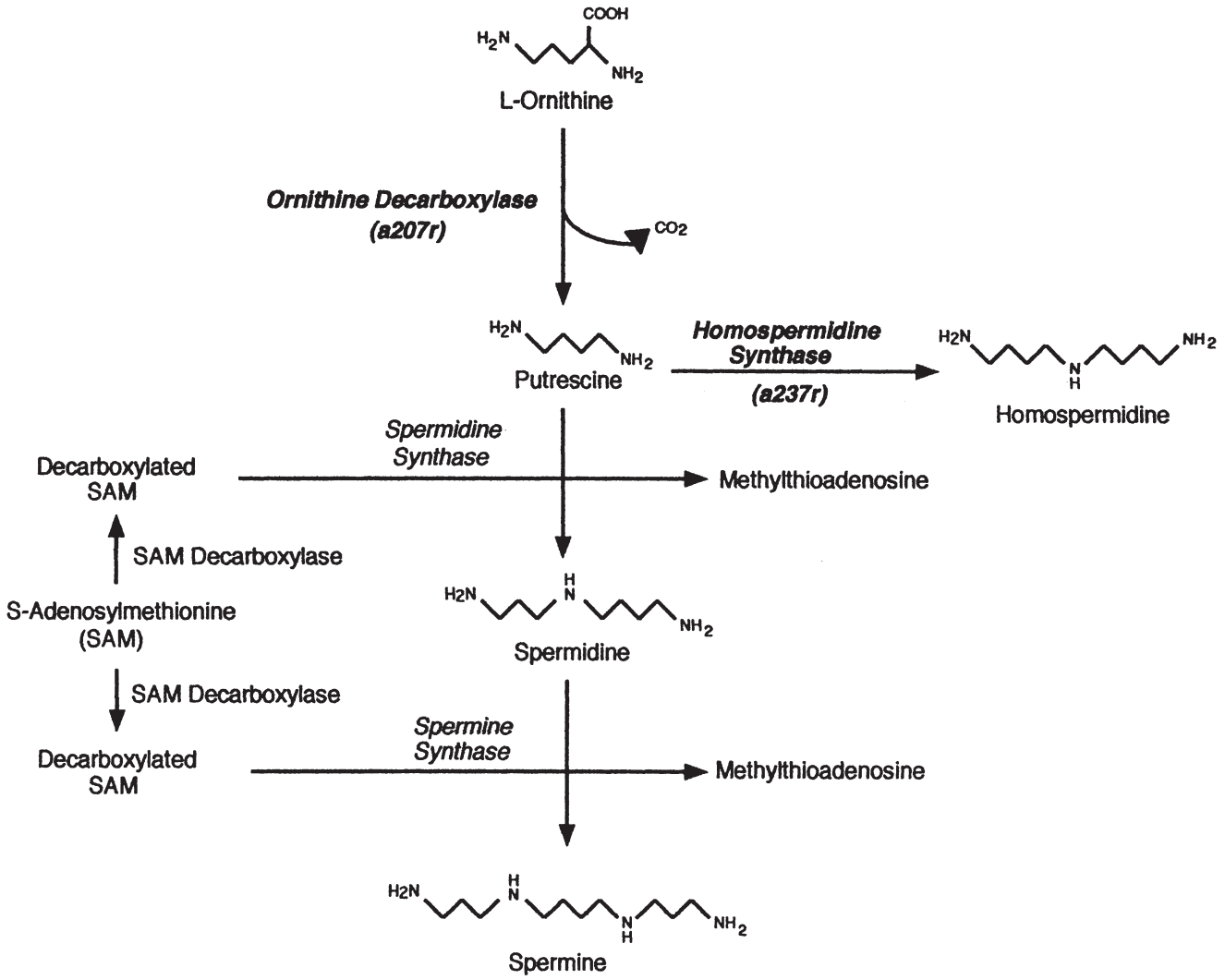

Figure 1. Polyamine biosynthetic pathway. The two enzymes, ODC and homospermidine synthase, encoded by chlorella virus PBCV-1 are highlighted.

of the genus *Chlorovirus* (family *Phycodnaviridae*)(Van Etten *et al.,* 1991; Van Etten and Meints, 1999; Van Etten, 2000), revealed an open reading frame (ORF) encoding a putative family 2 ODC (Lu *et al.,* 1996). To our knowledge, this is the first report of a virus-encoded ODC. Furthermore, the PBCV-1-encoded protein is the smallest ODC reported to date. Herein, we demonstrate that the PBCV-1-encoded protein has ODC activity.

RESULTS

PBCV-1 ORF A207R resembles ODCs

Comparing the predicted 375 virus PBCV-1 protein encoding ORFs to sequences in the databases identified an ORF (A207R) with 37–41% amino acid identity with ODCs from various organisms including humans, *T. brucei,* and *S. cerevisiae* (Fig. 2). A207R has both motifs that characterize family 2 ODCs (Prosite-PDOC00685) including a Lys^{48} in the first motif, the predicted PLP binding site. Two additional key amino acids involved in PLP binding to ODC have been identified from the crystal structure of the *T. brucei* protein (Grishin *et al.,* 1999). The PBCV-1 protein contains both of these amino acids, Glu^{252} and Arg^{255} (Fig. 2). A207R also contains 6 of 9 amino acids in the conserved WGPTCDG(L/I)D sequence in which Cys^{324} is the difluoromethylornithine (DFMO) binding site (Poulin *et al.,* 1992).

