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Chlorella Virus PBCV-1 Encodes a Functional Homospermidine Synthase

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Abstract

Sequence analysis of the 330-kb genome of chlorella virus *Paramecium bursaria* chlorella virus 1 (PBCV-1) revealed an open reading frame, A237R, that encodes a protein with 34% amino acid identity to homospermidine synthase from *Rhodopseudomonas viridis*. Expression of the *a237r* gene product in *Escherichia coli* established that the recombinant enzyme catalyzes the NAD^+-dependent formation of homospermidine from two molecules of putrescine. The *a237r* gene is expressed late in PBCV-1 infection. Both uninfected and PBCV-1-infected chlorella, as well as

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PBCV-1 virions, contain homospermidine, along with the more common polyamines putrescine, spermidine, and cadaverine. The total number of polyamine molecules per virion (~539) is too small to significantly neutralize the virus double-stranded DNA (>660,000 nucleotides). Consequently, the biological significance of the homospermidine synthase gene is unknown. However, the gene is widespread among the chlorella viruses. To our knowledge, this is the first report of a virus encoding an enzyme involved in polyamine biosynthesis.

Keywords: polyamines; homospermidine; homospermidine synthase; ornithine decarboxylase; dsDNA virus; chlorella viruses; PBCV-1; Phycodnaviridae.

Introduction

Homospermidine [$H_2N(CH_2)_4NH(CH_2)_4NH_2$], unlike the more common polyamines putrescine, spermidine, and spermine, has been detected in only a few, widely diverse organisms (Cohen, 1998). Homospermidine is used as a chemotaxonomic marker in certain bacterial taxa, such as the α subclass of the proteobacteria, which include the photosynthetic bacteria *Rhodopseudomonas* (Hamana *et al.,* 1985) and *Agrobacterium* (Hamana *et al.,* 1989b). It is also common in nitrogen-fixing bacteria such as *Rhizobium* (Smith, 1977; Fujihura and Harada, 1989) and nitrogen-fixing cyanobacteria (Hamana *et al.,* 1983; Hamana and Matsuzaki, 1992), as well as some methanogenic archaebacteria (Scherer and Kneifel, 1983). Homospermidine also occurs in a few algae, including *Chlorella* species (Kneifel, 1977; Hamana and Matzusaki, 1982), mosses, lichens, ferns (Hamana and Matsuzaki, 1985), some higher plants (Kuttan *et al.,* 1971; Yamamoto *et al.,* 1983; Hamana *et al.,* 1992), and animal tissues (Matsuzaki *et al.,* 1982).

Bacteria synthesize homospermidine from two molecules of putrescine by an NAD^+-dependent enzyme named homospermidine synthase (HSS) (**Fig. 1**); no other substrates or cofactors are required. HSS, a homodimer composed of two 52-kDa subunits, has been characterized from two bacteria: *Rhodopseudomonas viridis* (Tait, 1979; Tholl *et al.,* 1996) and *Acinetobacter tartarogenes* (Yamamoto *et al.,* 1993). The *R. viridis hss* gene is the only one that has been isolated and characterized (Tholl *et al.,* 1996). The amino acid sequence of the *R. viridis* HSS enzyme resembled the predicted amino acid sequence encoded by an open reading frame (ORF A237R) of unknown function in chlorella virus *Paramecium bursaria* chlorella virus 1 (PBCV-1).

PBCV-1 is the prototype of a genus (*Chlorovirus*, family Phycodnaviridae) of large (175–190 nm in diameter) polyhedral,

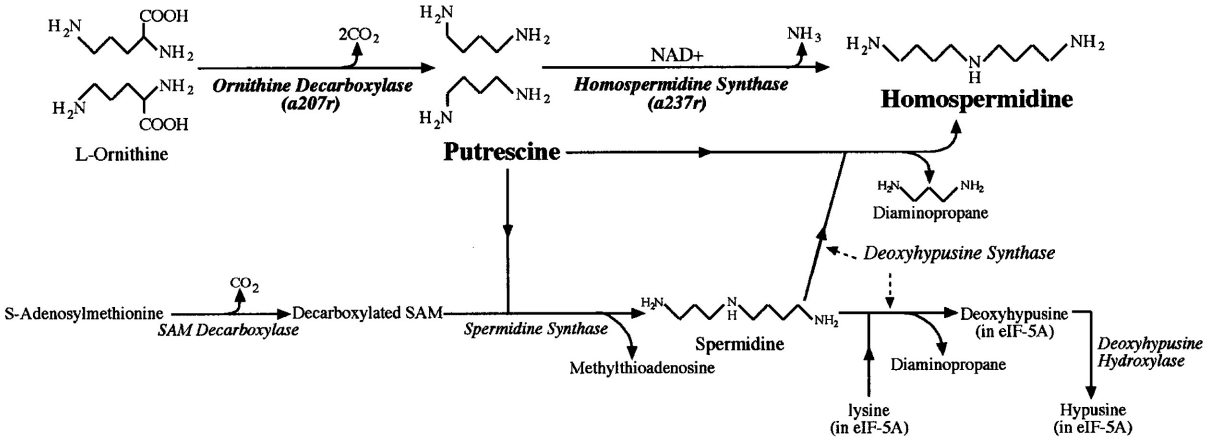

FIG. 1. Biosynthesis of homospermidine in bacteria (starting with ornithine) and plants (starting with ornithine and *S*-adenosylmethionine). Virus PBCV-1 encodes ornithine decarboxylase (*a207r*) and HSS (*a237r*) genes.

double-stranded DNA, plaque-forming viruses that infect certain unicellular, eukaryotic chlorella-like green algae (Van Etten *et al.,* 1991; Van Etten, 1995). The 330-kb PBCV-1 genome has been sequenced and is predicted to contain 377 protein encoding genes and 10 tRNA genes (Lu *et al.,* 1995, 1996; Li *et al.,* 1995, 1997; Kutish *et al.,* 1996). Many of the proteins encoded by PBCV-1 are unexpected, and their functions in the virus life cycle are unknown; among these are the enzymes hyaluronan synthase (DeAngelis *et al.,* 1997, Graves *et al.,* 1999), glutamine:fructose-6-phosphate amidotransferase (Landstein *et al.,* 1998), UDP-glucose dehydrogenase (Landstein *et al.,* 1998), and, as described here, a protein with HSS activity. To our knowledge, PBCV-1 is the first virus to encode a polyamine biosynthetic enzyme.

