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Characterization of Φ13, a Bacteriophage Related to Φ6 and Containing Three dsRNA Genomic Segments

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Received June 12, 2000; accepted July 2, 2000

The three dsRNA genomic segments of bacteriophage Φ13 were copied as cDNA and the nucleotide sequences were determined. The organization of the genome is similar to that of Φ6, and there is significant similarity in the amino acid sequences of the proteins of the polymerase complex and one of the membrane proteins, P6. There is little or no similarity in the nucleotide sequences. Several features of the viral proteins differ markedly from those of Φ6. Although both phages are covered by a lipid-containing membrane, the protein compositions are different. The host attachment protein consists of two peptides rather than one and the phage attaches directly to the LPS of the host rather than to a Type IV pilus. Despite the differences in the structure of the membranes, the two viruses can successfully exchange the genes for host attachment proteins and thereby change their host specificities. © 2000 Academic Press

Key Words: bacteriophage; dsRNA; *Pseudomonas.*

Bacteriophage Φ13 was isolated from the leaves of the radish plant (*Raphanus sativum*) (Mindich *et al.,* 1999). It is similar in structure to bacteriophage Φ6 which contains a genome of three segments of double-stranded RNA (Semancik *et al.,* 1973) packaged inside a procapsid which is covered by a shell of protein P8 and a lipid-containing membrane containing additional viral proteins (Vidaver *et al.,* 1973; Mindich, 1988). The genome of Φ6 has been cloned and sequenced and the life cycle and structure of the phage have been the subjects of considerable investigation (Butcher *et al.,* 1997; de Haas *et al.,* 1999; Mindich, 1999). Φ6 infects pseudomonads by attaching to a Type IV pilus. The viral membrane fuses with the outer membrane of the host. The nucleocapsid is then found in the periplasmic space. A viral muramidase digests the cell wall and the nucleocapsid enters the cell wherein it transcribes its genome. The viral procapsid composed of proteins P1, P2, P4, and P7 packages plus-strand transcripts in a process that results in one copy of each of the three dsRNA genomic segments, S, M, and L, in each virion. The filled procapsid acquires a shell of protein P8 and then a lipid-containing membrane that is assembled within the cell. The procapsid of Φ6 has the ability to package RNA, synthesize minus strands to make dsRNA, and then to transcribe the genome. All the reactions can be carried out *in vitro* under defined conditions. Until recently, Φ6 has been alone in the family Cystoviridae and alone in the genus *Cystovirus* (Murphy, 1995). It is now clear that this group is composed of many more phages, some like Φ7 very similar to Φ6; some, like Φ8, rather distantly related (Mindich *et al.,* 1999); and some like Φ13, somewhat intermediate. These are the only isolated bacteriophages with genomes composed of dsRNA. Their structure and replicative strategies show many similarities to the Reoviridae, dsRNA viruses that infect eukaryotic cells.

We present, in this paper, the cDNA cloning, sequencing, and manipulation of the genome of Φ13 and point out the similarities that exist between this virus and Φ6 as well as the marked differences in both structure and sequence.

RESULTS AND DISCUSSION

RNA sequence

The cDNA copies of the Φ13 genomic segments were sequenced and arranged into three groups corresponding to the three dsRNA genomic segments. The arrangement of the genes and the unique restriction sites of the cDNA copies are shown in Fig. 1. The sizes of the three segments were found to be 6458, 4213, and 2981 bp for segments L, M, and S, respectively. This compares with the corresponding sizes of 6374, 4063, and 2948 in Φ6 (Mindich, 1988). The base composition of the segments is 58.4, 57.9, and 56.2% GC, respectively, for L, M, and S as compared to about 56% GC in Φ6 (Mindich, 1988). Whereas the genomic segments of Φ6 have an 18-base identity at the 5′ end, the Φ13 segments have an identity of only 11 bases and they are different from those of Φ6; G(G/U)AAAAAAACUUUAUAUA

