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Characterization of ϕ12, a Bacteriophage Related to ϕ6: Nucleotide Sequence of the Large Double-Stranded RNA

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The isolation of additional bacteriophages besides ϕ6 containing segmented double-stranded RNA genomes (dsRNA) has expanded the *Cystoviridae* family to nine members. Comparing the genomic sequences of these viruses has allowed evaluation of important genetic as well as structural motifs. These comparative studies are resulting in greater understanding of viral evolution and the role played by genetic and structural variation in the assembly mechanisms of the cystoviruses. In this regard, the large double-stranded RNA genomic segment of bacteriophage ϕ12 was copied as cDNA and its nucleotide sequence determined. This genome's organization is similar to that of the large segment of bacteriophages ϕ6, ϕ8, and ϕ13. In the amino acid sequence of the viral RNA-dependent RNA polymerase (P2), similarity was found to the comparable proteins of ϕ6, ϕ8, and ϕ13. Amino acid sequence similarity was also noted in the nucleotide triphosphate phosphorylase (P4) to the comparable proteins of ϕ8 and ϕ13. © 2002 Elsevier Science (USA)

INTRODUCTION

Bacteriophage ϕ12 was isolated from the leaves of the Sweet Basil plant (*Ocimum basilicum*) (Mindich *et al.,* 1999), and it is among the nine known members of the *Cystoviridae* family. Bacteriophage ϕ6, which until recently has been alone in the genus cystovirus (Van Regenmortel *et al.*, 1999), was isolated from bean straw infested with *Pseudomonas syringae* pv. *phaseolicola* (Vidaver *et al.,* 1973). ϕ12 is similar in structure to bacteriophage ϕ6 in that it also contains a genome of three segments of double-stranded RNA (dsRNA) (Semancik *et al.,* 1973) packaged inside a procapsid that is covered by a protein shell and a lipid-containing membrane with additional proteins (Vidaver *et al.,* 1973). It has recently been shown that the ϕ6 active polymerase subunit is very similar in structure to that of the polymerase of hepatitis C virus, suggesting an evolutionary link between dsRNA viruses and the flaviviruses (Butcher *et al.,* 2001).

The genome of ϕ6 has been cloned and sequenced and the replication cycle and structure of the virus have been extensively investigated (Butcher *et al.,* 1997; de Haas *et al.,* 1999; Mindich, 1999). Study of genomic packaging in ϕ6 led to the development of a detailed packaging model (Mindich, 1999; Onodera *et al.,* 1998; Qiao *et al.,* 1997). Specifically, it is proposed that the binding of the small (plus sense) single-stranded RNA segment (s) to sites on the outside of an empty procapsid and positioning its 5′ end at an entry portal initiate packaging. When segment s is packaged, the binding sites are lost and then new sites for the middle segment (m) appear on the outside of the particle. The packaging of segment m results in the loss of its binding sites, and sites for the large segment (l) are revealed. Each of the viral RNA segments contains a packaging sequence of about 200 nucleotides near the 5′ ends of the plus strands (Gottlieb *et al.,* 1994).

The isolation of additional bacteriophages other than ϕ6 containing three segments of dsRNA revealed some to be very similar to ϕ6 and others only distantly related (Mindich *et al.,* 1999). Eight additional cystoviruses, ϕ7 to ϕ14, were isolated and are being characterized, particularly in regard to their relationship to ϕ6. The close relatives of ϕ6 include ϕ7, ϕ9, ϕ10, and ϕ11. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of this group with primers derived from ϕ6 sequences was possible, and subsequent genomic sequence analyses demonstrated 80 to 85% sequence similarity. Within open-reading frames (*orfs*), the base sequence changes were concentrated in the third base of codon triplets so that the amino acid sequences remained highly conserved. The 5′ ends of the genomic plus strands were found to contain the *pac* sequences, which are about 200 nucleotides in length and unique and specific for the packaging of each segment. The sequences of the *pac*