Results

Similarity between R. viridis HSS enzyme and PBCV-1 ORF A237R

The deduced 518-residue protein encoded by chlorella virus PBCV-1 ORF A237R has 34% amino acid identity (and a FASTA score of 541) with the 477-residue *R. viridis* HSS enzyme (**Fig. 2**). The most striking identity (44%) between the two proteins occurs between amino acid residues 205 and 410. Currently, databases contain no

```
R. viridis                          MTD..WPV YHRI..... .DGPIVMIGF  19
PBCV-1       MYMNSKKSNR DVNVNSNGAN SNVKNNRFVN ANKLNFSVDL GDRRILQVGC  50

             GSIGRGTLPL IERHFAFDRS KLVVIDP.SD EARKLAEA.R GVRFIQQAVT  67
             GGVGASMPPL YKRHLKFSSG NIIIIDKNRT KIDKFAEKYP TMKFINTEVT 100

             RDNYRELLVP LLTAGPGQGF CVNLSVDTSS LDIMELARFN GALYIDTVVE 117
             KNNYKNIIDQ YLKKG...DV FVDLAWYMNT KDLLRYCHEK GIHFVNTAIE 147

             PWLGFYFDPD LKPEARSNYA L....RETVL AARPNKPGGT TAVSCCGANP 163
             SWYG...EED CKAKTKECET LYRHQHDVRE LAKSWGNKGP TAVVGHGANP 194

             GMVSWFVKQA LVN....LAA DLGVTGEEPT T..REEWARL ...AMDLGVK 204
             GWVSHAMKIG IQDWVDYLSK KNSSDSNVKK AKEWLAKGKY NEAAKLLNIQ 244

             GIHIAERDTQ RASFPKPFDV FVNTWSVEGF VSEGLQPAEL GWGTFERWMP 254
             VIHISERDTQ ITNDPKKVGE FVNTWSPTGL IEEGSLPAEL GWGTHET.MK 293

             DNARGHDSGC GAGIYLLQPG ANTRVRSWTP TAMAQYGFLV THNESISIAD 304
             QYVKHFSKGP GNEVYIPKSM AMNTTVKSVV PSGEIVGCVI PHEEANSISY 343

             FLTVRDAAGQ AVYRPTCHYA YHPCNDAVLS LHEMFG.SGK RQSDWRILDE 353
             FLTT.TKGGK ATYRPTVHYA YMLPDVAIAS LQEYQADGCP DFLKKERVLK 392

             TEIVDGIDEL GVLLYGHGKN AYWYGSQLSI EETRRIAPDQ NATGLQVSSA 403
             DDIISGKDEL GVLMMSPKYG KWWTGSLLDI ETSRKLVPHQ SATIVQVSAS 442

             VLAGMVWALE NPNAGIVEAD DLDFRRCL.E VQTPYLGPVV GVYTDWTP.L 451
             VLAAIIYALK YSNLGPIFPE DMDSDWIMKK LIMPYLGEWR SAKIVWEPSL 492

             AGRPGLFPED IDTSDPWQFR NVLVRD 477
             SNVPKKYHKT KDLIFEKFLI NPPVME 518
```

FIG. 2. Amino acid alignment of HSS enzymes from *R. viridis* and chlorella virus PBCV-1. Identical amino acids are highlighted.

other *hss* genes. The G + C content of the A237R ORF is 44%, similar to the 40% G + C content of the entire PBCV-1 genome.

Expression of recombinant PBCV-1 HSS enzyme

ORF A237R has two potential start codons: codon 1 and codon 3 (Fig. 2). Therefore, two pET-15b-based plasmids were constructed to produce A237R-His-tagged recombinant proteins in *Escherichia coli*. Plasmids pBB21 and pBB23 were designed so that the first and third ATGs, respectively, of ORF A237R served as the translational start codons. At 3 h after induction, a fusion protein of the expected size, 58 kDa, composed ~8% of the soluble protein in extracts from bacteria containing each of the plasmids (**Fig. 3**, lane 4; only the results with cells expressing pBB23 are shown in Fig. 3). Before isopropyl-b-D-thiogalactopyranoside (IPTG) addition, uninduced cells contained small

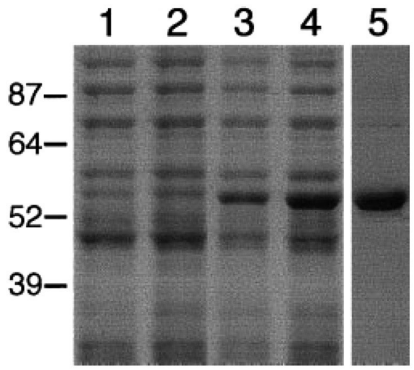

FIG. 3. SDS–PAGE analysis of soluble protein from *E. coli* BL21 (DE3) expressing the PBCV-1 A237R protein. *E. coli* with pET-15b before (lane 1) and 3 h after IPTG induction (lane 2) and *E. coli* with pBB23 before (lane 3) and 3 h after IPTG induction (lane 4). Recombinant protein after passage over a His-binding column (lane 5).

amounts of the 58-kDa protein, indicating that uninduced cells produce a basal level of T7 RNA polymerase activity (Studier *et al.,* 1990). *E. coli* cells with vector alone produced no 58-kDa protein.

