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Unique properties of the inner core of bacteriophage ϕ8, a virus with a segmented dsRNA genome

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Abstract

The inner core of bacteriophage ϕ8 is capable of packaging and replicating the plus strands of the RNA genomic segments of the virus in vitro. The particles composed of proteins P1, P2, P4, and P7 can be assembled in cells of *E. coli* that carry plasmids with cDNA copies of genomic segment L. The gene arrangement on segment L was found to differ from that of other cystoviruses in that the gene for the ortholog of protein P7 is located at the 3′ end of the plus strand rather than near the 5′ end. In place of the normal location of gene 7 is gene H, whose product is necessary for normal phage development, but not necessary for in vitro genomic packaging and replication. Genomic packaging is dependent upon the activity of an NTPase motor protein, P4. P4 was purified from cell extracts and was found to form hexamers with little NTPase activity until associated with inner core particles. Labeling studies of in vitro packaging of ϕ8 RNA do not show serial dependence; however, studies involving in vitro packaging for the formation of live virus indicate that packaging is stringent. Studies with the acquisition of chimeric segments in live virus indicate that ϕ8 does package RNA in the order *s/m/l*. The inner core of bacteriophage ϕ8 differs from that of its relatives in the Cystoviridae in that the major structural protein P1 is able to interact with the host cell membrane to effect penetration of the inner core into the cell.

Keywords: Bacteriophage; dsRNA; Pseudomonas; phi8; Genomic packaging

Introduction

Bacteriophage ϕ8 belongs to the family Cystoviridae, bacteriophages with genomes of three dsRNA molecules inside a polyhedral inner core enveloped by a lipid-containing membrane (Mindich et al., 1999). The first isolated member of this family, ϕ6, has been studied in detail (Butcher et al., 2001; Mindich, 1999; Vidaver et al., 1973). Until this report, ϕ6 was the only segmented dsRNA virus for which in vitro packaging worked. In vitro packaging also can be done with ϕ8, but it appears to be less stringent than ϕ6. ϕ6 attaches to a pilus produced by its host *Pseudomonas syringae* and subsequently fuses its membrane with the outer membrane of the host. The nucleocapsid, which consists of the inner core with proteins P1, P2, P4, and P7 covered by a shell of P8, enters the cell. P8 is lost and the inner core begins transcription of the genome to produce plus-strand transcripts, which serve as messengers and replication templates. Segment L codes for the inner core proteins P1, P2, P4, and P7 and these assemble to form a dodecahedral structure which is then capable of recognizing and packaging the plus strands in the order *s:m:l* (Mindich, 1999). Minus-strand synthesis begins when all three genomic segments are packaged. Late in infection, protein P8 covers the inner core particle and finally the particle is enveloped in a lipid-containing membrane inside the infected cell. Lysis follows, releasing about 100 mature virions.

ϕ8 differs from ϕ6 in several important ways, which will be detailed in this report. First of all, there is virtually no sequence similarity between the amino acid sequences of the ϕ8 proteins and those of the other members of the Cystoviridae. There are also major structural differences between ϕ8 and the other family members in that ϕ8 protein

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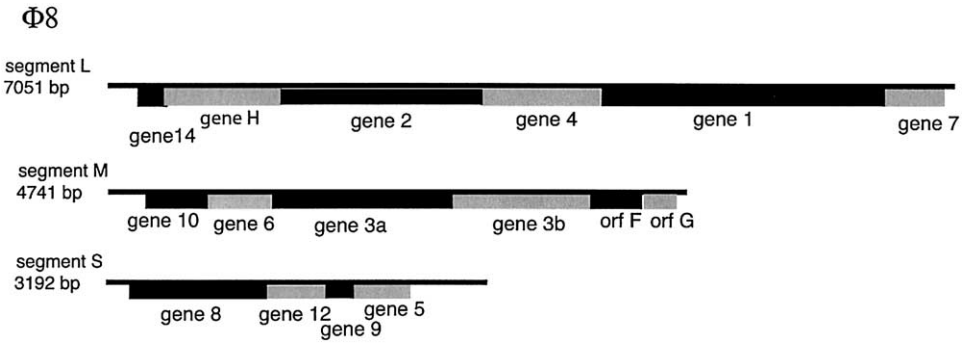