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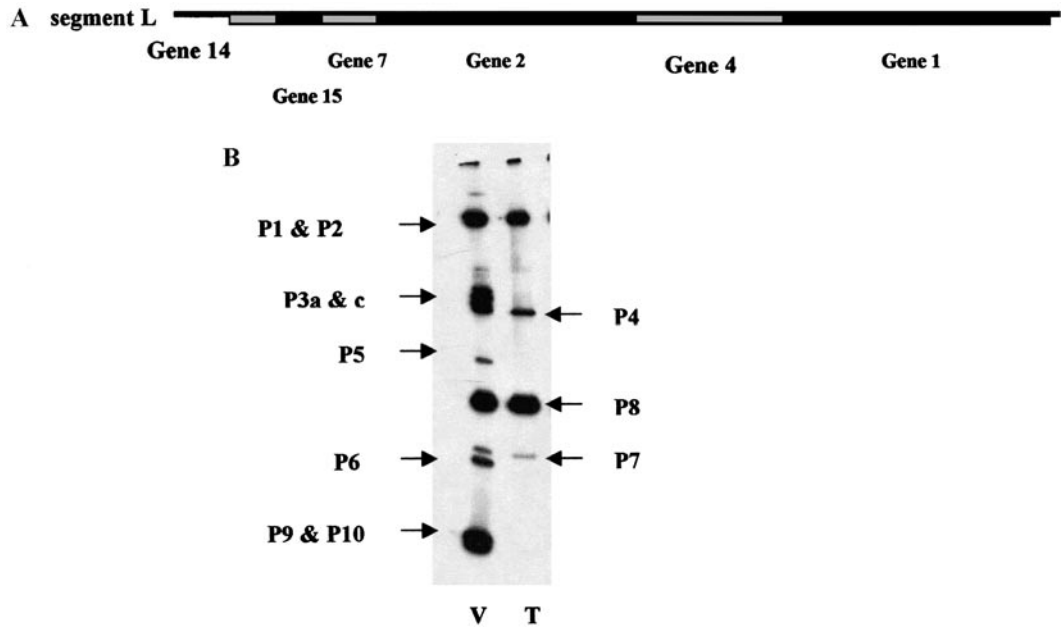

FIG. 1. (A) cDNA copy of the L genomic segment of ϕ12. The genes are numbered to correspond to those of ϕ6, ϕ8, and ϕ13. (B) Autoradiogram of PAGE analysis of whole virus (V) and Triton X-100-treated virus (T).

regions in this group of phages were about 90% identical, and it was also found that they were capable of accepting both the m and s segment of ϕ6 (Mindich *et al.,* 1999).

Bacteriophages ϕ8, ϕ12, and ϕ13 were judged to be distantly related to ϕ6 (Mindich *et al.,* 1999). These phages, while unable to infect the normal host of ϕ6, *P. syringae* pv. *phaseolicola* HB, can infect a mutant, LM2333. This latter strain is resistant to several common phages, most of which contain DNA genomes. The three phages were also able to infect both a derivative of LM2333 lacking type IV pili and a rough strain of *P. syringae,* Ro49da1. It is likely that these phages attach directly to the LPS. RT-PCR on the RNA segments of these phages using primers derived from ϕ6 was unsuccessful (Mindich *et al.,* 1999). Previous studies have determined the nucleotide sequence of both the ϕ8 and ϕ13 genomes, showing that the overall genetic organization is similar to ϕ6, although for the most part there is no similarity in either the nucleotide sequences or amino acid sequences. An exception is that the amino acid motifs characteristic of viral RNA polymerases are present in the protein sequences of the polymerase complex. In the case of bacteriophage ϕ8, it was noted that protein P8, which constitutes a shell around the procapsid in ϕ6, is part of the membrane in this phage. The ϕ8 and ϕ13 host attachment proteins consist of two peptides rather than the one found in ϕ6 (Hoogstraten *et al.,* 2000; Qiao *et al.,* 2000).