Homospermidine in E. coli-expressing ORF A237R

Polyamine contents were measured in wild-type *E. coli* and recombinant *E. coli* expressing either the *R. viridis hss* gene or both forms of the *a237r* gene. Wild-type cells contained putrescine, cadaverine, spermidine, and spermine but no detectable homospermidine (**Table 1**). In contrast, *E. coli* cells expressing the *R. viridis hss* gene contained homospermidine (930 nmol/g fresh weight), as did *E. coli* cells expressing pBB21 or pBB23, albeit at lower levels (24 and 141 nmol/g fresh weight, respectively). Because pBB23-containing cells produced about five times more homospermidine than pBB21-containing cells, pBB23-containing *E. coli* was used for the remainder of the experiments. The growth medium of pBB23- containing cells dramatically influenced the ratio of spermidine to homospermidine, which varied from 13:1 in cells grown in LB medium to 0.3:1 in cells grown in a polyamine-free medium. The presence of homospermidine in the polyamine fraction from cells expressing the *a237r* gene was confirmed by both gas chromatography– mass spectroscopy and liquid chromatography–mass spectroscopy. In these confirmatory experiments, cells were grown in polyamine-free medium.

Table 1 Polyamine Content of Wild-Type Strain *E. coli* BL21 (DE3), and Recombinant *E. coli* Expressing the *R. viridis hss* Gene (PAK) and Two Forms of the Virus PBCV-1 *hss* Gene (pBB21 and pBB23)

Plasmid	*Polyamine*	*Concentration (nmol/g_{fw})*
pET-15b	Putrescine	1293
Cadaverine	315	
Spermidine	3052	
Spermine	36	
pAK *R. viridis*	Putrescine	1114
Cadaverine	286	
Spermidine	1396	
Homospermidine	930	
Spermine	265	
pBB21	Putrescine	1726
Cadaverine	427	
Spermidine	2827	
Homospermidine	24	
Spermine	89	
pBB23	Putrescine	1375
Cadaverine	490	
Spermidine	1853	
Homospermidine	141	
Spermine	84	

Note. The cells were grown in LB broth.

The incorporation of exogenous ^{14}C-putrescine into homospermidine in pBB23 containing cells was also monitored at various times after IPTG induction. At 3 and 8 h after induction, the cells took up 7.5% and 18% of exogenous ^{14}C-putrescine, respectively, and of the ^{14}C-putrescine taken up by the cells, 30% and 54%, respectively, was incorporated into homospermidine (**Table 2**). Together, the results in Tables 1 and 2 establish that *E. coli* cells expressing *a237r* synthesize homospermidine.

Recombinant A237R protein has HSS activity

Protein extracts were prepared from *E. coli* expressing *a237r*, and after purification over a His-binding column (Fig. 3, lane 5), the recombinant protein was assayed for HSS activity. NAD^+ was required

Table 2 Incorporation of ^{14}C-Putrescine into Homospermidine in *E. coli* Expressing the PBCV-1 *hss* Gene (pBB23)

Time (h)	*^{14}C-Putrescine remaining in the medium (cpm)*	*Incorporation of ^{14}C-putrescine into homospermidine (%)*
0	3427	0
3	3171	30
8	2826	54

for activity, and no polyamines other than putrescine were required. Maximum HSS activity occurred at 20°C in 50 mM K^+ (**Fig. 4**). Increasing the incubation temperature to 25°C reduced the recombinant HSS activity by ~50%, and no activity was detected at 37°C. Protein extracts from wild-type *E. coli* BL21 (DE3) cells had no HSS activity.

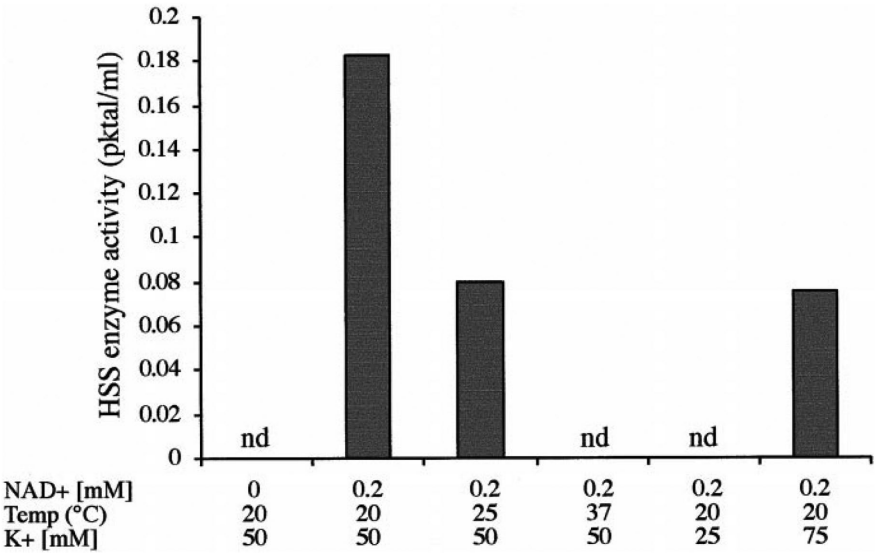

Fig. 4. Characteristics of the recombinant PBCV-1-encoded HSS. The recombinant protein was assayed at the indicated temperatures and K^+ concentrations. Enzyme reactions were incubated for 2 h and then terminated by applying 20-μl aliquots onto TLC plates. The amount of product was calculated from the ratio of labeled putrescine to homospermidine obtained by radioscanning with a TLC multichannel analyzer. nd means no homospermidine was detected.