The predicted size of the PBCV-1 protein, 372 amino acids, is smaller than all ODCs in the databases. The ODC from *Drosophila* consists of 394 amino acid residues (Rom and Kahana, 1993); however, ODCs from most organisms have 420 or more amino acid residues (Table 1). To rule out the possibility that the small size of the PBCV-1 ODC resulted from a DNA sequencing error, a 1217-nucleotide region encompassing the *a207r* gene was amplified from PBCV-1 DNA by polymerase chain reaction (PCR) and resequenced. The nucleotide sequence was identical to the original report (Lu *et al.,* 1996).

An amino acid alignment of A207R with ODCs from

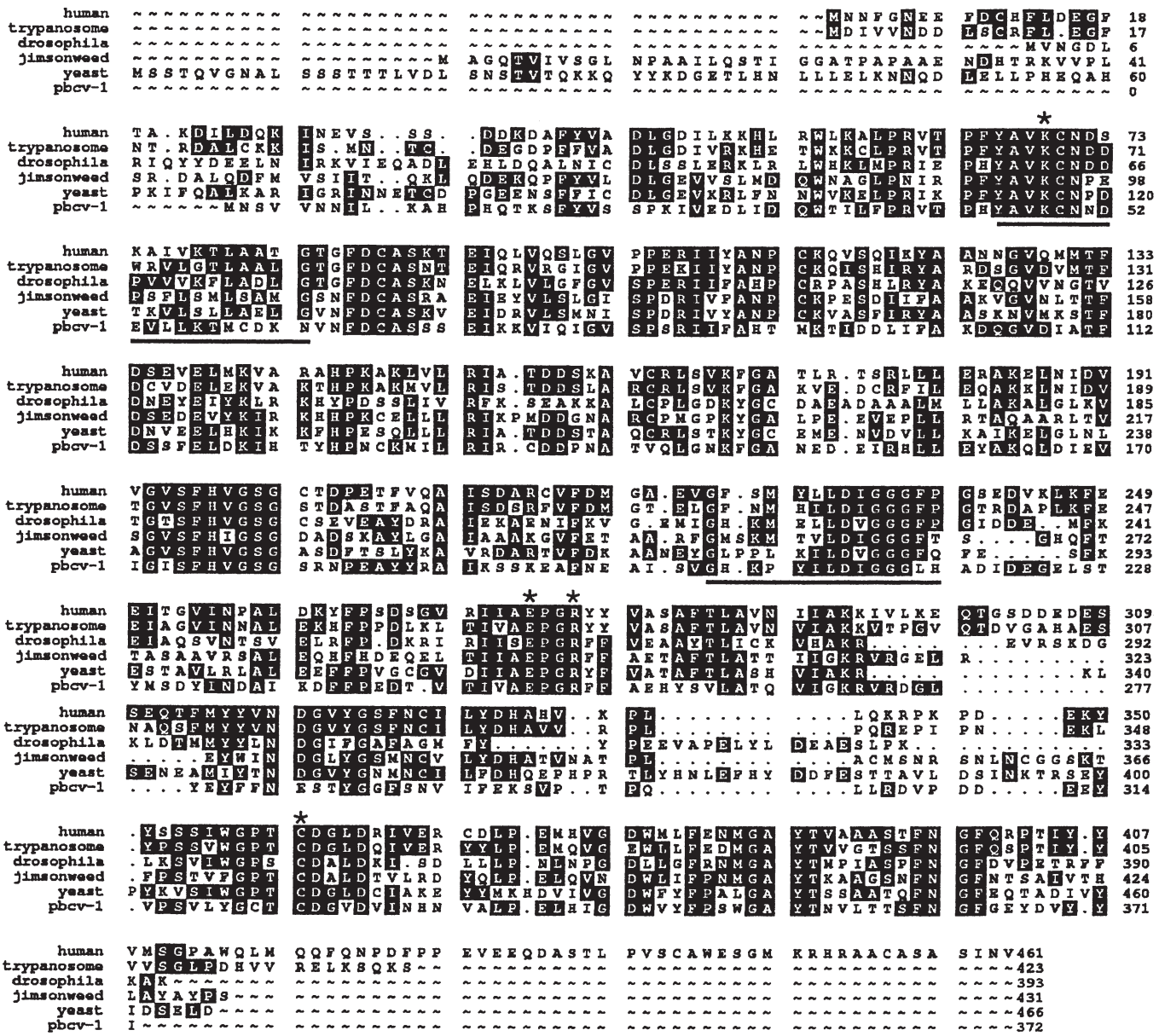

Figure 2. Amino acid alignment of ODCs from human, *T. brucei, Drosophila,* jimsonweed, *S. cerevisiae,* and PBCV-1. Alignments were done with the Wisconsin GCG Pileup program. The two motifs used to distinguish family 2 ODCs (Prosite PDOC00685) are underlined. Important amino acids are indicated with asterisks: Lys^{48} is the binding site for the cofactor PLP and Glu^{252} and Arg^{255} are also involved in PLP binding; Cys^{324} is the binding site for the irreversible suicide inhibitor DFMO. Key residues are numbered according to the PBCV-1 amino acid sequence.