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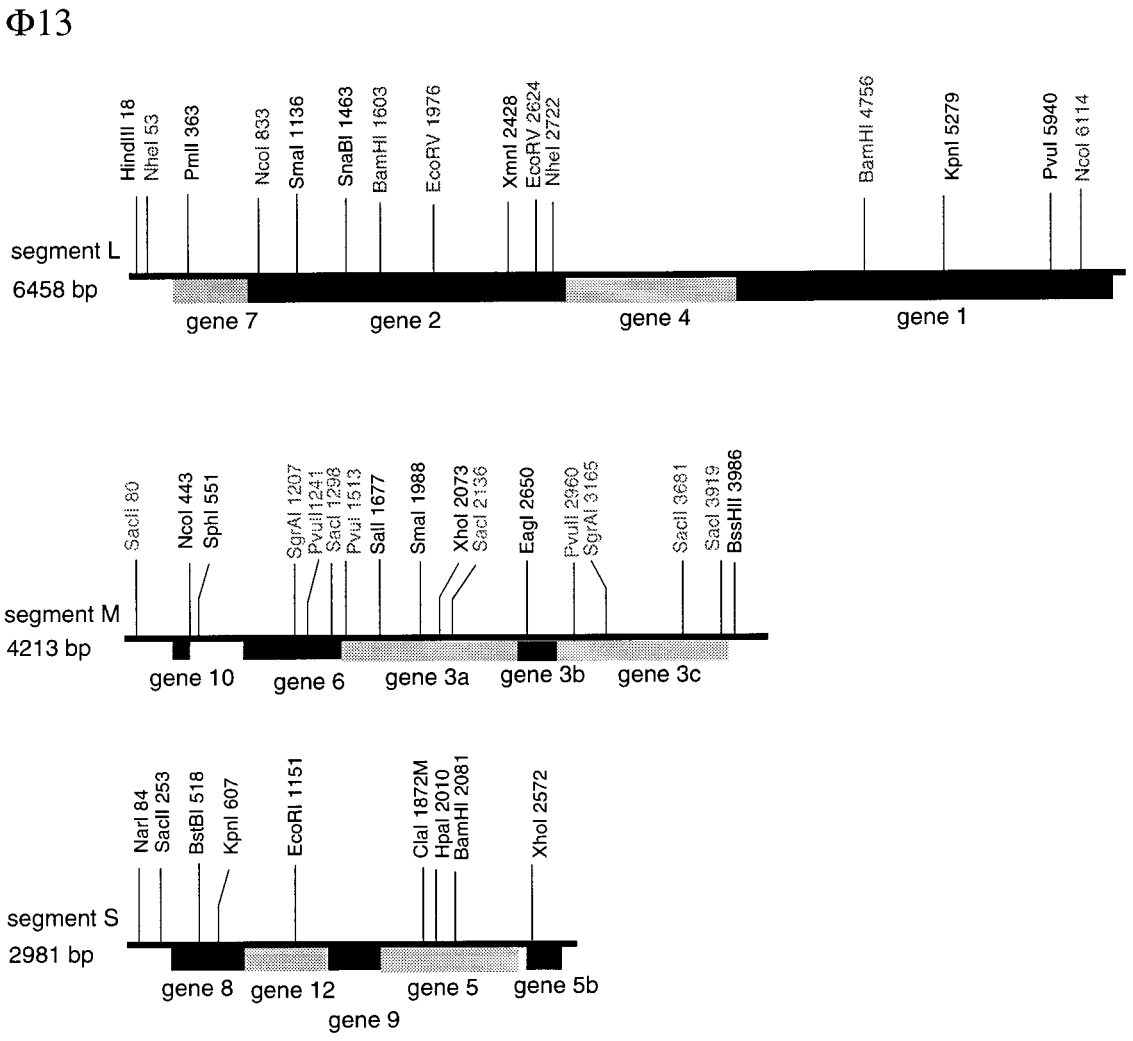

FIG. 1. Restriction map of the cDNA copies of the three genomic segments of Φ13. The genes are numbered so as to correspond to those of Φ6.