Fig. 1. Genetic maps of the ϕ8 genomic segments. Plus-strand transcripts have *pac* sequences near the 5′ ends upstream of the proximal genes. Note that the position of gene 7 is different from that found in the other cystoviruses.

P8 does not form a shell around the inner core. In this case it is a minor component of the membrane (Hoogstraten et al., 2000). Whereas ϕ6 enters the periplasmic space as a nucleocapsid covered by P8, ϕ8 enters as an inner core with P8 left behind as part of the membrane fused to the host outer membrane.

ϕ8 virions bind directly to the outer membrane of host cells. They have no affinity for the pilus that binds ϕ6 and its closest relatives; however, two other members of the Cystoviridae, ϕ12 and ϕ13, also bind directly to the outer membrane of the host cells (Mindich et al., 1999). These two phages have an arrangement of protein P8 similar to that found in ϕ6.

Among the consequences of the differences in structure between ϕ8 and its relatives is the finding that the inner core of ϕ8 is able to accept the lipid envelope directly without first forming a shell of P8. The inner core of ϕ8 can also infect spheroplasts of the host cells, whereas inner cores of ϕ6 must be covered by P8 in order to infect spheroplasts (Olkkonen et al., 1991).

Other differences are found in the behavior of protein P4, the NTPase that is the motor for genomic packaging. In ϕ6, P4 forms hexamers in the presence of ATP and these hexamers show NTPase activity (Juuti et al., 1998). In ϕ8, P4 forms hexamers in the absence of ATP and these hexamers have little NTPase activity until associated with empty inner cores (procapsids).

Additionally, the gene for the minor inner core protein P7 of ϕ6 and its relatives is located near the 5′ end of the segment L plus strand. The gene for the ortholog of this protein in ϕ8 is found at the 3′ end of segment L. A gene designated H is found in the normal position of gene 7. The protein coded by gene H is not a component of the inner core of ϕ8.

Results

Assembly of procapsids in E. coli

Procapsids were assembled in *E. coli* JM109 when plasmids containing cDNA copies of segment L (Fig. 1) were expressed. The particles, containing P1, P2, P4, and P7, could be purified from lysates by zonal centrifugation in sucrose gradients (Fig. 2). Upon further purification in sucrose gradients by density equilibrium centrifugation the particles were found to be highly pure but substantially diminished in the amount of P4 relative to the other proteins of the procapsid. Even the particles purified by the single zone sedimentation seemed to have less P4 than particles produced by ϕ6.

Position of gene 7

The members of cystoviridae have *orfs* near the 5′ end of segment L that are not necessary for phage development under laboratory conditions. These *orfs* are generally designated gene 14 (Mindich et al., 1999). They might play a role in regulation of expression of gene 7 (Casini and Revel, 1996), but they are not necessary for productive infection. In some of the phages closely related to ϕ6, namely ϕ7 and ϕ9, there are two *orfs* in front of gene 7, designated gene E and gene 14. Gene 7 is, on its own, polar on gene 2 (Mindich et al., 1988). The product of gene 2 is the viral polymerase (Gottlieb et al., 1990; Mindich et al., 1988) and P7 is an accessory protein involved in packaging and both

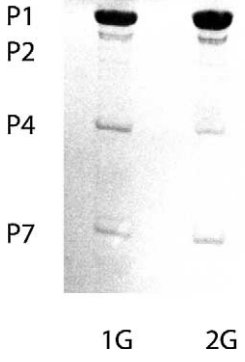

Fig. 2. Coomassi-stained acrylamide gel of ϕ8 procapsid proteins. Procapsids were produced in *E. coli* and purified by zonal centrifugation in sucrose gradients, (1G) or by zonal and equilibrium centrifugation (2G). Note that P4 is stained more intensely than P7 in 1G, but less so in 2G.