We have recently determined the nucleotide sequence of the small and middle segments of bacteriophage ϕ12 and have seen that the host cell attachment proteins (P6, P3a, P3b, and P3c) have marked similarity to the comparable proteins of ϕ13. In contrast, we found significant similarity of the ϕ12 lysis cassette proteins (P5 and P10) to those of bacteriophage ϕ6 (Gottlieb *et al.,* 2002). In this paper, we present the cDNA cloning and complete sequencing of the large genomic segment of ϕ12. We discuss the implications of this work on the evolutionary history of the cystoviruses.

RESULTS AND DISCUSSION

RNA sequence

The cDNA copies of the ϕ12 large genomic segment were sequenced and the arrangement of the genes was determined. The size of the L segment was found to be 6751 bp (Fig. 1A) and its base composition is 54.1% GC. The corresponding size of the L segment in ϕ6 is 6374 bp with a base composition of 55.5% GC (Mindich, 1988). The sizes of the corresponding segment in ϕ8 and ϕ13 were found to be 7051 and 6458 bp at 54.0 and 58.4% GC, respectively (Hoogstraten *et al.,* 2000; Qiao *et al.,* 2000).

Identification of genes

The genes in the L segment were named for the genes in the same position as ϕ6 and are also arranged in a similar manner to those of ϕ8 and ϕ13 (Fig. 1A and Table 1). The assignments were made on the basis of chromosomal position, size, and amino acid similarity or identity with those of the other cystoviruses. The gene assignments were also integrated with the results of SDS-polyacrylamide gel analysis (SDS-PAGE) of the viral proteins, both from purified virions and detergent-extracted

TABLE 1

List of *orfs* and Ribosome-Binding Sites for the ϕ12 L Segment

orf	Start	End	aa number	MW[a]	IEP[b]	Ribosome-binding sites
						Complement of the 16s rRNA 3′ end UAAGGAGGUGAUC
P14	410	709	99	11.5	8.79	GACUAACAGGGAAUCCAUUAUGCGA
P15	714	1016	100	11.1	8.85	GUAAUCAAGGAGUAAACGGAUGUUC
P7	1019	1528	169	18.5	4.32	AUGAAUACCUGGGGUAACUAUGGAC
P2	1512	3491	659	75.4	6.75	UUCGACCACGGAGCGAUCGAUGAUG
P4	3488	4483	331	35.1	6.34	AUUACAAAGGUAAUUACGUAUGAUC
P1	4497	6668	723	79.0	5.70	AACUGAAGGAUAGUUCAUCAUGGCA

[a] Molecular weight in kDa.
[b] Predicted isoelectric point of the protein.

virions. In addition N-terminal analysis of proteins transferred from the SDS-PAGE gels to PVDF filters and subjected to Edman degradation helped in gene assignments.

The viral nucleocapsid proteins were readily identifiable by SDS-PAGE of Triton X-100 treated ^{35}S-Met–labeled virions in that the detergent removed the envelope-associated proteins (Fig. 1B). We were able to visualize three of the four proteins that constitute the procapsid. P2, the viral polymerase, is not visible on this SDS-PAGE analysis and presumably migrates extremely close to protein P1. The polymerase protein was judged to be present in the virus in a lower amount than that of P1 and therefore both proteins appear as one band in the autoradiogram.

Gene 7 is the first procapsid *orf* in the L segment near the 5′ end known to encode a protein (Fig. 1A). The AUG initiating codon starts at position 1019 and is preceded by a ribosome-binding site (Woese *et al.,* 1984) (Table 1). N-terminal analysis of the first 5 amino acids of the protein that had been excised from a blot of the gel bands confirmed its identity. The molecular weight of P7 was calculated to be 18.5 kDa. Two genes that we call 14 and 15, respectively, precede gene 7 (Fig. 1A). In ϕ6, gene 14 is thought to play a role in the expression of gene 7, and it is also found to be present in the L segment of ϕ8. In ϕ12, both genes 14 and 15 have a calculated molecular weight of 11.5 and 11.1 kDa, respectively, and are preceded by a ribosome-binding site (Woese *et al.,* 1984) (Table 1), and it remains to be seen whether they are expressed and are part of the virion.