for Φ6 vs G(GA/AU)AAAAACUU for Φ13. The sequences at the 3′ ends of the segments show somewhat similar predicted secondary structures, but they show less identity to each other than that found in Φ6 (Fig. 2). A startling finding is that the 3′ structure of segment M is virtually identical to that of Φ6. It is so similar that it seems likely that it is the result of an interaction with Φ6 or a very close relative. The structures of the 3′ regions of segments M in Φ13 and Φ6 are shown in Fig. 2. The boxed region is the conserved identity in Φ6. It can be seen that the sequence similarity between the two phages is greater than that between the Φ6 segments. It is also striking that the terminal sequences of the Φ13 segments are all different. In Φ6 and the other related phages, the terminal sequences are identical. The 3′ region is not involved in packaging specificity, but is important for polymerase recognition. We had previously found that sequences such as CCC could suffice for Φ6 polymerase recognition in contrast to the normal CUCU-CUCU. Apparently, Φ13 can accommodate the three different terminal sequences.

The sequences of the first 300 nucleotides in each plus-strand transcript of the genome are necessary and sufficient for packaging in Φ6 (Gottlieb *et al.,* 1994). This region folds into a complex of stem-loop structures called the *pac* region (Mindich, 1999). The same regions in Φ13 can also be folded into a similar complex, although with completely different sequence and structure. In Φ6, the *pac* sequences terminate about 50 nucleotides before the first *orf.* The spacing in Φ13 is consistent with this.

Identification of genes

The genes of segment L are arranged in a similar manner to those of Φ6 (Fig. 1, Table 1). The assignments could be made on the basis of chromosomal position, size, and amino acid identity or similarity with Φ6. Amino acid similarity was present in genes 1, 2, 4, and 7 on the basis of a blastP 2.09 comparison (Tatusova and Madden, 1999). Gene 2, which codes for the polymerase, showed 65% similarity to gene 2 of Φ6 and this involved