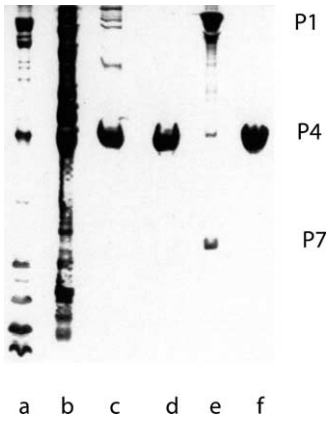

Fig. 3. Purification of protein P4. Lane a shows purified ϕ6 virus and lane e shows purified procapsid. Lane b contains the proteins precipitated with 57.5% saturation with ammonium sulfate. Lane c is the fraction isolated from zonal centrifugation in a sucrose gradient. Lanes d and f show the protein fraction eluted from the Hitrap QHP column.

plus-strand and minus-strand RNA synthesis (Gottlieb et al., 1990; Juuti and Bamford, 1997).

In ϕ8, the gene that we had originally designated gene 7 does not appear to code for a protein that is a component of the virion. Instead, a gene that lies at the 3′ end of segment L codes for a protein that is the size of P7 in ϕ6 and is a component of the procapsid and assembles onto P1 in the absence of P2 and P4. N-terminal amino acid analysis of the 19-kDa protein in the procapsid shows MTDPIT which matches the N terminus of the product of the gene at the 3′ end of L, which we have now designated gene 7 (GenBank Accession No. NC 003299). Analysis of the sequence of gene 7 shows that there is low but apparent similarity to the amino acid sequence of P7 of ϕ13. P7 of ϕ13 has a high degree of similarity (60%) to P7 of ϕ6, but ϕ8 P7 has no discernable similarity to P7 of ϕ6. We have renamed the gene upstream of gene 2 gene H. Gene H is polar on gene 2. The role of the product of gene H in ϕ8 is not yet clear. If it were a component of the virion it would be in very low copy number, since it is not seen at all in particles. The limit of detection is about three or four molecules per virion. We have prepared mutants lacking 85 amino acids at the N terminus of protein H and have found that they are very defective but can benefit from suppressor mutations in segment L. Complete deletions of gene H are not viable but can be complemented. Particles assembled in cells that carry plasmids missing gene H do not differ in composition or behavior from particles formed in cells carrying complete copies of segment L.

Properties of P4

P4 of ϕ8 was produced in *E. coli* BL21 DE3 with plasmid pLM2900. The purification scheme is described under Materials and Methods and shown in Fig. 3. The protein sediments as a multimer in sucrose gradients, similar to the behavior of ϕ6 multimers of P4, even in the absence of ATP or ADP. P4 of ϕ6 sediments as a monomer in the absence of NTP (Juuti et al., 1998). However, in contrast to the behavior of ϕ6 P4, ϕ8 P4 shows little ATPase activity as a multimer. The multimer appears as a hexamer in the electron microscope (Fig. 4). Rotation analysis shows the hexameric nature of the multimer.

Procapsids show considerable ATPase activity, but upon further purification, the amount of P4 in the particles is diminished (Fig. 2) and the amount of ATPase activity decreases by about 50 percent. When purified P4 is added to these procapsids, the ATPase activity shows a dramatic increase back to that of the one-gradient-purified particles. The single-gradient procapsids show activity for both ATP and GTP. The apparent K_m for ATP is 0.3 mM, but concentrations higher than 0.5 mM inhibit enzyme activity. This is also true for GTP. The minimal reaction rate for ϕ6 P4 is 3 to 4 μmol/min/mg protein (Paatero et al., 1995). The specific activity for ϕ8 P4 on procapsids is about the same. Mitochondrial F1 ATPase has a V_{max} of about 100 μmol/min/mg protein (Pullman et al., 1960). The ATPase activity is maximum at 45°C; neither magnesium nor calcium ions promote activity.