human (Hickok *et al.,* 1987), jimsonweed (Michael *et al.,* 1996), *Drosophila* (Rom and Kahana, 1993), *T. brucei* (Phillips *et al.,* 1987), and *S. cerevisiae* (Fonzi and Sypherd, 1987) indicates that the smaller size of A207R can be attributed to the absence of amino acids at both the N-terminus and the C-terminus, as well as three internal gaps of 14, 12, and 5 amino acids beginning at amino acid residues 277, 303, and 311, respectively. Each of these internal gaps also occurs in at least one of the other ODCs included in Fig. 2. The G + C content of the *a207r* gene is 41%, essentially identical to the 40% G + C content of the PBCV-1 genome (Van Etten *et al.,* 1985). Several stretches of adenines and thymidines occur within the 50 nucleotides upstream of the ATG start codon and could serve as the *a207r* transcriptional start site.

Expression of recombinant PBCV-1 ODC

To determine if *a207r* encodes a functional ODC, a plasmid, pODCTM9, was constructed to express a His-tagged recombinant protein in *E. coli.* Cells containing pODCTM9 produced the expected 43-kDa recombinant fusion protein after induction with isopropyl-β-D-thiogalactopyranoside (IPTG). About 50% of the His-A207R

Table 1

Comparative Properties of ODC from Several Organisms

Organism	Amino acids	M.W. (kDa)	K_m ornithine (mM)	Specific activity (μmol/min/mg)[a]
PBCV-1	372	41.9	0.78	100
Jimsonweed	431	46	NA[b]	NA
T. brucei	423	46.8	0.33	43.5
Mouse	461	51	0.13	17
S. *cerevisiae*	466	52	0.091	0.52
Human	461	51	NA	NA
Drosophila	393	44	NA	NA
Xenopus	460	50.8	NA	NA

[a] μmol ornithine decarboxylated/min/mg protein.
[b] NA, not available.

protein was in the soluble fraction of the bacterial extract. The protein was purified to apparent homogeneity over a Ni-NTA column; about 2 mg of recombinant protein was obtained per liter of *E. coli* cells. We did not remove the N-terminal His residues prior to characterizing the enzyme for two reasons. First, a His-tag had no effect on recombinant ODC activity from mouse, *T. brucei,* or *Leishmania donovani (Osterman et al.,* 1994) and second, as described below, the His-tagged A207R protein had excellent enzyme activity.

Characterization of PBCV-1 recombinant ODC

After a 5- to 10-min lag the recombinant His-tagged A207R protein decarboxylates L-[1-^{14}C]ornithine to putrescine in a linear fashion for at least 45 min (Fig. 3A) and the rate varies with protein concentration (Fig. 3B). The enzyme requires PLP as a cofactor and maximum activity occurs with 2.5 mM dithiothreitol (DTT) (results not shown). The enzyme has maximum activity at pH 9.0 (Fig. 3C). The temperature optimum for the enzyme is 42°C (Fig. 3D), which is considerably higher than the 25°C optimum temperature for growing host and virus.

The PBCV-1 ODC has a K_m for ornithine of 0.78 mM (inset Fig. 3A) and a specific activity of 100 μmol ornithine decarboxylated/min/mg protein at 42°C. Both of these values are higher than ODCs from other organisms (Table 1). Storage of the enzyme at –80°C in the presence of 0.5 mg/ml bovine serum albumin (BSA), DTT, and PLP resulted in ~30% loss of activity after 8 weeks.

Effect of inhibitors on PBCV-1 ODC activity

L-arginine and L-lysine often compete with L-ornithine for binding to the ODC active site. The PBCV-1 ODC was inhibited by both amino acids; enzyme activity was inhibited ~50% by 0.25 mM arginine (Fig. 4A) or by 1 mM lysine (Fig. 4B). Neither 0.4 mM D-ornithine nor 10 mM L-diaminopimelic acid effected enzyme activity (results not shown).

Surprisingly, PBCV-1 ODC was relatively insensitive to DFMO, a specific and irreversible inhibitor of ODCs (Metcalf *et al;* 1978). Typically, micromolar concentrations of DFMO inhibit family 2 ODCs. However, the PBCV-1 enzyme required ~2.5 mM DFMO for 50% inhibition (Fig. 4C). In contrast, the virus ODC was more sensitive to difluoromethylarginine (DFMA): 50% inhibition occurred between 0.1 and 0.25 mM DFMA (Fig. 4D).

Expression of the *a207r* gene in PBCV-1-infected cells

RNA was extracted from viral infected cells at various times after infection and hybridized to a single-stranded antisense *a207r* probe to determine if the gene is transcribed during PBCV-1 replication. The *a207r* probe hybridized to a 1.2-kb RNA transcript extracted from viral infected cells at 15–45 min p.i.; hybridization decreased at 60 min p.i. and increased again at 90–180 min p.i. (Fig. 5). This RNA transcript is the expected size for a 372-amino-acid protein. Since PBCV-1 DNA synthesis begins 60–90 min p.i. (Van Etten *et al.,* 1984), *a207r* is expressed as both an early and a late gene.