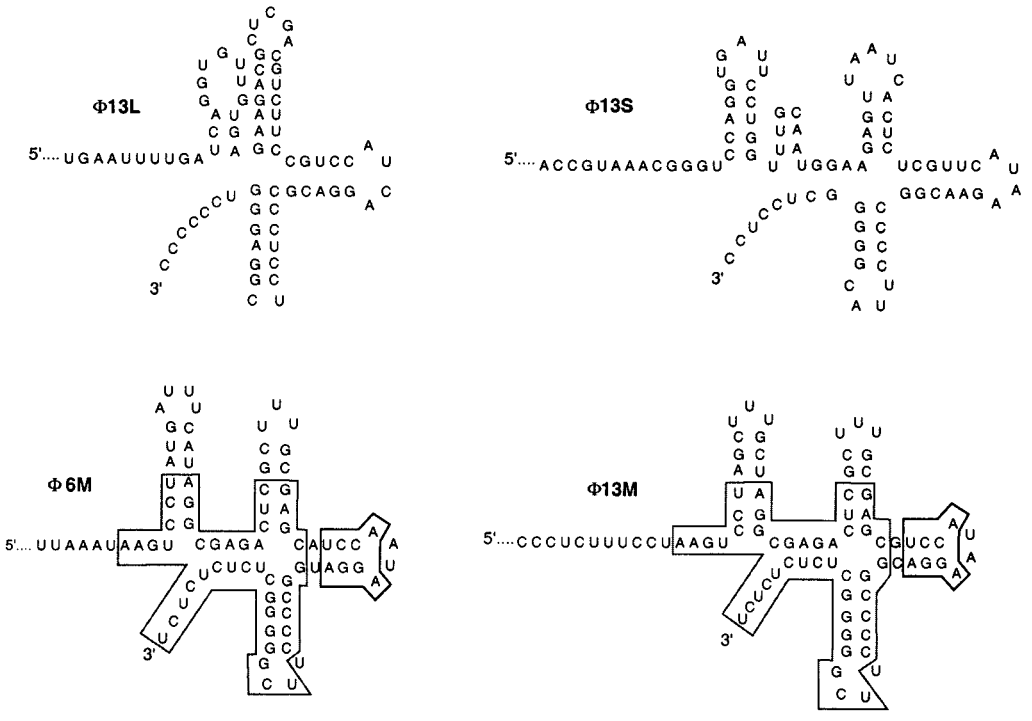

FIG. 2. Secondary structure predicted for the 3′ ends of the three plus strands of the Φ13 genome. The boxed sequence in segment M is the consensus sequence in the 3′ region of the three genomic segments of Φ6. The structure of the 3′ region of the Φ6 segment M is also shown. Note that the identity of the two M sequences is even greater than those of the boxed sequences.

50% identity in amino acids. The aspartate sequence GDD in motif VI (Koonin *et al.,* 1989) was present in the Φ13 gene as SDD, similar to the case for Φ6. Proteins P1, P4, and P7 showed about 50% similarity and 30% identity with corresponding Φ6 proteins. Gene 4 of Φ13 had a Walker motif A (Walker *et al.,* 1982) for ATP binding which was GGTGAGKS as contrasted to GATGSGKS in Φ6. Protein P4 is the NTPase necessary for genomic packaging in Φ6 (Gottlieb *et al.,* 1992). Plasmids expressing genes 1, 2, 4, and 7 of Φ13 in *Escherichia coli* formed procapsids of a lower quality to those found with Φ6 procapsids (unpublished results).

Genes in segments S and M were named for genes in the same position in Φ6; however, the products of genes

TABLE 1

List of orfs for Φ13

orf	Segment	Start	End	aa[a]	MW[b]	IEP[c]
P1	L	3947	6352	801	86.6	6.98
P2	L	719	2860	713	79.7	6.09
P3a	M	1488	2573	361	40.1	4.82
P3b	M	2580	2858	92	9.9	4.02
P3c	M	2845	3945	366	38.7	7.62
P4	L	2857	3933	358	37.7	6.70
P5	S	1542	2279	245	27.1	7.44
P5b	S	2497	2799	100	10.5	11.31
P6	M	785	1477	230	24.1	10.88
P7	L	252	722	156	16.6	4.63
P8	S	335	790	151	16.3	5.32
P9	S	1293	1505	70	7.6	10.59
P10	M	326	442	38	4.0	10.36
P12	S	794	1291	165	17.3	4.74

[a] The number of amino acids in the orf.
[b] Molecular weight in kDa.
[c] The isoelectric point of the protein product.

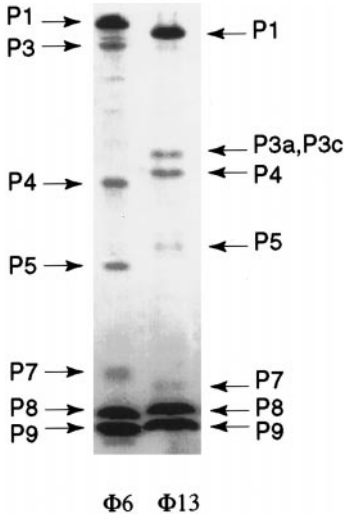

FIG. 3. Coomassie-stained gel of PAGE analysis of whole virus of Φ13 and Φ6. The bands for proteins P6 do not appear.

3, 5, 9, and 10 showed no similarity to the corresponding proteins in Φ6. Proteins P6, P8, and P12 did show 28, 22, and 22% identity to the corresponding Φ6 proteins using the BlastP 2.0.9 program (Tatusova and Madden, 1999). The gene assignments could also be integrated with the results of gel analysis of protein samples of purified virions (Fig. 3), detergent extracted virions, and radioactively labeled infected cells. All the gene products of Φ13 segments S and M were found in the membrane of the virion with the exception of proteins P12 and 3b.