In vitro genomic packaging and replication

ϕ6 procapsids are capable of packaging plus strands of genomic segments in the order *s:m:l* in the presence of ATP or other NTPs. Minus-strand synthesis starts after all three segments are packaged (Frilander et al., 1992).

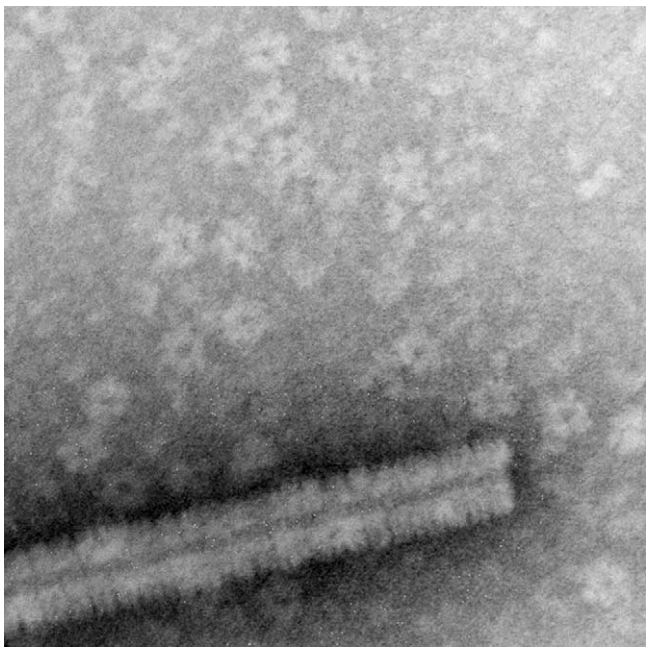

Fig. 4. Multimers of protein P4. Purified P4 was stained with sodium vanadate and viewed in the STEM at Brookhaven National Laboratory. The diameter of the multimer is calculated to be about 11 nm based upon the 18-nm diameter of TMV.

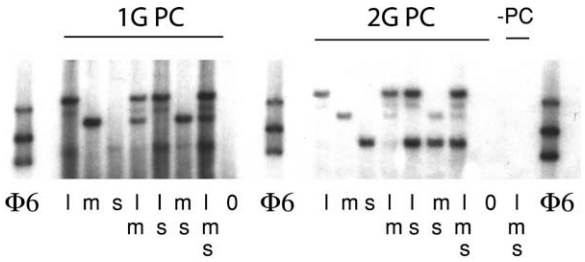

Fig. 5. In vitro minus-strand synthesis. Nonradioactive plus strands were incubated with procapsids in various combinations with all four NTPs. UTP was labeled with α-^{32}P. Note that different patterns are seen with the particles purified with a single sucrose gradient (1GPC) and those purified by means of an additional equilibrium gradient (2GPC). The single-gradient particle appears to package/replicate segment s poorly, while the two-gradient particle appears to package/replicate segment m poorly. There is no significant evidence for cooperativity. Radioactive ϕ6 dsRNA is present as a size standard.

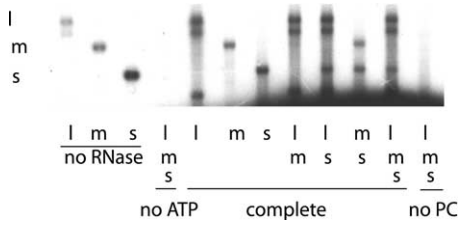

Fig. 6. In vitro packaging of radioactive plus strands. Radioactive RNA was incubated with procapsids, treated with RNase I, and electrophoresed on agarose gels. Control lanes show the migration of untreated RNA, and the lack of protection when ATP or procapsids (PC) are absent from the complete mixture. The *l* transcript contains truncated species that are also packaged. Note that segment *l* seems to inhibit the packaging of segment m. There is no evidence for cooperativity in packaging.