Occurrence of the *a207r* gene in other chlorella viruses

To determine if the *a207r* gene is common among the chlorella viruses, genomic DNAs from 42 chlorella viruses isolated from diverse geographical regions were hybridized to the *a207r* probe used in the Northern analyses (results not shown). DNA from 32 of the viruses that infect *Chlorella* NC64A hybridized strongly with the probe; weak or no hybridization occurred with 5 viruses—NYs-1, IL-5-2s1, MA-1D, NY-2B, and NY-2A—that infect the same host (NC64A viruses). No hybridization was detected with DNA from the *Chlorella* NC64A host or DNA from 4 of the 5 viruses which infect *Chlorella* Pbi (Pbi viruses). However, shotgun DNA sequencing has identified an *odc* gene in NC64A virus NY-2A and Pbi virus CVM-1 (unpublished results). These two *odc* genes differ sufficiently from the PBCV-1 *odc so* that the PBCV-1 probe gives no detectable signal. These findings indicate that *odc* genes are common in the chlorella viruses.

Phylogenetic analyses

Neighbor-joining analyses of PAM distances depict the PBCV-1 enzyme arising near the ancestral origin of the ODC clade (Fig. 6), sharing a common origin with the ODC gene of *Selenomonas ruminatum,* and branching prior to the divergence of the other ODC genes. Ninety percent of the neighbor-joining bootstrap replicates, but only 45% of the more conservative maximum likelihood

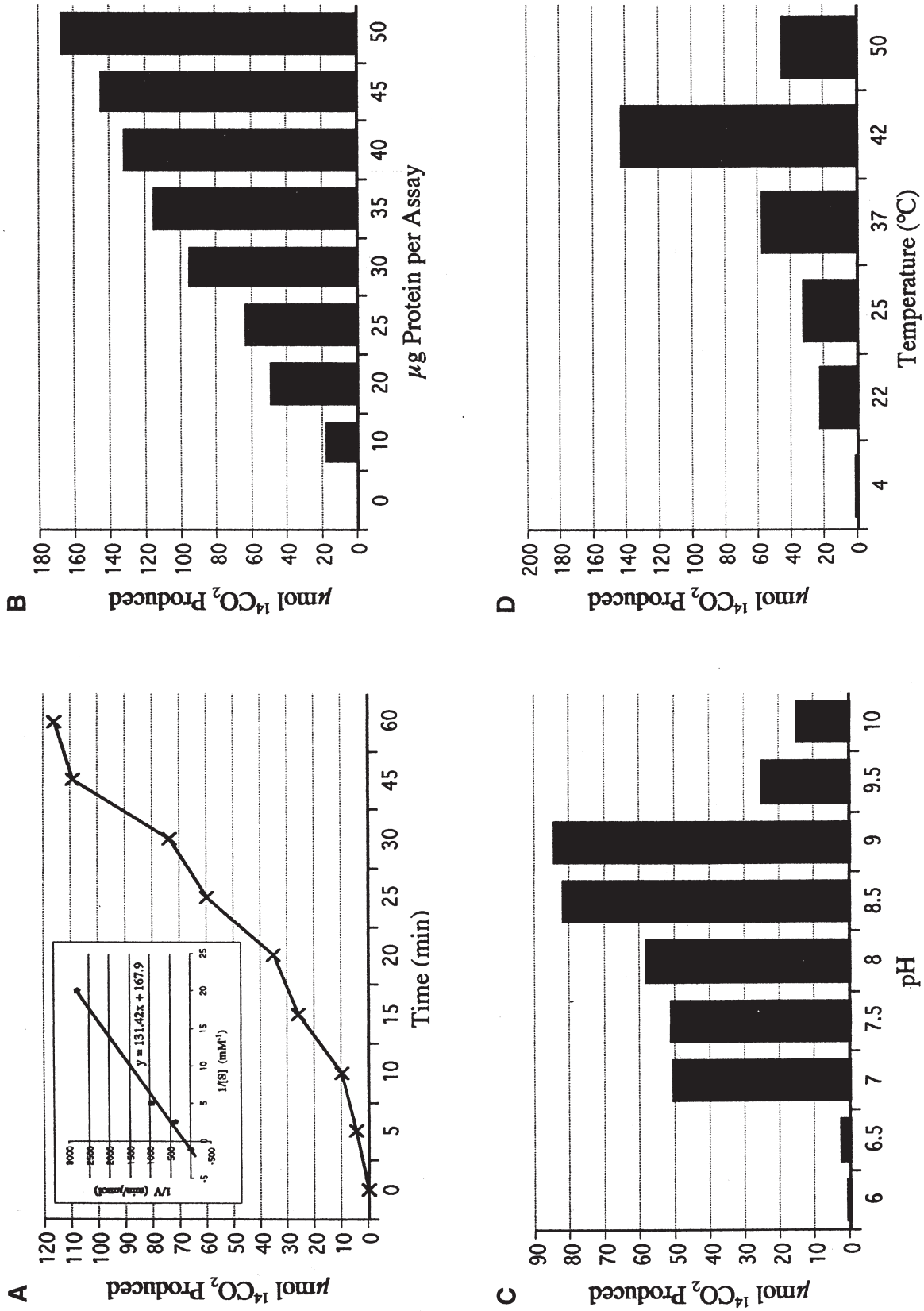

Figure 3. Effect of time (A), protein concentration (B), pH (C), and temperature (D) on PBCV-1 recombinant ODC activity. (A) Insert: Lineweaver-Burk plot of PBCV-1 ODC.