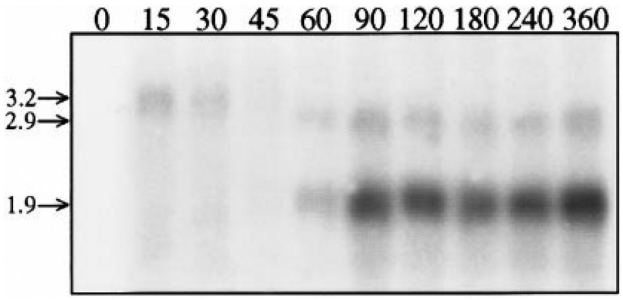

Fig. 5. Northern blot analyses of RNA isolated from uninfected (0) and PBCV-1-infected chlorella cells at the indicated times p.i. The RNAs were hybridized with an antisense *a237r* gene probe.

Expression of the a237r gene in PBCV-1-infected cells

RNA was extracted from cells at various times after virus infection and hybridized to an antisense *a237r* gene probe to determine whether the gene was transcribed during PBCV-1 infection. The probe hybridized strongly to a 1.9-kb RNA extracted from cells at 60–360 min post-infection (p.i.) (**Fig. 5**). This RNA size is sufficient for a protein of 518 amino acid residues. Because PBCV-1 DNA synthesis begins ~60 min p.i. (Van Etten *et al.,* 1984), the *hss* gene is a late gene. However, the probe also hybridized slightly to another late RNA of ~2.9 kb and an early RNA of ~3.2 kb. The nature of these other RNAs is unknown, but complex transcription patterns have been observed with other PBCV-1 genes, such as glutamine:fructose-6-phosphate amidotransferase (Landstein *et al.,* 1998).

The recombinant PBCV-1 HSS protein reacted with a polyclonal antibody to *R. viridis* HSS (data not shown). However, attempts to detect the PBCV-1 HSS protein in cell extracts, made at various times after virus infection, by Western blotting were unsuccessful; presumably, the PBCV-1 HSS protein does not accumulate to levels sufficient for immunochemical detection by this antiserum.

Polyamines in virions and uninfected and infected chlorella cells

The polyamine content of uninfected chlorella as well as cells at 60, 150, and 240 min after PBCV-1 infection was determined (**Table 3**). Healthy and infected cells contained homospermidine, putrescine,

Table 3 Polyamine Content of Uninfected and PBCV-1-Infected Chlorella Cells and PBCV-1 Virions

	Polyamine (nmol/3.3 × 10^{10} cells) (~1 g_{fw})				
	Putrescine	*Cadaverine*	*Spermidine*	*Homospermidine*	*Spermine*
Cell					
Uninfected	0.76	3.90	5.82	1.33	ND[a]
60 min p.i.	0.76	3.42	5.28	1.99	ND
150 min p.i.	1.54	1.48	3.33	0.62	ND
240 min p.i.[b]	2.52	2.17	3.28	0.52	ND
Virus	(nmole/1.3 × 10^{13} virus particles) (0.11 g)[c]				
PBCV-1	6.0	0.68	4.22	0.72	ND

a. ND, not detected.
b. Virus assembly is complete, and most virus particles are released between 420 and 480 min p.i.
c. Approximate number of virus particles produced from 3.3 3 1010 cells.

cadaverine, and spermidine. However, polyamine concentrations changed after virus infection, especially between 60 and 150 min p.i. The putrescine level increased ~2-fold, and the other three polyamines decreased 50–60%. Infection by another chlorella virus, SC-1A, produced similar changes in the polyamine levels of infected cells (Xing, 1996). These same four polyamines were also detected in purified PBCV-1 virions (Table 3). The low concentration of homospermidine relative to spermidine in the virions was independently confirmed with a new procedure for quantifying polyamines using ion spray–mass spectroscopy (Furuumi *et al.,* 1998).

Using the data in Table 3, the number of polyamine molecules per virus particle was calculated to determine whether they might be involved in neutralizing the virus DNA. Virions on average contain 539 polyamine molecules with a combined charge of 1308 (**Table 4**). Because the total number of nucleotide phosphates in the double-stranded DNA of a virus particle exceeds 660,000 (Li *et al.,* 1997), the polyamines could only neutralize ~0.2% of DNA phosphates.

In separate experiments, virion polyamines proved to be freely exchangeable. With our standard virus purification protocol (Van Etten *et al.,* 1981, 1983), which uses a Tris buffer, we found that Tris (a monoamine) could replace >.95% of the viral polyamines. Likewise, extensive washing of purified PBCV-1 with polyamine-free buffer (e.g., PBS) displaced both Tris and viral polyamines.

Table 4 Polyamine Content of a PBCV-1 Virion

Polyamine	*Molecules/ virion*	*Total polyamine charge*	*Phosphate residues neutralized (%)*
Putrescine	277	554	0.08
Cadaverine	32	64	0.01
Spermidine	196	588	0.09
Homospermidine	34	102	0.02
Total	539	1308	0.20

The a237r gene is widespread in the chlorella viruses

To determine whether the *hss* gene is common among the chlorella viruses, DNA from 42 chlorella viruses and host *Chlorella* NC64A were hybridized to the *a237r* gene probe used in the Northern analyses. DNA from all of the viruses infecting *Chlorella* NC64A hybridized to various levels with the probe (**Fig. 6**). No hybridization was detected with DNA from the host or with DNA from five 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, the *hss* gene is widespread among the NC64A viruses.