ϕ8 procapsids show minus-strand synthesis when exposed to either transcripts obtained from ϕ8 virions or SP6 polymerase transcripts of cDNA plasmids. There are some differences in the ϕ8 system and those found with ϕ6. Additionally, there are differences in behavior between core particles purified through one versus two gradients. In the case of SP6 transcripts of cDNA plasmids, we find that in two-gradient particles minus-strand synthesis on templates *l* and *s* is strong, but weak on segment *m* even though the concentration of plus strand for *m* is raised to several-fold above that of *s* and *l* (Fig. 5). In one-gradient particles, the synthesis of S is weaker than that of M. A dramatic difference between the packaging behavior in ϕ8 compared to ϕ6 is the affinity of RNA in the standard 5% PEG 4000 environment. ϕ6 packaging is saturated at about 500 μg RNA/ml, while ϕ8 packaging is not saturated at 10 times that amount. ϕ8 packaging can be saturated at about 500 μg RNA/ml in 10% PEG 4000, while this level of PEG is inhibitory to ϕ6 packaging.

In ϕ6, in the absence of manganese ions, minus-strand synthesis is dependent upon the packaging of all three genomic segments, although very high concentrations of RNA can result in minus-strand synthesis of individually packaged segments, particularly segment *l* (Frilander et al., 1995). In ϕ8, we find that in vitro minus-strand synthesis does not depend upon the packaging of all three genomic segments. The individual segments are replicated as well as the mixtures of all three (Fig. 5). Packaging of plus-strand RNA is not serially dependent in vitro and segment *l* appears to compete with segment *m* packaging (Fig. 6).

In addition, packaging and minus-strand synthesis takes place in vitro with transcripts that lack proper *pac* sequences. These molecules can compete with normal plus strands for packaging and minus-strand synthesis. When a plus-strand copy of segment S with a deletion from N9 to N158, pLM3042, is incubated with a truncated plus strand of S, pLM3001, that has a normal *pac* sequence, we can see that the molecule with the abnormal *pac* sequence is able to compete with the normal molecule (Fig. 7). The same is true for pLM3041, which has a smaller deletion in *pac*. A molecule that has the 5′ sequence of ϕ6S and the 3′ sequence of ϕ8S, pLM3040, is also able to compete with pLM3001. None of these molecules with abnormal *pac* sequences can be incorporated into live virus.

In order to determine whether the *pac* region of segment M was responsible for its poor packaging and replication, we prepared a construct with the *pac* region of L replacing the *pac* region of M. The transcript (3018) showed active minus-strand synthesis (Fig. 8) and was able to compete with the plus strands of S and L for minus-strand synthesis while the normal M plus strand (2669) did not package well nor compete. However, the 3018 transcript could be acquired by a mutant of ϕ8 with a change in protein P1. This analysis is the subject of a separate report.

Although the packaging and minus-strand synthesis with ϕ8 procapsids is much less efficient than that seen with ϕ6, it is still possible to package and replicate RNA in vitro and infect spheroplasts with the product. Whereas a similar experiment with ϕ6 procapsids would yield thousands of plaques, the ϕ8 transfection yielded an average of 150