Following gene 7 is gene 2 (Fig. 1A). The aspartate sequence GDD found in conserved segment III of viral RNA-dependent RNA polymerases was present in protein P2 (Koonin *et al.,* 1989). This motif is SDD in ϕ6 and ϕ13 (Mindich *et al.,* 1988; Qiao *et al.,* 2000) but is also GDD in ϕ8 (Hoogstraten *et al.,* 2000). In segment L in ϕ6, ϕ8, and ϕ13, translation stop mutations in gene 7 are polar on gene 2. It has been seen in these other three cystoviruses that the AUG initiating codon for gene 2 overlaps (ϕ6), directly follows (ϕ8), or directly precedes (ϕ13) the stop codon for P7 (Mindich *et al.,* 1988; Hoogstraten *et al.,* 2000; Qiao *et al.,* 2000). In gene 2 of both ϕ6 and ϕ8, no ribosome-binding site was found to precede the gene (Mindich *et al.,* 1988; Hoogstraten *et al.,* 2000). A surprising finding was that the initiating codon for gene 2 of ϕ12 was found 15 bp prior to the stop codon for gene 7 (Table 1). The gene itself has a ribosome-binding site (Woese *et al.,* 1984) before it (Table 1), and it remains to be determined whether translation stop mutations in gene 7 are polar on gene 2 in this cystovirus.

Gene 4 of ϕ12 codes for protein P4, which is the NTPase found to be necessary for genomic packaging in ϕ6 (Gottlieb *et al.,* 1992). N-terminal analysis of the first 8 amino acids confirmed P4's identity. It was found to have a Walker motif A (Walker *et al.,* 1982) for NTP binding that had the amino acid sequence GKGNSGKT. In ϕ6, ϕ8, and ϕ13, this motif is GATGSGKS (Mindich *et al.,* 1988), GTAGGKT (Hoogstraten *et al.,* 2000) and GGTGAGKS (Qiao *et al.,* 2000), respectively. The molecular weight of the protein was calculated to be 35.1 kDa (Table 1), and a ribosome-binding site (Woese *et al.,* 1984) precedes the gene.

Gene 1 follows gene 4 (Fig. 1A), and its identity was also confirmed by N-terminal analysis of the first 5 amino acids of the protein. The initiating codon, AUG, begins at position 4497 and this *orf* ends at position 6668 (Table 1), with the stop codon 83 bp from the 3′ end of the RNA segment.

Structural similarity of the proteins to those of other cystoviruses

The sequence of the L segment was analyzed using both the BLAST X and BLAST P software provided at the National Center for Biotechnology Information's web site. We found that the ϕ12 polymerase P2 displayed significant identity to the comparable proteins of bacteriophages ϕ6, ϕ8, and ϕ13, at 21, 24, and 20%, respectively. Qiao *et al.* report that the ϕ13 P2 has 50% identity to the polymerase of ϕ6 and Hoogstraten *et al.* describe the ϕ8 P2 as having 20% identity to the ϕ6 P2 (Qiao *et al.,* 2000;

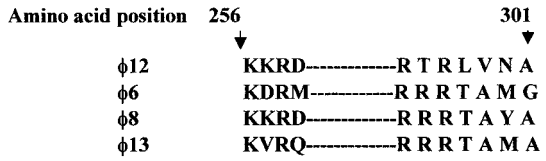

FIG. 2. Amino acids in the immediate vicinity of the key basic residues within the substrate pore of the viral polymerase P2. The amino acid position numbering is that of φ12.