Discussion

The polyamines putrescine, spermidine, and spermine are common in cells and also are structural components of many viruses, where they are believed to aid in neutralizing viral nucleic acid (Tyms *et al.*, 1990; Cohen, 1998). PBCV-1 virus particles, as well as uninfected and virus-infected *Chlorella* NC64A cells, contain putrescine, cadaverine, spermidine, and homospermidine. However, it is unlikely that these polyamines are important in the neutralization of PBCV-1 DNA because the number of polyamine molecules per PBCV-1 virion is so low that they could neutralize only ~0.2% of the virus phosphate residues. Presumably, the viral DNA is neutralized by either positively charged ions (e.g., Mg^{2+}), small peptides, or even proteins; Yamada *et al.* (1996) demonstrated the presence of DNA-binding proteins in the virion of a related chlorella virus. Furthermore, the functional significance of polyamines in the PBCV-1 particle must be limited because they are associated only loosely with the virion since they can be replaced by

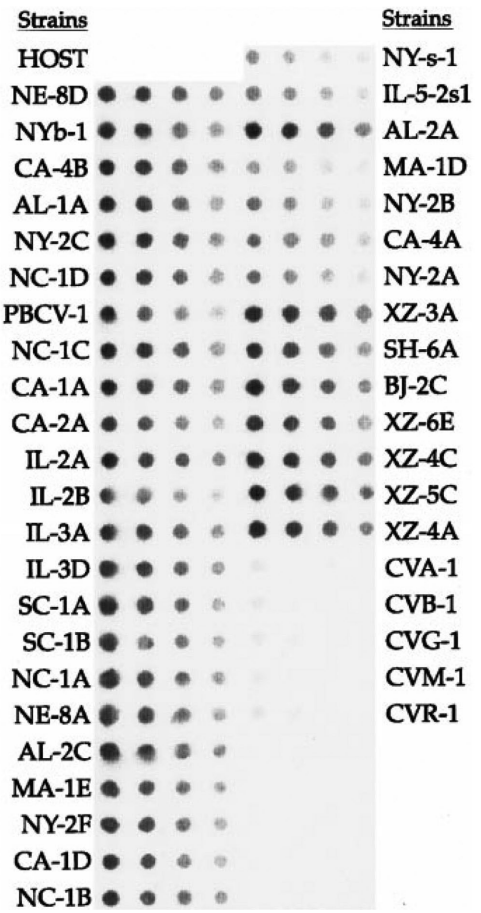

Fig. 6. Hybridization of the PBCV-1 *a237r* gene to DNA isolated from the host *Chlorella* NC64A and 37 *Chlorella* NC64A viruses and 5 viruses (CVA-1, CVB-1, CVG-1, CVM-1, and CVR-1) that infect *Chlorella* Pbi. The blots contain 1, 0.5, 0.25, and 0.12 μg of DNA, left to right, respectively.

Tris or displaced by washing the particles in a polyamine-free buffer without affecting virus infectivity.

The impetus for measuring PBCV-1 polyamine content was the discovery that the virus encodes an ORF that has 34% amino acid identity with an HSS enzyme from *R. viridis*. The PBCV-1 *hss* gene encodes a protein with NAD-dependent HSS enzyme activity. To our knowledge, this is the first report of a virus-encoded enzyme involved in polyamine biosynthesis. Interestingly, PBCV-1 also encodes a protein that resembles ornithine decarboxylase (Lu *et al.,* 1996), a key enzyme in polyamine biosynthesis and one of the most highly regulated enzymes in eukaryotic organisms (e.g., Davis *et al.,* 1992; Cohen, 1998). Ornithine decarboxylase converts ornithine to putrescine, the substrate for the bacterial and PBCV-1 HSS enzymes (Fig. 1). Preliminary experiments with a recombinant PBCV-1 ornithine decarboxylase indicate that the protein has the expected enzyme activity (T. J.

Morehead and J. L. Van Etten, unpublished results). Thus PBCV-1 encodes the complete biosynthetic pathway for the synthesis of homospermidine from ornithine.

It is important to note that the PBCV-1-encoded HSS catalyzes the NAD-dependent formation of homospermidine from two molecules of putrescine and that spermidine does not participate in the reaction. In contrast, spermidine is a required precursor of homospermidine synthesis in higher plants (Bottcher *et al.,* 1994). The difference between homospermidine synthesis in bacteria and plants derives from the discovery that eukaryotic translation initiation factor eIF-5A contains a hydroxyputrescine- lysine adduct called hypusine [N^6-(4-amino-2-hydroxybutyl)-2,6-hexanoic acid] in the ratio of one hypusine per molecule of protein (Park *et al.,* 1993; Cohen, 1998). Hypusine is formed by attaching a 2-hydroxybutylamine moiety to the ϵ-amino group of a single lysine side chain of the eIF-5A precursor protein. Hypusine synthesis requires two enzymes (Fig. 1). The first enzyme, deoxyhypusine synthase, takes the butylamine portion of spermidine and adds it to the lysine side chain while liberating 1,3-diaminopropane. The second enzyme, deoxyhypusine hydroxylase, adds the 2-hydroxy group. In plants, deoxyhypusine synthase also can transfer butylamine from spermidine to putrescine, resulting in homospermidine (Kaiser, 1999). Consequently, deoxyhypusine synthase also functions as an HSS.