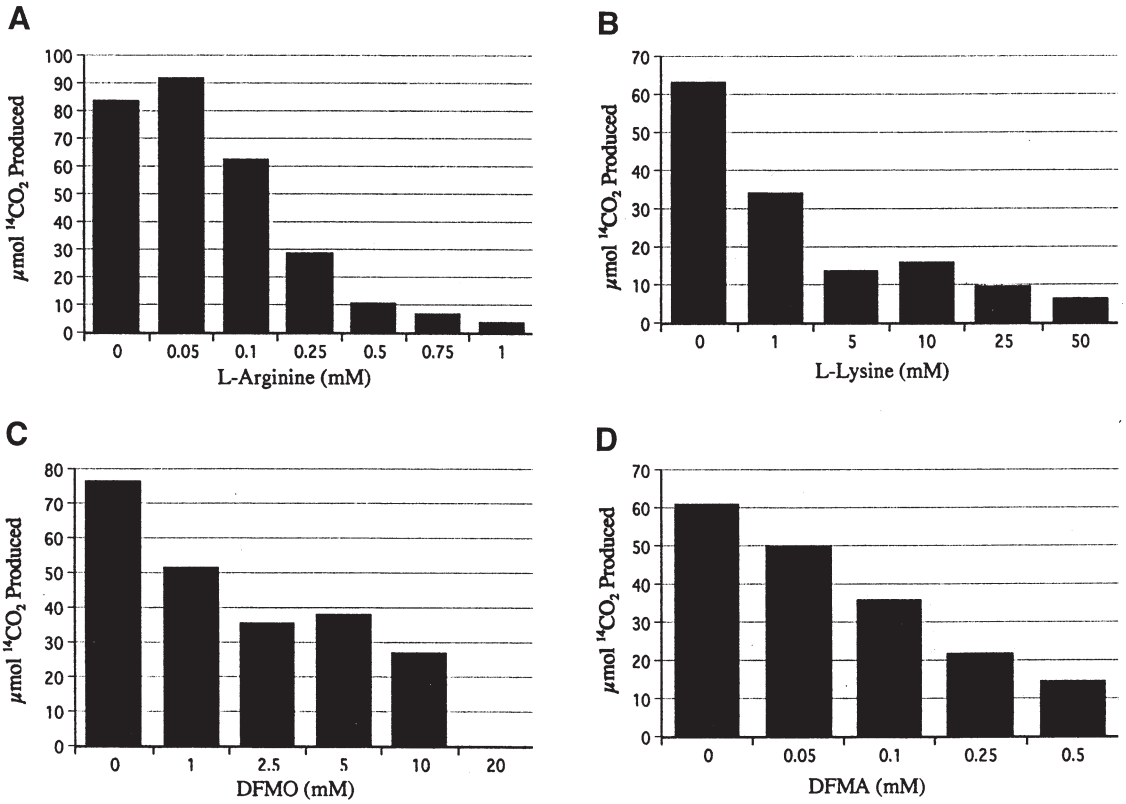

Figure 4. Effect of L-arginine (A), L-lysine (B), DFMO (C), and DFMA (D) on PBCV-1 recombinant ODC activity. The inhibitors were added to the enzyme 15 min prior to starting the reaction.

bipartitions, support this relationship. The ODC gene lineage appears to be monophyletic, supported by neighbor-joining bootstrap and quartet puzzling metrics (100 and 83%, respectively).

DISCUSSION

This report establishes that despite its small size, 372 amino acids with a predicted molecular weight of 41,969 Da, the chlorella virus PBCV-1 ODC has properties comparable with other ODCs. The specific activity of the PBCV-1 enzyme is higher than ODC from other organisms (Table 1). However, the high K_m (0.78 mM) of PBCV-1 ODC indicates relatively weak binding to ornithine (Table 1). The virus ODC prefers a pH of 9.0 and has a temperature optimum of 42°C, higher than optima for host cell growth and viral replication. Possibly the most surprising findings were that arginine was a strong competitive inhibitor of PBCV-1 ODC and that the enzyme was relatively insensitive to the irreversible inhibitor DFMO. The arginine affinity is consistent with strong inhibition by DFMA (Fig. 4).

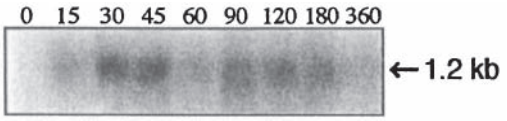

Figure 5. Northern hybridization of RNAs isolated from uninfected and PBCV-1-infected *Chlorella* NC64A at 0, 15, 30, 45, 60, 90, 120, 180, and 360 min p.i. RNA was electrophoresed on a 1.2% agarose gel, transferred to a nylon membrane, and probed with an antisense ^{32}P-single-stranded *a207r* probe.