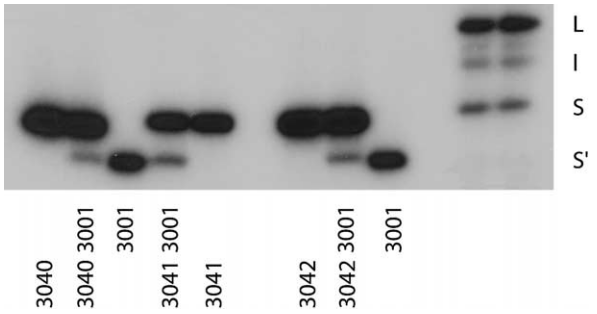

Fig. 7. Minus-strand synthesis demonstrating the ability of RNA without a proper *pac* sequence to compete with RNA that has a normal *pac* sequence. Transcript 3001 is missing 922 nucleotides from its interior but has a normal S *pac* sequence. Transcript 3042 is a normal S plus strand missing nucleotides 9 to 158 from the *pac* sequence. 3041 is missing nucleotides 35 to 158 and 3040 has exchanged the ϕ8 *pac* sequence for that of ϕ6. 3001 is present at 50 μg/ml, while the competing RNAs are at 10-fold higher concentration.

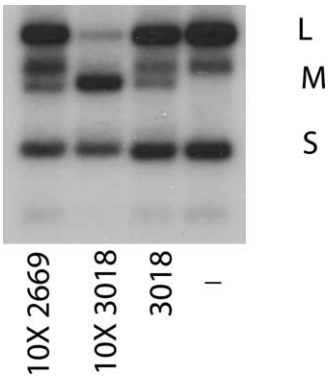

Fig. 8. Minus-strand synthesis comparing plus strand of M with a normal *pac* sequence (2669) and the *pac* sequence of L. All reactions contain 50 μg/ml of normal L and S plus strands.

plaques from a 25-μl reaction. A major difference, however, is that the ϕ8 system does not need protein P8, whereas the ϕ6 particles are completely uninfective in the absence of P8 (Olkkonen et al., 1991). Clearly, there is a great difference in the properties of the major core protein P1 between ϕ8 and ϕ6. Although the conditions for genomic packaging and minus-strand synthesis for transfection are virtually the same as those for radioactive labeling studies of packaging or minus-strand synthesis, we found that molecules with incomplete or missing *pac* sequences, pLM3040 and pLM3042, did not compete with normal plus strands when assayed for plaque formation.

In vivo acquisition of genomic segments

Although the in vitro packaging and minus-strand synthesis in ϕ8 does not show the serial dependence that was observed for ϕ6, experiments with the in vivo acquisition of genomic segments suggest that serial dependence is also operating in ϕ8. In ϕ6, a chimera could be made from the S and M segments. If the plus strand of this construct had the *pac* site of S at the 5′ end, it could be maintained in live virus along with the normal L segment to form a two-segment genome. If the plus strand of this construct had the *pac* sequence of M at the 5′ end, it could be maintained only if a normal or truncated S segment was also included in the genome along with the normal L segment.

We prepared chimeric genomic segments of S and M in ϕ8. Acquisition of the chimeric segments was promoted in several ways. In the first case, we electroporated plasmids with T7 promoters into cells carrying plasmids that express T7 RNA polymerase. The cDNA plasmids do not replicate in this strain, but they do transcribe. The combinations were L+M+S, which yielded plaques; L+SM chimera, which yielded plaques; L+ MS chimera or L+ MS chimera+ S, or L+MS +micro S, which did not yield plaques. The SM chimera has the *pac* sequence of S at the 5′ end, while the MS chimera has that of M.

In the second case we electroporated the plasmids producing chimeric transcripts into cells that were infected with a virus that carried a deletion of genes F and G in segment M. The virus was able to acquire the SM transcript in several hundred plaques, but it could not acquire the MS transcript. Finally, we prepared shuttle plasmids that could be maintained in pseudomonads or *E. coli* with inserts that could produce the SM or MS transcripts. Cells carrying these plasmids were infected with the FG deletion phage and the progeny were screened for acquisition of the transcripts. The SM transcript was acquired easily; however, a few plaques that had acquired the MS transcript were found. Analysis of the RNA in these virions showed that normal S was present and that the MS transcript was truncated (Fig. 9). This finding is consistent with the idea that the MS transcript can be acquired by the virus, but that the maintenance of S is necessary. The RNA content of ϕ8 cannot be increased beyond a particular point; therefore the only viable construct is one where the MS chimera is somewhat truncated.