The PBCV-1-encoded HSS is clearly related to bacterial enzymes rather than to plant enzymes; this conclusion is supported by the amino acid similarity between A237R and *R. viridae* HSS, as well as the cross-reactivity of the PBCV-1 enzyme with rabbit polyclonal antisera prepared against *R. viridae* HSS. Presumably the host alga synthesizes homospermidine from spermidine and putrescine by deoxyhypusine synthase and late in PBCV-1 infection the virus produces homospermidine from two putrescines. The inability of the PBCV-1 *hss* gene to hybridize with the host "*hss*" gene(s) and the 3-fold increase in putrescine observed after PBCV-1 infection are consistent with this scenario.

Even if this scenario is correct, it leads to two questions. Why is homospermidine biosynthesis important for PBCV-1 replication? Why does the infected cell need two pathways to synthesize homospermidine; especially when so little of the compound is packaged in the virion? With respect to the first question (i.e., why PBCV-1 contains the genes for ornithine decarboxylase and HSS), we suggest two possibilities. (1) Spermidine and homospermidine are functionally distinct;

that is, the extra methylene in homospermidine allows it to carry out a function that spermidine cannot. (2) Spermidine and homospermidine are functionally interchangeable, but the carbon flow through their respective biosynthetic pathways is determined by the nutritional/physiological status of the host cell. The two homospermidine synthesis pathways differ in one important feature. Homospermidine synthesized by the virus-encoded enzyme requires two molecules of putrescine, and thus its synthesis depends only on cellular levels of carbon and nitrogen. In contrast, the propylamine in spermidine comes from *S*-adenosylmethionine and thus is dependent on cellular levels of carbon, nitrogen, and sulfur. This distinction suggests that the homospermidine concentration in the infected cell might increase if the host chlorella is grown under limiting sulfur conditions. This possibility will be explored in the future.

Materials and methods

Strains and culture conditions

The growth of PBCV-1 host *Chlorella* strain NC64A on MBBM medium, the production and purification of PBCV-1, and the isolation of PBCV-1 DNA have been described (Van Etten *et al.,* 1981, 1983). *E. coli* strains INVαF (InVitrogen, Carlsbad, CA), XL1 Blue (Stratagene, La Jolla, CA), and BL21 (DE3) (Novagen, Madison, WI) were grown in LB medium (Samrook *et al.,* 1989). In some experiments, *E. coli* expressing the recombinant HSS enzyme were grown on a polyamine-free medium consisting of 0.4% glucose, 2.0 g/l citric acid, 3.5 g/l $NaPO_4$, 0.2 g/l $MgSO_4$, 0.002% thiamine, and 5 mg/l calcium pantothenate (Vogel and Bonner, 1956).

Cloning and expression of the recombinant PBCV-1 hss gene

PBCV-1 ORF A237R has two potential translational start codons: codon 1 and codon 3 (Fig. 2). Therefore, two versions of the PBCV-1 A237R ORF were cloned from PCR amplified viral DNA using the following oligonucleotide primers: 59 primer 1, TGTGCATATGTATATGAATTCAAAAAAG; 59 primer 3, ACTGCATATGAATTCAAAAAAGAGTAAC; and 39 primer, TTTTGGATCCTTATTCCATAACAGGAGG. The underlined bases indicate the 59 and 39 restriction sites for *Nde*I and

*Bam*HI endonucleases, respectively, that were used for cloning. The PCR reaction (25 µl) contained 770 ng of PBCV-1 genomic DNA, 480 pmol of each primer, 50 µmol of each dNTP, 1.5 mM $MgCl_2$, and 5 units of *Taq* DNA Polymerase (Qiagen, Hilden, Germany) with the appropriate buffer. Amplification was performed in a Perkin–Elmer Thermocycler (Foster City, CA) using 35 cycles of 97°C for 1 min, 60°C for 1 min, and 72°C for 1 min. PCR fragments of the expected size were digested with *Nde*I and *Bam*HI and then inserted into *Nde*I–*Bam*HI sites of the expression plasmid pET-15b (Novagen). The resulting plasmids, pBB21 and pBB23, were transformed into *E. coli* XL1 Blue for maintenance or into *E. coli* BL21 (DE3) (Studier and Moffat, 1986) for expression. The *a237r* gene was expressed by growing cells overnight at 37°C in 200 ml of LB medium containing 100 µg/ml ampicillin to an A_{600} of 1.5. Then 50 ml of fresh LB medium was added to the culture (final volume of 250 ml), and the cells were induced with 4 mM IPTG 1 h later. Aliquots (1 ml) of cells were harvested by centrifugation at hourly intervals. The cells were washed with lysis buffer (50 mM Tris–HCl, pH 8.0, 2 mM EDTA), centrifuged, and resuspended in 400 µl of lysis buffer containing 100 µg/ml lysozyme. After incubation on ice for 15 min, cells were disrupted by two 30-s exposures to sonication, and the samples were centrifuged at 4°C.

Then 10 µl of supernatant was added to an equal volume of 2× dissociation buffer (Sambrook *et al.,* 1989) and boiled for 5 min. SDS–PAGE was performed in the buffer system of Laemmli (1970) on 10% acrylamide gels. Proteins were stained with Coomassie Brilliant Blue R 250. The remainder of the sample was loaded onto a His-trap column (Amersham Pharmacia, Uppsala, Sweden) in 5 mM imidazole, 0.5 M NaCl, and 20 mM Tris–Cl, pH 7.9, and washed with 60 mM imidazole, 0.5 M NaCl, and 20 mM Tris–Cl, pH 7.9, and the recombinant protein was eluted with 1 M imidazole, 0.5 M NaCl, and 20 mM Tris–Cl, pH 7.9. The sample was then applied to an NAP 10 column (Pharmacia, Piscataway, NJ) and eluted with 1.5 ml of ice-cold elution buffer (50 mM KPO_4 and 2 mM dithiothreitol, pH 8.5); 100-µl fractions were collected and assayed for HSS activity.