Polar relationships

Most genes in the Φ13 genome have recognizable Shine-Delgarno ribosome-binding sequences (Table 2). However, as in the case of Φ6, a number of genes do not have these motifs and are dependent upon upstream genes for their ribosome loading and are consequently subject to polarity. In segment L, gene 2 translation depends upon that of gene 7. In Φ6, gene 7 is preceded by gene 14. Gene 14 is not indispensable, but might play a role in the expression of gene 7. In Φ13, gene 14 is missing. Gene 7 is polar on gene 2. As in the case of Φ6, both genes 4 and 1 have their own ribosome-binding sites. Gene 3a in segment M has no discernible ribosome-binding site; yet it is expressed well. Genes 3b and 3c have binding sites, but it is not clear that 3b is expressed. Gene 8 is polar on gene 12 as in the case of Φ6; and gene 9 is polar on gene 5. Gene 5b has a good ribosome-binding site, but its expression is not clear (Table 2).

Exchanges between Φ13 and Φ6

We had previously shown that Φ6 could acquire plasmid transcripts that include proper 5′ and 3′ sequences embedded in their structure (Onodera *et al.,* 1995). These transcripts are trimmed *in vivo* to form exact copies of the genomic segments and replace them in the phage genome. We now find that Φ13 can acquire plasmid transcripts of Φ6 segment M, even though the *pac* sequence of Φ13M is completely different from that of Φ6. Φ6 cannot acquire segment M of Φ13 except in the case where the *pac* sequence of Φ6 precedes the coding regions for the genes of Φ13M. The genomic packaging for Φ13 is apparently less stringent than that for Φ6. Φ13 propagates on strains that have rough LPS, with or without Type IV pili; Φ6 propagates on strains that have a specific Type IV pilus. Therefore it is possible to carry out highly selective screening for the acquisition of either Φ6 or Φ13 attachment proteins. The membranes of these phages are formed primarily by protein P9 and phospholipid under the guidance of morphogenetic protein P12 (Johnson and Mindich, 1994). The exchanges between Φ6 and Φ13 show that membrane proteins P6 and P10 can recognize viral membranes despite the lack of sequence similarities in the respective P9 and P12 proteins. Once we had a strain of Φ13 with the host specificity of Φ6, it was possible to screen for the acquisition of Φ13 genes again, but with reporter genes such as *kan* or *lac* inserted into the noncoding region near the 3′ end of segment M. These phages were useful in demonstrating the ability of Φ13 to establish carrier state infections in pseudomonads, *E. coli* and *S. typhimurium,* even though the phage could not form plaques on strains of the latter two species (Mindich *et al.,* 1999). These experiments also demonstrated that the cDNA clones of segment M were accurate, since the genes were expressed successfully.

It was also possible to use the powerful selection to acquire plasmid transcripts that contained chimeric constructions of segment S of Φ13 along with segment M of Φ6 (see Materials and Methods) (Fig. 4). This demon-

TABLE 2

Ribosome-Binding Sites for Φ13 Genes

UAAGGAGGUGAUC	Revised composition of 16S RNA
CUCUAAUUAGGAGCUGUUUAUGUUG	P1
CAAUCACAGGCGCUGAAGUAUGACU	P2
ACGGCGUAACUUUCAAACAAUGAUC	P3a
GCUAAUGCUCUGAGGUCGUAUGGAC	P3b
AUUGCACGAGGAGACGCCGAUGGAC	P3c
CCGUUACGAGGGAUGUCAGAUGACU	P4
UUCCGUUCUUCCGCCAGUAAUGGCG	P5
GCUCGUCUGGAGCAGCAAGAUGCGA	P5b
CCAGCUCUCAGGAGGCUUAAUGGGC	P6
UUUACGAUUGGAGAUUCACAUGCUU	P7
ACACACGUAAGGAAUACGUAUGGCC	P8
ACGUAUAAGGAUAUAUGACAUGGGU	P9
UCAGUCGUAAGGAUACGAUAUGAAC	P10
CAAACGGACGGUCUAAGACAUGCUC	P12

Note. The nucleotide sequences around the initiating codons for each of the Φ13 proteins. The initiating codons AUG and the presumed binding sequences are underlined.