Hoogstraten *et al.,* 2000). Recently, the 2-Å X-ray structure of the active polymerase subunit of φ6 was determined, and sequence motifs characteristic of RNA polymerases were noted (Butcher *et al.,* 2001). The key aspartic acid residues found at positions 324, 453, and 454 in the φ6 P2 are also found at positions 349, 469, and 470 of the φ12 polymerase. The second and third of these conserved amino acids are within the conserved segment III of viral RNA-dependent RNA polymerases (Koonin *et al.,* 1989). The structural analysis of the φ6 polymerase indicates that tyrosine 630 found within the polymerase-template complex might form a platform in which an initiation complex could be constructed (Butcher *et al.,* 2001), and this tyrosine is conserved in the φ12 P2 at position 621. φ13 and φ8 have a tryptophan and tyrosine at this site, respectively (Hoogstraten *et al.,* 2000; Qiao *et al.,* 2000). Crystal soaking experiments of the φ6 P2 with NTPs demonstrated a binding site in the substrate pore that orders triphosphate moieties by attachment to the key basic residues lysine 223 and arginines 225, 268, and 270 (Butcher *et al.,* 2001). The latter three arginine residues are conserved in the φ12 P2 at positions 258, 295, and 297. The φ12 lysine residue is located at position 256 (Fig. 2). The amino acid sequences of the polymerases from φ8 and φ13 show conservation of these basic residues (Hoogstraten *et al.,* 2000; Qiao *et al.,* 2000). The amino acids in the immediate vicinity of the basic residues of the substrate pore are also of interest in defining the relationships among the characterized cystoviruses. Although this region would be expected to be highly conserved, the φ12 sequence near arginines 295 and 297 is most distant in comparison to the analogous regions of the others, yet at the lysine 256, arginine 258 region it resembles that of φ8 (Fig. 2).

The φ12 NTPase, P4, displayed significant identity to the comparable proteins of φ6, φ8, and φ13 showing 23% identity to that of φ6 and 27% to both φ8 and φ13. No significant similarities of the φ12 P1 or P7 proteins with those of the comparable proteins from the other three characterized cystoviruses were noted. Protein P1 is the major structural protein of the procapsid and may be the major determinant of viral RNA packaging specificity. Protein P7 plays a stimulatory role in RNA packaging along with plus- and minus-strand synthesis (Mindich, 1999). Their divergence in φ12 could reflect the variation in the RNA genomic sequences. In φ8, amino acid similarity is reported to be absent in genes 1, 4, and 7 when compared to those proteins of φ6 (Hoogstraten *et al.,* 2000). However, the φ13 proteins P1, P4, and P7 showed about 50 and 30% identity with the corresponding φ6 proteins (Qiao *et al.,* 2000).

The observation that some of the components of the phage can display undetectable levels of similarity in proteins with analogous function warrants further investigation. In regard to the RNA polymerase and NTPase of the procapsid, the data suggest that φ12 is closer to φ8 and φ13 than to φ6. Previously, we have shown that the φ12 lysis cassette is related to that of φ6, while the attachment specificity proteins appear closer to those of φ13 (Gottlieb *et al.,* 2002). Therefore, in total, these results are interesting in that evolution of form and function in φ12 may have occurred by acquisition in the exchange of large pieces of genetic information from more than one other virus followed by genetic drift of that sequence. We see that the sequences of P1 and P7 are unrelated to any of the known cystoviruses and may be related to as yet undiscovered members of this virus family.

MATERIALS AND METHODS

Bacterial strains, phage, and plasmids

P. syringae pv. *phaseolicola* HB10Y (HB) is the host of φ6 and was utilized as a phenotypic screen in that φ12 is noninfectious on it. LM2333 is a mutant of HB which φ12 productively infects (Mindich *et al.,* 1999). *Escherichia coli* strain XL1-Blue Supercompetent Cells (*recA1, end1, gyrA96, thi-1, hsdR17, supE44, relA1,* [F′, *proAB, lac1*^{q}, *ZΔM15,* Tn*10* (Tet^{r})]) (Stratagene, La Jolla, CA) were used as hosts for cDNA cloning.