HSS enzyme activity

Recombinant HSS enzymes were assayed at 20°C in a total volume of 125 µl of S buffer (50 mM KPO_4, 2 mM dithiothreitol, pH 8.5),

containing 3.7 μM ^{14}C-putrescine (0.5 μCi/assay), 1.0 mM putrescine, and 0.2 mM NAD^+ (Tholl *et al.,* 1996). Enzyme reactions were incubated for either 2 or 16 h and then terminated by applying 20-μl aliquots onto thin-layer chromatography (TLC) plates (Silica gel 60 F254; Merck, Rahway, NJ). Putrescine (Rf 0.45) and homospermidine (Rf 0.11) were separated in a solvent system consisting of acetone/methanol/25% NH_4OH (4:3:2 by volume). The amount of product was calculated from the ratio of labeled putrescine to homospermidine obtained by radioscanning with a TLC multichannel analyzer (Raytest, Wilmington, DE) (Bottcher *et al.,* 1993).

The in vivo conversion of ^{14}C-putrescine to homospermidine by recombinant E. coli

Forty ml of actively growing *E. coli* cells containing pBB23 was induced with 0.4 mM IPTG. Simultaneously, 1 μCi of ^{14}C-putrescine was added to the culture, and 10-ml samples were centrifuged 3 and 8 h later. The bacterial pellets were frozen in liquid nitrogen, resuspended in 1 ml of hot methanol (50–60°C), and incubated for 30 min. After centrifugation for 20 min at 13,000 rpm, a 10-μl aliquot of the supernatant was applied to a Silica gel 60 F254 TLC plate and chromatographed as described above.

Polyamine analysis

E. coli cells and uninfected and PBCV-1-infected chlorella cells (1.9×10^{10} cells/sample) at 60, 150, and 240 min p.i. were harvested by centrifugation and lyophilized, and polyamines were extracted according to the protocol of Redmond and Tseng (1979). Polyamines also were extracted from 2 mg of lyophilized PBCV-1 virions by the same procedure.

Polyamines were analyzed either as benzoyl derivatives by HPLC (Redmond and Tseng, 1979), as dabsyl derivatives by HPLC (Koski *et al.,* 1987), as *N*-carbomethoxyderivatives by gas chromatography–mass spectrometry (Husek *et al.,* 1992), or as heptafluorobutyryl derivatives by ion spray ionization–mass spectrometry (Furuumi *et al.,* 1998).

Northern, Southern, and Western analyses

Chlorella cells (1 × 109) 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 as described (Landstein *et al.,* 1996). RNA was hybridized to an antisense ^{32}P− single-stranded DNA probe (Graves and Meints, 1992) at 65°C in 50 mM $NaPO_4$, 1% BSA, and 2% SDS. Virus DNAs used for dot blots were denatured and applied to nylon membranes (Micron Separation Inc., Westborough, MA), fixed by UV cross-linking, and hybridized with the same probes used for the Northern analysis.

Proteins were transferred to nylon membranes using a Novablot Multiphore II apparatus (Pharmacia) as described (Sambrook *et al.,* 1989). Immunodetection was carried out with an Immuno-Blot Kit (Bio-Rad, Hercules, CA). The polyclonal antiserum used in Western blot experiments was prepared in rabbits against the *R. viridis* HSS enzyme.

Other procedures

DNA and putative protein sequences were analyzed with the use of the University of Wisconsin Computer Group package of programs (Genetics Computer Group, 1997). The GenBank accession number for PBCV-1 ORF A237R is U42580.

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References

Bottcher, F., Adolph, R. D., and Hartmann T. (1993). Homospermidine synthase, the first pathway-specific enzyme in pyrrolizidine biosynthesis. *Phytochemistry* **32**, 679–689.

Bottcher, F., Ober, D. J., and Hartmann, T. (1994). Biosynthesis of pyrrolizidine alkaloids: Putrescine and spermidine are essential substrates of enzymatic homospermidine formation. *Can. J. Chem.* **72,** 80–85.

Cohen, S. S. (1998). "A Guide to the Polyamines." Oxford University Press, New York, p. 595.

Davis, R. H., Morris, D. R., and Coffino, P. (1992). Sequestered end products and enzyme regulation: The case of ornithine decarboxylase. *Microbiol. Rev.* **56,** 280–290.

DeAngelis, P. L., Jing, W., Graves, M. V., Burbank, D. E., and Van Etten, J. L. (1997). Hyaluronan synthase of chlorella virus PBCV-1. *Science* **278,** 1800–1803.

Fujihara, S., and Harada, Y. (1989). Fast-growing root nodule bacteria produce a novel polyamine, aminobutylhomospermidine. *Biochem. Biophys. Res. Commun.* **165,** 659–666.

Furuumi, N., Amano, D., Xu, Y. J., Samejima, K., Niitsu, M., and Shirahata, A. (1998). Ion spray ionization-mass spectrometric separation and determination of heptafluorobutyryl derivatives of polyamines. *Anal. Biochem.* **265,** 253–259.

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

Graves, M. V., Burbank, D. E., Roth, R., Heuser, J., DeAngelis, P. L., and Van Etten, J. L. (1999). Hyaluronan synthesis in virus PBCV-1 infected chlorella-like green algae. *Virology* **257,** 15–23.

Graves, M. V., and Meints, R. H. (1992). Characterization of the major capsid protein and cloning of its gene from algal virus PBCV-1. *Virology* **188,** 198–207.

Hamana, K., Kamekura, M., Onishi, H., Akazawa, T., and Matsuzaki, S. (1985). Polyamines in photosynthetic eubacteria and extreme-halophilic archaebacteria. *J. Biochem.* **97,** 1653–1658.