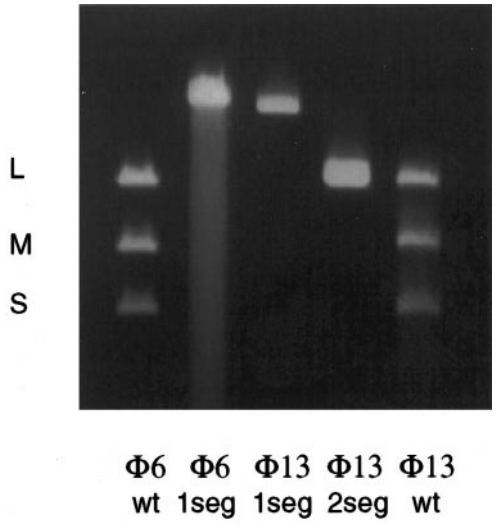

FIG. 4. Agarose gel electrophoresis of dsRNA extracted from virions of Φ13 and Φ6. RNA was extracted from wild-type virus as well as from virus containing single genomic segments (1seg) of Φ6 (Onodera *et al.,* 1998) and Φ13 . A sample of Φ13 containing a normal L segment and a chimeric segment of the genes of S and M (2seg) is also shown; the two genomic segments run as a doublet.

strated that segment S was accurate. It was then possible to produce a plasmid that produced a transcript that contained the genes of Φ13 S and L segments along with those of Φ6 M. This plasmid gave rise to plaques without the need for helper phage. The new phage had a single genomic segment (Fig. 4). The cDNA copy of segment L was therefore also accurate.

The finding that Φ13 can function well with the entire genome arrayed in a single RNA molecule instead of the normal three segments suggests that the tripartite genomic structure is not necessary. An argument has been put forward that the normal arrangement is useful in offering the possibility of easy genetic exchange (Chao *et al.,* 1997). However, our preference on the basis of the work presented here and previously with Φ6 (Onodera *et al.,* 1998) is that the disadvantages of the single genomic segment are primarily the difficulty in packaging the resulting 13.5 kb-plus strand and a loss in the control of gene expression. Although the amount of each segment as dsRNA is equal, amounts of message are very unequal (Coplin *et al.,* 1975).

MATERIALS AND METHODS

Bacterial strains, phage, and plasmids

LM2489 is a rough derivative of *P. syringae* pv. *phaseolicola* HB10Y (HB) (Vidaver *et al.,* 1973) and was used as the primary host for plating Φ13 and Φ6. Φ6 plates on HB but Φ13 does not (Mindich *et al.,* 1999). LM2509 is a derivative of LM2489 that lacks pili and is resistant to Φ6, but sensitive to Φ13. Strain ERA is an isolate of *P. pseudoalcaligenes.* S4 is a derivative of ERA that contains a nonsense suppressor mutation (Mindich *et al.,* 1976).

Plasmid pLM1454 is a derivative of the cloning vector pT7T3 19U (Pharmacia). It was used for the cloning of cDNA copies of phage DNA produced by RT-PCR.

Media, enzymes and chemicals

The media used were LC and M8 (Sinclair *et al.,* 1976). Ampicillin plates contained 200 μg of ampicillin/ml in LC agar. All restriction enzymes, T4 DNA ligase, T4 DNA polymerase, and T4 polynucleotide kinase, were purchased from Promega, New England Biolabs, and Boehringer Gmbh, Mannheim.

Preparation of pure virions of Φ13

Three hundred milliliters of fresh lysate was used to infect 1 to 3 L of fresh LM2489 culture with a density of 2×10^8 cells/ml, at a multiplicity of 10. After lysis the culture was spun at 7000 rpm for 10 min at 4°C. NaCl (0.5 M) and 10% PEG-6000 were added to the supernate to precipitate the phage. The suspension was centrifuged, and the pellet was resuspended in 20–30 ml of buffer A overnight at 4°C. Buffer A is composed of 10 mM $KHPO_4$ and 1 mM $MgCl_2$, pH 7.5.