Plasmid pT7T319U (Pharmacia, Peapack, NJ) was used as the cloning vector for the cDNA copies of phage cDNA produced by reverse transcription. Plasmid pCR vector purchased from Invitrogen (Carlsbad, CA) or a pGEM-T vector from Promega Corp. (Madison, WI) were both utilized for the rapid cloning and sequence analysis of the RT-PCR produced cDNA derived from the viral genomic RNA. The pCR recombinants were constructed as directed in the product manual and isolated using Qiagen miniprep columns. The clones using the pGEM-T vector were constructed as described below.

Media, enzymes, and chemicals

The media used were LB and M9. Ampicillin plates contained 200 μg/ml in LB agar supplemented with 40 μg/ml of isopropyl-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indoly-β-D-galactoside(Xgal) (Maniatis, 1982). Restriction enzymes were purchased from Promega. Poly(A) polymerase was purchased from Perkin-Elmer (Boston, MA). The reagents used for cDNA synthesis of the phage RNA and its cloning to a plasmid vector were all supplied in The Universal Riboclone cDNA Synthesis

System also purchased from Promega. Ready to Go RT-PCR Beads were utilized for the RT-PCR amplification of select portions of the RNA segment (Amersham Pharmacia Biotech, Piscataway, NJ). Buffer ACN was used for the suspension of purified bacteriophage and contains 10 mM KPO_4 (pH 7.5), 1 mM $MgSO_4$, 200 mM NaCl, and 0.5 mM $CaCl_2$.

Preparation of pure virions of ϕ12

Twenty to 30 plate lysates of ϕ12 were prepared by plating phage dilutions into soft agar with an overnight grown culture of LM2333. These plates were incubated overnight at room temperature. The next day, phage-containing top agar was collected and the cell debris and agar were removed by centrifugation in a Sorvall SS-34 rotor at 15,000 rpm, 15 min at 4°C. Phage was collected by centrifugation in a Beckman TI-75 rotor at 33,000 rpm, 2 h at 4°C. The pellet was suspended in 1 ml of buffer ACN. Purification of the phage was by equilibrium centrifugation in CsCl, average density 1.28 g/ml, using a Beckman SW 50.1 rotor at 33,000 rpm, overnight at 4°C. The next day the phage band was located by light scattering, collected by tube puncture, and dialyzed overnight in buffer ACN. The dialyzed sample was centrifuged in a Beckman TI-75 rotor at 33,000 rpm, 2 h at 4°C, and the collected phage suspended in 300 μl buffer ACN.

In order to isolate nucleocapsids (NC) free of the lipid envelope, the purified phage were treated with 2% Triton X-100 and the preparation was centrifuged at 33,000 rpm in a Beckman TI-75 rotor, 90 min at 4°C. The pellet was suspended in buffer ACN and stored at −80°C.

Isolation and *in vitro* transcription of the ϕ12 dsRNA

dsRNA was isolated from viral particles by phenol:chloroform (1:1) extractions. The RNA was precipitated with 10% 7.5 M NH_4Ac and 2.5 vol of ethanol. The RNA was pelleted and resuspended in 50 μl of sterile water.

Nucleocapsids isolated from virions by Triton X-100 treatment were utilized in *in vitro* transcription, in the presence of manganese ions, to synthesize complete transcripts of the three viral RNA segments (Emori *et al.,* 1983). The synthesized transcripts were extracted with phenol:chloroform, ethanol-precipitated, pelleted, and resuspended in H_2O. dsRNA from both the whole phage and synthesized transcripts served as templates for the cDNA synthesis.

Preparation of cDNA

Poly(A) tailing. Either dsRNA or viral transcripts were denatured by boiling for 5 min and rapidly cooled in a dry ice/ethanol bath. A 5× poly(A) polymerase buffer was added to the RNA along with ATP and yeast poly(A) polymerase. The mixture was incubated for 1 min at 30°C, transferred to ice, and brought to a volume of 50 μl with TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). The poly(A)-tailed RNA was then extracted with phenol/chloroform, ethanol-precipitated, pelleted, and resuspended in sterile water.