In other organisms, ODC is subject to a variety of control mechanisms and the protein typically turns over rapidly *in vivo* (Cohen, 1998). Several years ago studies with mammalian cells showed that putrescine induces synthesis of a heat-labile, trypsin-sensitive protein that decreases ODC activity in a noncompetitive manner (Fong *et al.,* 1976; Heller *et al.,* 1976; Hayashi *et al.,* 1996; Cohen, 1998). This inhibitor protein was named antizyme and was shown to target ODC for proteolysis by the 26S proteasome (Fong *et al.,* 1976; Heller *et al.,* 1976; Cohen, 1998). Regions rich in Pro, Glu, Ser, and Thr residues (PEST regions) often exist in the C-terminal portion of ODCs and these PEST regions target proteins for cellular proteases (Loetscher *et al.,* 1991; Cohen, 1998). For example, mammalian ODCs contain two PEST sequences, one in the C-terminus that influences protein stability (Ghoda *et al.,* 1989) and an internal PEST sequence (Li and Coffino, 1992). Removal of 37 amino acid residues from the C-terminus stabilized the protein (Ghoda *et al.,* 1989; Cohen, 1998), indicating that the ODC carboxyl-terminal region is required for basal degradation (Li and Coffino, 1992). The internal PEST region serves as the an-

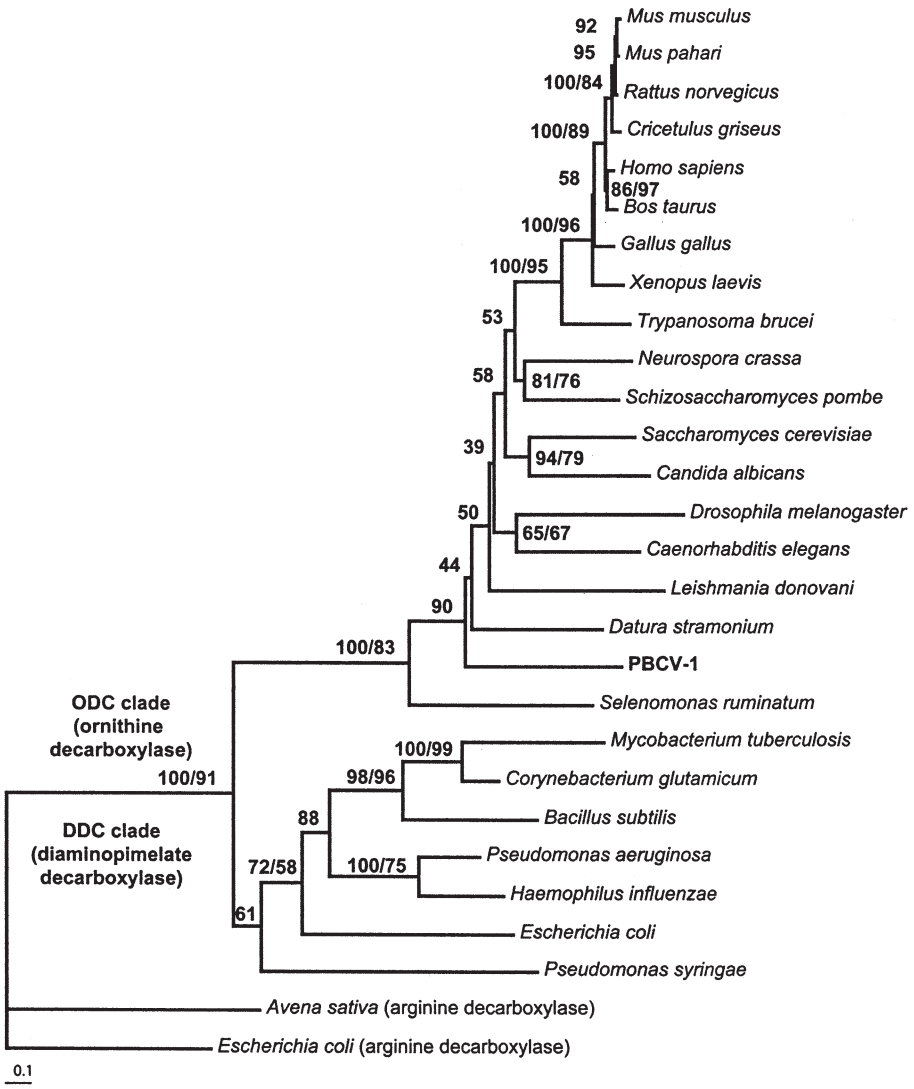

Figure 6. Gene genealogy of PBCV-1 ODC and other group IV decarboxylases. Tree was produced using neighbor joining of PAM distances. Partition frequency indices follow bootstrap support values where nodes of neighbor-joining and maximum likelihood trees are concordant. Scale bar references branch length as frequency of changes per site.

tizyme-binding site required for polyamine-mediated ODC degradation.