Chimeric structures were also built with segments M and L. These were in plasmids pLM3050 and pLM3052, whose transcripts began respectively with the 5′ region of segment M and L. These plasmids were electrophoresed along with plasmid pLM2755 which contains the S segment, into *P. syringae* strain LM3313, which expresses SP6 polymerase. The number of plaques obtained was seven with the ML transcript, and one with the LM transcript. The resulting phages were propagated and their RNA was analyzed. In both cases the phage carried two segments, the normal S and the chimeric ML or LM. RT-PCR analysis and restriction analysis of the chimeric segments confirmed their structures. However, the phage resulting from the LM chimera segment contained a mutation in gene 1 that was similar to the mutation found in the gene 1 of the phage that was able to package an M segment with an L *pac* sequence.

Discussion

The cystoviridae are a family of bacteriophages with three dsRNA genomic segments packaged within a polyhe-

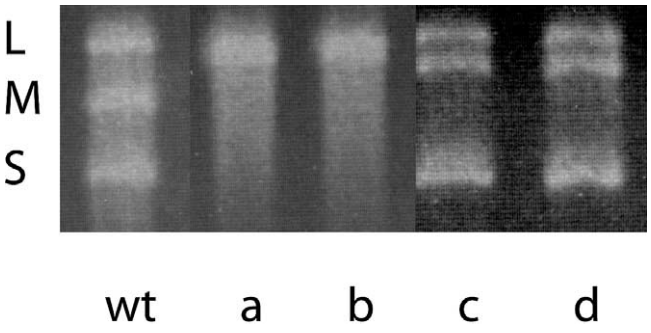

Fig. 9. dsRNA isolated from wild-type ϕ8 (wt); from two-segment virus containing normal L and the SM chimeric segment (a, b); and from three-segment virus containing normal L and S and a truncated chimeric MS segment (c, d).

dral core of four proteins. The core is within a lipid-containing membrane. The members of this family have similar gene arrangements and similar structure. However, ϕ8 stands at the furthest end of the similarity distribution. Its proteins have less sequence similarity to the other phages than the rest of the family. ϕ8 also differs from the others in that it does not form the shell of P8 over the inner core. The P8 shell in ϕ6 appears to play a role in the interaction with membrane, both for the acquisition of the viral membrane to form the mature virion and for the passage of the core particle through the cytoplasmic membrane and into the host cell. In ϕ8, the major structural protein of the inner core, P1, seems to play the roles of P8 in addition to its function as the major structural protein of the inner core and as the determinant of packaging specificity.

The inner core, composed of proteins P1, P2, P4, and P7 in all Cystoviridae, is the machine for the packaging and replication of the genome. We show, in this report, that the inner core particle is capable of packaging and replicating plus-strand transcripts of the ϕ8 genome. There are indications that the rules for genomic packaging in ϕ8 are not as stringent as those for ϕ6. The stringency of the packaging rules appears to be minimal when assayed by the incorporation of radioactive material. We have found that in vitro packaging and minus-strand synthesis do not show the serial dependence found in ϕ6 and that molecules that lack proper *pac* sequences can be packaged and serve as templates for minus-strand synthesis. It is worth noting that the packaging in ϕ6 can be perturbed by changing plus-strand RNA concentration, buffer conditions, or the conditions of inner core purification, so that serial dependence is compromised. It might be that the in vitro packaging conditions for ϕ8 are far enough from those found in the infected cell that the normal behavior of the system is not seen. The molecules with improper *pac* sequences are not acquired by live virus (unpublished results). The experiments with the in vivo acquisitions of chimeric segments are consistent with serial dependence of packaging. Although SM chimeras function satisfactorily, MS chimeras require the presence of segment S. ML chimeras seem to function as well; however, LM chimeras are acquired only with a concomitant mutation in gene 1. The packaging of RNA for transfection also shows high specificity. It may be that some factor is missing from the in vitro system that is important for precision in packaging. This might be a host factor or even a product of the viral genome. The dramatic difference in affinity for RNA shown by ϕ8 procapsids compared to those of ϕ6 suggests that there may be another factor necessary for normal packaging. It is possible that the inner core particles that we prepare are composed of two populations, one stringent and capable of producing live virus and the other less restrictive and incapable of producing live virus. However, we have also found that transcripts with no *pac* sequences at all can be picked up by virus in vivo, although at very low frequencies (Onodera et al., 2001).