The resuspended Φ13 was treated with DNase for 10 min at room temperature to make the suspension less viscous. It was then spun at 23,000 rpm for 45 min in a zone gradient of 10–30% sucrose in buffer A. The phage band was isolated and treated with PEG to precipitate the virions. The pellet was resuspended in buffer A and applied to a gradient of 40–60% sucrose in buffer A and spun at 23,000 rpm overnight at 4°C in the SW41 rotor. The phage band was isolated and concentrated.

Isolation of the Φ13 dsRNA

RNA was isolated from the virus by phenol:chloroform (1:1) extractions. The RNA was then precipitated with 10% 7.5 M NH_4Ac and 2.5 vol of ethanol and resuspended in 50 μl TE buffer.

Preparation of cDNA: Poly(A) tailing

The RNA was denatured by boiling for 5 min and rapidly cooling with dry ice/ethanol. A 5× poly(A) polymerase buffer was added to the RNA along with ATP and yeast poly(A) polymerase (Amersham). The mixture was incubated at 30°C for 1 min and transferred to ice and the reaction stopped with EDTA. The poly(A) RNA was then extracted with phenol/chloroform and precipitated and resuspended in water.

First-strand synthesis

Of phosphorylated oligo(dT) 1 μl was added to 10 μl of poly(A) RNA. After 5 min at 70°C the sample was cooled on ice for 5 min. Then 4 μl of 5× first-strand buffer, 3 μl

H_2O, 40 u RNase inhibitor (RNasin), and 30 u AMV reverse transcriptase were added and incubated at 42°C for 1 h. All products needed for the first- and second-strand synthesis were provided by the Promega cDNA kit (Universal Riboclone cDNA Synthesis System). The reaction products were stored at −70°C overnight.

Second-strand synthesis

After thawing the reverse-transcribed RNA, 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. After the second-strand synthesis proceeded for 3 h at 16°C, the *E. coli* DNA polymerase I was inactivated at 70°C for 10 min. Then T4 DNA polymerase was added for 10 min at 37°C to blunt the ends of the cDNA. The sample was then treated with phenol/chloroform, ethanol precipitated, and resuspended in 2.5 μl dH_2O.

Preparation of the vector used for cloning

pLM1454 was cut with *Hin*cII, dephosphorylated with shrimp alkaline phosphatase, purified by electrophoresis, electroeluted, precipitated, and resuspended in 20 μl TE buffer. The ligation mixture was composed of 2.5 μl Φ13 cDNA, 0.5 μl vector, 0.5 μl 10× ligation buffer, 0.5 μl 10 mM ATP, and 2.5 u T4 DNA ligase. All products are provided by the Promega cDNA kit. Incubation was overnight at 16°C. The ligation mixture was used to transform supercompetent Epicuran *E. coli* (Stratagene). The cells were plated out on LC plates with 40 μg/ml X-gal and 200 μg/ml ampicillin. White colonies were picked and small DNA preparations were made. The plasmids were cut with restriction enzyme *Pvu*II and promising candidates were sequenced first with M13 primers and then with oligonucleotides prepared on the basis of the sequence found. At the point where it seemed that the ends of the segments were identified, we prepared cDNA copies by using RT-PCR with oligonucleotides having sequences found in the obtained copies. Sequencing was done at the New York University Medical Center Sequencing Facility. The sequences were assemble using the GCG program GelStart. The sequence of the Φ13 genome was submitted to GenBank and the accession numbers for segments L, M, and S are AF261668, AF261667, and AF261666, respectively.

Preparation of complete cDNA plasmids

The cDNA pieces were assembled to form complete copies of the three genomic segments. In many cases, the connections could be made by using unique restriction sites made evident by the sequencing project (Fig. 1). The ends of segments were prepared by using oligonucleotides with convenient restriction sites as primers for PCR. Three plasmids were prepared, pLM2200, pLM2196, and pLM2202. They contain exact complete copies of genomic segments L, M, and S, respectively, in plasmid pT7T3 19U. The sequences start at the first nucleotide of the T7 RNA polymerase transcript. If these plasmids are cut and ligated to plasmid pKT230, they can be propagated and express in *Pseudomonas* strains.