The PBCV-1 ODC lacks a C-terminal PEST sequence and a comparison of the 24-amino-acid antizyme-binding region of the mouse ODC (residues 117–140) with the PBCV-1 enzyme indicates only 7 common amino acids. Thus the virus enzyme lacks both PEST sequences that control ODC levels in mammalian cells. Searching the PBCV-1 genome for ODC antizyme ORFs found in mammals (Hayashi *et al.,* 1996) and in the fission yeast *Schizosaccharomyces pombe* (Chattopadhyay *et al.,* 2001; Ivanov *et al.,* 2000) failed to detect such a protein. Therefore, we conclude that PBCV-1 ODC levels are not controlled by antizymes. However, this conclusion may be premature. A recent report indicates that ODC levels in the baker's yeast *S. cerevisiae* are also controlled by an antizyme-like mechanism. This is surprising since no antizyme homologue has been detected in *S. cerevisiae* either *in vitro* or *in silico* (Gupta *et al.,* 2001). It would seem worthwhile to determine if the PBCV-1 ODC gene complements the *S. cerevisiae* ODC gene, and if so, whether virus protein levels are regulated like the yeast protein.

Another possibility is that the PBCV-1 enzyme is a primitive ODC that lacks regulatory regions. This suggestion is supported by phylogenetic analyses; the PBCV-1 ODC resides near the origin of the clade containing eukaryotic ODCs (Fig. 6). This scenario implies that the chlorella viruses and their genes have a long evolutionary history. This hypothesis is supported by phylogenetic analysis of eukaryotic DNA polymerases, which indicates that the phycodnavirus DNA polymerases reside near the origin of all eukaryotic δ DNA polymerases (Villarreal, 1999; Villarreal and DeFilippis,

2000), supporting the theory that the chlorella viruses are ancient.

In addition to encoding ODC, PBCV-1 encodes a functional homospermidine synthase (Kaiser *et al.,* 1999). Homospermidine synthase catalyzes the synthesis of the rare polyamine homospermidine from two molecules of putrescine. Thus PBCV-1 encodes the complete pathway for the synthesis of homospermidine from ornithine (Fig. 1). Consequently, one might expect polyamines, including homospermidine, to play an important role(s) in PBCV-1 replication or serve structural roles in the virus particle. Indeed, polyamines are structural components of many viruses, helping to neutralize viral nucleic acids (Sheppard *et al.,* 1980; Tyms *et al.,* 1990; Cohen, 1998). However, these expectations are not fulfilled for PBCV-1. We reported previously that uninfected and PBCV-1-infected *Chlorella* NC64A cells, as well as virus particles, contain putrescine, cadaverine, spermidine, and homospermidine and that the ratios of these polyamines change during virus infection; however, the total polyamine concentration remains relatively constant during virus replication (Kaiser *et al.,* 1999). Also it is unlikely that polyamines neutralize PBCV-1 DNA because there are only ~0.002 polyamine molecules per virus phosphate residue (Kaiser *et al.,* 1999). Furthermore, the polyamines are easily displaced from the particles by washing virions in a polyamine-free buffer without affecting virus infectivity. Therefore, the biological function of the polyamines and associated enzymes remains a mystery

MATERIALS AND METHODS

Strains and culture conditions

The production and purification of the chlorella viruses and the isolation of their DNAs have been described previously (Van Etten *et al.,* 1981, 1983). *E. coli* strains DH5αMCR (*E. coli* Genetic Stock Center, New Haven, CT) and BL21(DE3) (Novagen, Madison, WI) were grown in LB medium (Sambrook *et al.,* 1989).

Cloning and expression of PBCV-1 ODC

PBCV-1 *a207r* was cloned from PCR-amplified viral DNA using the following oligonucleotide primers:

5' Primer: AAATTGCTCGAGATGAATTCTGTTGTAAATAAC

3' Primer: ATTATTTTGTCATTTGGGATCCTCATTTAAATGTAGT

The underlined bases indicate the 5' and 3' restriction sites for *Xho*I and *Bam*HI endonucleases that were used for cloning. The PCR contained 1.2 μg of PBCV-1 genomic DNA, 26 pmol of each primer, 0.2 mmol of each dNTP, 5 mM $MgCl_2$, 5 units of *Taq* DNA polymerase (Sigma, St. Louis, MO), 0.6 units of Vent DNA polymerase (New England Biolabs, Beverly, MA), and 1× PCR buffer in a 50-μl reaction volume. Amplification was carried out with an Ericomp Thermocycler (Deltacycler II) using 25 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2.5 min. PCR fragments of the expected size were digested with *Xho*I and *Bam*HI and inserted into the *Xho*I–*Bam*HI sites of the pET-15b expression vector (Novagen). This process produced a 6-His residue tag at the N-terminus of the target protein. The resulting plasmid, pODCTM9, was transformed into either *E. coli* strains DH5αMCR for maintenance or BL21(DE3) for expression.