Hamana, K., and Matsuzaki, S. (1982). Widespread occurrence of norspermidine and norspermine in eukaryotic algae. *J. Biochem.* **91,** 1321–1328.

Hamana, K., and Matsuzaki, S. (1985). Distinct difference in the polyamine compositions of Bryophyta and Pteridophyta. *J. Biochem.* **97,** 1595–1601.

Hamana, K., and Matsuzaki, S. (1992). Polyamines as a chemotaxonomic marker in bacterial systematics. *CRC Crit. Rev. Microbiol.* **18,** 261–283.

Hamana, K., Matsuzaki, S., Niitsu, M., and Samejima, K. (1989). Polyamine distribution and the potential to form novel polyamines in phytopathogenic agrobacteria. *FEMS Microbiol. Lett.* **65,** 269–274.

Hamana, K., Matsuzaki, H., Niitsu, M., and Samejima, K. (1992). Distribution of unusual polyamines in leguminous seeds. *Can. J. Bot.* **70,** 1984–1990.

Hamana, K., Miyagawa, K., and Matsuzaki, S. (1983). Occurrence of *sym*-homospermidine as the major polyamine in nitrogen-fixing cyanobacteria. *Biochem. Biophys. Res. Commun.* **112,** 606–613.

Husek, P. Huang, Z. H., and Sweely, C.C. (1992). Gas chromatographic determination of amines, amino alcohols and acids after treatment with alkyl-chloroformates. *Anal. Chim. Acta* **259,** 185–192.

Kaiser, A. (1999). Cloning and expression of a cDNA encoding homospermidine synthase from *Senecio vulgaris* (Asteraceae) in *Escherichia coli*. *Plant J.* **19,** 1–7.

Kneifel, H. (1977). Untersuchungen uber Inhaltsstoffe von Algen. *Chemiker Zeitung* **101,** 165–168.

Koski, P., Helander, M., Sarvas, M., and Vaara, M. (1987). Analysis of polyamines as their dabsyl derivatives by reversed-phase high-performance liquid chromatography. *Anal. Biochem.* **164,** 261–266.

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.

Kuttan, R., Radhakrishnan, A. N., Spande, T., and Witkop, B. (1971). *Sym*-homospermidine, a naturally occurring polyamine. *Biochemistry* **10,** 361–365.

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

Landstein, D., Graves, M. V., Burbank, D. E., DeAngelis, P., and Van Etten, J. L. (1998). Chlorella virus PBCV-1 encodes functional glutamine:fructose-6-phosphate amindotransferase and UDP-glucose dehydrogenase enzymes. *Virology* **250,** 388–396.

Landstein, D., Mincberg, M., Arad, S., and Tal, J. (1996). An early gene of the chlorella virus PBCV-1 encodes a functional aspartate transcarbamylase. *Virology* **221,** 151–158.

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.

Matsuzaki, S., Hamana, K., Imai, K., and Matsuura, K. (1982). Occurrence in high concentrations of N1-acetylspermidine and *sym*-homospermidine in the hamster epididymis. *Biochem. Biophys. Res. Commun.* **107,** 307–313.

Park, M. H., Wolff, E. C., and Folk, J. E. (1993). Is hypusine essential for eukaryotic cell proliferation? *Trends Biochem. Sci.* **18,** 475–479.

Redmond, J. W., and Tseng, A. (1979). HPLC determination of putrescine, cadaverine, spermidine and spermine. *J. Chromatogr.* **170,** 479–481.

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.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Scherer, P., and Kneifel, H. (1983). Distribution of polyamines in methanogenic bacteria. *J. Bacteriol.* **154,** 1315–1322.

Smith, T. A. (1977). Homospermidine in *Rhizobium* and legume root nodules. *Phytochemistry* **16,** 278–279.

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

Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185,** 60–89.

Tait, G. H. (1979). The formation of homospermidine by an enzyme from *Rhodopseudomonas viridis*. *Biochem. Soc. Trans.* **7,** 199–200.

Tholl, D., Ober, D., Martin, W., Kellermann, J., and Hartmann, T. (1996). Purification, molecular cloning and expression in *Escherichia coli* of homospermidine synthase from *Rhodopseudomonas viridis*. *Eur. J. Biochem.* **240,** 373–379.

Tyms, A. S., Clarke, J. R., Ford, L., and Edoranta, T. (1990). Polyamines and the growth of pathogenic viruses. *In* "The Biology and Chemistry of Polyamines" (Goldenberg, S. H., and Algranti, J. D., Eds.), pp. 121–133, IRL Press, Oxford.

Van Etten, J. L. (1995). Minireview: Giant chlorella viruses. *Mol. Cells* **5,** 99–106.

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.

Vogel, H. J., and Bonner, D. M. (1956). Acetylornithinase of *Escherichia coli*: Partial purification and some properties. *J. Biol. Chem.* **218,** 97–106.

Yamada, T., Furukawa, S., Hamazaki, T., and Songsri, P. (1996). Characterization of DNA-binding proteins and protein kinase activities in chlorella virus CVK2. *Virology* **219,** 395–406.

Yamamoto, S., Aoyama, Y., Kawaguchi, M., Iwado, A., and Makita, M. (1983). Identification and determination of *sym*-homospermidine in root of water hyacinth *Eichhornia crassipes* Solms. *Chem. Pharm. Bull.* **31,** 3315–3318.

Yamamoto, S., Nagata, S., and Kusaba, K. (1993). Purification and characterization of homospermidine synthase in *Acinetobacter tartarogenes* ATCC 31105. *J. Biochem.* **114,** 45–49.

Xing, W. Master's thesis, University of Nebraska, 1996.