First-strand synthesis. Phosphorylated oligo(dT) (1–2 μg) was added to 10 μl of poly(A) tailed RNA. The sample was left at 70°C for 5 min, after which it was cooled on ice for 5 min. Four microliters of 5× first-strand buffer, 3 μl H_2O, 40 u RNAse inhibitor (RNasin), and 30 u of AMV reverse transcriptase were added and incubated at 42°C for 1 h.

Second-strand synthesis. To the reverse-transcribed RNA sample, 40 μl 2.5× second-strand buffer, 37.6 μl H_2O, 0.8 u RNaseH, and 23 u *E. coli* DNA polymerase I were added and the second-strand synthesis proceeded for 4 h at 14°C. The polymerase I was inactivated at 70°C for 10 min. T4 DNA polymerase was added for 10 min at 37°C to blunt the ends of the cDNA. The reaction was stopped by bringing it to 20 mM EDTA. The sample was then treated with phenol:chloroform, ethanol-precipitated, pelleted, and suspended in 2.5 μl dH_2O.

Preparation of the vector utilized for cloning. PT7T3 19U was digested with *Sma*I and dephosphorylated with shrimp alkaline phosphatase. The vector was then treated with phenol:chloroform, ethanol-precipitated, and suspended in 10 μl of H_2O. The ligation mixture contained 5 μl of the cDNA sample, 0.5 μl vector, 1 μl 10× ligation buffer (containing ATP), and 2.5 u of T4 DNA ligase. Incubation was overnight at 14°C. The ligation mixture was used to transform supercompetent XL-1 Blue *E. coli.* Transformed cells were spread on LB plates containing 40 μg/ml of X-gal, 40 μg/ml IPTG, and 200 μg/ml of ampicillin. White colonies were picked by toothpick and small plasmid preparations prepared using QIAprep Spin Miniprep columns (Qiagen, Stanford, CA). The plasmids were cut with restriction endonuclease *Pvu*II and plasmids containing inserts were sequences first with both the T3 and M13r primers. Subsequent sequencing of the cloned cDNA inserts was with oligonucleotide primers (Integrated DNA Technologies, Coralville, IA) designed from the sequences derived using the initial primer set.

RT-PCR synthesis of cDNA. cDNAs of all three dsRNA segments were amplified using a modification of the Lambden method (Lambden *et al.,* 1992). The three amplified cDNAs were separated on a 1% agarose gel and purified using the gel extraction kit of Qiagen. The purified PCR products were then cloned into the pGEM-T vector and positive clones were identified based on insert size and restriction enzyme patterns.

Preparation of radioactively labeled bacteriophage

LM2333 was infected with ϕ12 at a multiplicity of infection of 20 in M9 medium supplemented with amino acids, metal ions, and glucose. ^{35}S-Met (10 μCi/ml) was

added and the culture was allowed to proceed to lysis. The phage was purified as described above.

cDNA sequence analysis

cDNA sequencing was performed at both The Protein/DNA Technology Center of Rockefeller University, New York City, and the RCMI Facility at The City College of New York. The sequences were assembled using the AlignIR Assembly and Alignment Software, Li-Cor Biotechnology Division, Lincoln, Nebraska. The sequence of the L segment was submitted to GenBank and the accession number is AF408636.

Amino acid sequence analysis of the ϕ12 proteins

The amino acid sequences encoded on the L segment were analyzed for similarity to other protein amino acid sequences using the Basic Local Alignment Search Tool (BLAST X) provided by the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health (Bethesda, MD) (Altschul *et al.,* 1990).

Protein sequence

Proteins were sequenced from the N-terminus using automated Edman degradation on a PE Biosystems 494 protein sequencer at the Protein Chemistry Core Facility, Howard Hughes Medical Institute of Columbia University (New York, NY). PAGE-separated viral proteins were transferred to PVDF membranes by Western blotting and the proteins visualized with Ponceau stain. These were excised from the membrane and sent to the sequencing facility for the analysis.

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