The *a207r* gene was expressed by growing cells overnight at 25°C in 125 ml of LB medium containing 100 μg/ml ampicillin to an A_{600} of 1.0. Then, 12.5 ml of the overnight culture was subcultured into 500 ml LB medium containing 100 μg/ml ampicillin (5 liters total volume). The cultures were induced with 1 mM IPTG and incubated at 25°C overnight. The cells were harvested by centrifugation at 5000 rpm for 5 min and resuspended in 400 ml of phosphate-buffered saline which contained 100 μg/ml of lysozyme. After incubation on ice for 30 min, cells were disrupted by sonication for 3 min using a Tekmar sonic disruptor at 100% amplitude, in 5-s pulses. Samples were centrifuged at 10,000 rpm for 10 min to separate soluble and insoluble fractions.

Purification of recombinant enzyme

Addition of $(NH_4)_2SO_4$ to the soluble fraction resulted in ODC precipitation in the 35–65% fraction. The precipitated protein was collected by centrifugation, resuspended in 100 ml buffer A (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) containing 0.04 mM PLP (Sigma) and 2.5 mM DTT (Sigma) and applied to a NiNTA Superflow column (Qiagen, Hilden, Germany) equilibrated with buffer A, 0.04 mM PLP, and 2.5 mM DTT. The column was washed with 50 ml of buffer A, followed by 50 ml of buffer B (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 0.04 mM PLP, and 2.5 mM DTT, pH 8.0). The His-tagged protein was eluted with 50 ml of buffer C (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 0.04 mM PLP, and 2.5 mM DTT, pH 8.0) containing 0.5 mg/ml BSA. All column washes and subsequent elutions were performed at 4°C. The fractions containing the recombinant protein, monitored by their faint yellow color from the PLP, were pooled and dialized against 50 mM CAPSO, pH 9.0, containing 2.5 mM DTT and 0.04 mM PLP. Protein concentrations were determined by the Lowry method (Lowry *et al.,* 1951). Purified enzyme was stored in 100-μl aliquots at –80°C.

Enzyme assays

Enzyme assays were carried out in Warburg flasks at 42°C with shaking unless otherwise noted. Enzyme reactions contained 50 mM CAPSO buffer, pH 9.0, 0.04 mM PLP, 2.5 mM DTT, 0.125 μCi L-[1-^{14}C]ornithine hy-

drochloride (Amersham Biochemicals, Piscataway, NJ), 0.4 mM L-ornithine hydrochloride, and enzyme in a final volume of 250 μl. A Whatman 1-cm filter paper was soaked with 20 μl of 10% KOH and placed in the center chamber of the Warburg flasks to trap $^{14}CO_2$ released from the decarboxylation reaction. The assay mixture was placed in the bottom of the flasks while the enzyme was placed in the sidearms of the flasks. The flasks were sealed with serum stoppers and then tipped slightly to mix the enzyme with the substrate mixture. The reactions were stopped after 30 min with the addition of 500 μl of 6 N HCl and allowed to incubate an additional 30 min to trap residual $^{14}CO_2$. The filter papers were removed, placed into scintillation vials containing 10 ml of Optifluor (Packard, Downers Grove, IL), and counted in a liquid scintillation counter.

Northern and dot blot analyses

Chlorella cells (1×10^9) were collected at various times after PBCV-1 infection, frozen in liquid, nitrogen, and stored at —80°C. RNA was extracted, denatured with formaldehyde, separated on 1.2% agarose gels, and transferred to nylon membranes (Micron Separations Inc., Westborough, MA) as described (Graves *et al.,* 2001). RNA was hybridized to an antisense ^{32}P-single-stranded *odc* probe (Graves and Meints, 1992) at 65°C in 50 mM $NaPO_4$, 1% BSA, 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., Sunny-vale, 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.

Viral DNAs used for dot blots were denatured, applied to nylon membranes fixed by UV cross-linking, and hybridized with the same probes used for the Northern analysis.

Phylogenetic analyses

Amino acid sequences were aligned using Clustal X (Thompson *et al.,* 1997) and then optimized manually with MacClade 4.02 (Maddison and Maddison, 2001). Phylogenetic trees were constructed using maximum likelihood and neighbor-joining approaches.

The maximum likelihood analysis assumed the amino acid substitution model of Jones *et al.* (1992). Amino acid substitution frequencies were estimated empirically from the multiple sequence alignment. The model of rate heterogeneity assumed eight Γ-distributed rate categories. The distribution parameter of gamma (alpha) was 1.59, also estimated from the data set. The maximum likelihood tree was identified via 100,000 quartet puzzling steps using PUZZLE 4.0.2 (Strimmer and von Haeseler, 1996). Branch support for each node in the unrooted tree was inferred as the percentage of shared bipartitions from among the 20,475 analyzed quartets.

The neighbor-joining tree was constructed using the PAM matrix model of probabilities of change between amino acids (Dayhoff *et al.,* 1978) with the Protdist and neighbor programs in PHYLIP (Felsenstein, 1993). Tree building included subreplicates and random input order of taxa. Branch support was estimated using the same parameters, but from 1,000 bootstrap-resampled data sets generated using the Seqboot program of PHYLIP. All trees were rooted with the arginine decarboxylase genes of *E. coli* and *Avena sativa.*

Other procedures

DNA and putative protein sequences were analyzed with the University of Wisconsin Computer Group Version 10.1 package of programs (Genetics Computer Group, 2000). The GenBank Accession Numbers for PBCV-1 ORF A207R are U42580 and T17697.

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