Preparation of novel genomic constructions

Φ13 was propagated on a lawn of LM2489 carrying plasmid pLM1084 which contains a complete cDNA of segment M of Φ6. When this preparation was plated on strain HB, which does not support Φ13, plaques were obtained that had acquired the Φ6 segment M (Mindich *et al.,* 1999), but retained segments S and L of Φ13. This phage was named Φ2544.

A cassette for the α portion of β-galactosidase was inserted into the *Bss*H1 site in plasmid pLM2196 (Φ13 genomic segment M). Φ2544 was able to acquire this transcript by growth on a lawn of LM2489 carrying the plasmid. This resulted in phage, Φ2551, which was Lac^+ and carried the host attachment proteins of Φ13.

Plasmid pLM2196 has several *Pst*I sites. A 3′ terminal segment was removed that contained *Pst*I at N4113 and XbaI at the terminus of the cDNA insert. This piece was exchanged with a *Pst*I/*Xba*I fragment of plasmid pLM778 or pLM779, which contains a cDNA copy of Φ6M with *kan* inserted into the *Pst*I site, with a small deletion on the 3′ side (Onodera *et al.,* 1993). The new plasmids, pLM2336 or pLM2337, have a cDNA copy of Φ13 segment M, but with a *kan* insert at the *Pst*I site and the 3′ end of Φ6M. The transcripts of these plasmids could be acquired by Φ2544 to produce phage carrying the *kan* resistance gene in segment M. These phages were capable of setting up kanamycin-resistant carrier state infections in *Pseudomonas* LM2509, and also in *E. coli* JM109 and *S. typhimurium* 3789 (Mindich *et al.,* 1999).

Plasmids pLM2266 and pLM2267 were constructed by ligating the Φ13 genes of pLM2196 starting at the *Sac*II site at position 80 with the *Mlu*I site of the Φ6M cDNA plasmid pLM656 which is at position 452 (after the *pac* sequence). The resulting plasmids have the *pac* sequence of Φ6 and the genes of segment M of Φ13. Φ6 is able to acquire this transcript and express the host attachment proteins of Φ13 in its membrane. The resulting phage is Φ2554.

Plasmid pLM2202 contains the cDNA copy of Φ13 segment S. It was cut with *Xho*I and *Xba*I and the cDNA copy of Φ6 M from plasmid pLM656 was cut with *Hin*dIII and *Xba*I and ligated 3′ to the Φ13 sequence. This resulted in a plasmid, pLM2330, that produced a transcript with the *pac* sequence of Φ13 and the genes of Φ13S and Φ6M. A ligate of this plasmid with pKT230 could be propagated in LM2489 and enabled the acquisition of the transcript by Φ13. This phage, Φ2555 contains only two genomic segments, normal L and the chimeric SM.

The cDNA portion of plasmid pLM2200, from the

*Hin*dIII site to the 3′ end, which contains the genes of Φ13L, was ligated to a *Pst*I site near the 3′ end of pLM2330 to produce a plasmid, pLM2350, whose transcript contained the genes of Φ13S and L and those of Φ6M.

In vitro protein synthesis

Plasmids were cut with various restriction enzymes and then transcribed with T7 RNA polymerase. The transcripts were then added to lysates of *E. coli* prepared for *in vitro* protein synthesis in the presence of [^{35}S]methionine (Promega). The samples were precipitated with acetone and resuspended in sample cracking buffer and analyzed on 18% acrylamide gels. The gels were soaked in EnHance (New England Nuclear), dried, and incubated with film to produce autoradiograms.

Preparation of radioactively labeled phage

LM2489 was infected with Φ13 at a multiplicity of infection of 20 in M8 medium supplemented with amino acids, metal ions, and glucose. Of [^{35}S]methionine 10 μCi/ml was added and the culture was allowed to proceed to lysis. The phage was purified as described above except that only the zone sedimentation was performed.

ACKNOWLEDGMENT

This work was supported by Grant GM31709 from the National Institutes of Health.

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