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 ϕ8. LM128 was also used and LM2691 is LM128 carrying plasmid pLM1086 which is a derivative of pRK290 (Ditta et al., 1980) and pAR1219 (Parichehre et al., 1984) and expresses T7 RNA polymerase in pseudomonads. LM3313 expresses SP6 polymerase. LM2817 is *E. coli* JM109 carrying plasmid pLM2424, which is pT7T319U with a cDNA copy of ϕ8 segment L. Plasmids pLM2653, pLM2669, and pLM2755 are derivatives of pT7T319U with SP6 polymerase promoters and cDNA copies of ϕ8 segments L, M, and S, respectively. Plasmid pLM3001 contains a cDNA copy of segment S with a deletion of the sequence between *Bam*HI sites at 2006 and 2928. It is used for competition studies in packaging. Plasmid pLM3040 has a cDNA copy of ϕ8S with the 5′ sequence of ϕ6S. Plasmid pLM3041 has a copy of S with a deletion of nucleotides 35 to 158. Plasmid pLM3042 has a deletion from nucleotides 9 to 158. Details of the construction of the plasmids are available from the authors.

Media

The media used were LC and M8 (Sinclair et al., 1976). Ampicillin plates contained 200 μg of ampicillin/ml in LC agar.

Preparation of procapsids

Plasmid pLM2424 contains a cDNA copy of segment L of ϕ8. A culture of JM109 carrying pLM2424 was grown overnight at 24°C in LB broth with ampicillin at 200 μg/ml and 1 mM IPTG. Cells were harvested, washed with buffer A (10 mM potassium phosphate, pH 7.5, 1 mM magnesium chloride), and lysed with a French Press at 7000 lb/in^2. The lysate was spun at 12,000 rpm for 15 min and the supernate applied to a 10 to 30% sucrose gradient with 10 mM phosphate, pH 7.5, and spun for 45 minutes at 35,000 rpm. The procapsids formed a sharp band that was collected and designated one-gradient PC. Some of this material was applied to a 30 to 60% sucrose gradient and spun overnight at 23,000 rpm at 4°C. A sharp band was collected and designated two-gradient PC.

In vitro minus-strand synthesis

Minus-strand synthesis reactions were carried out at 28°C for 90 min in a volume of 12.5 μl containing buffer H3, macaloid, ammonium acetate, 5 or 10% PEG 4000, ATP, GTP, and CTP at 1 mM and UTP at 0.1 mM, 0.6 to 2 μg of each plus-strand RNA, 1 μg procapsid and 5 μCi uridine 5′-[α-^{32}P]triphosphate (Frilander et al., 1992; Got-

tlieb et al., 1990). The reaction products were analyzed with 1 μg of carrier RNA on 0.8% agarose gels containing 0.1% SDS in 0.5 × TBE buffer (Maniatis et al., 1982). The optimum conditions were similar to those for ϕ6, namely 100 mM ammonium acetate, 4 mM magnesium ions, 5 mM DTT, Tris buffer, pH 8.8; however, the PEG4000 concentration was found to be optimal at 10% rather than the 5% used for ϕ6.

In vitro genomic packaging

Frozen purified procapsid preparations were thawed and incubated for 60 min at 28°C in a 12.5 μl packaging reaction consisting of H3 buffer, 3 or 4 mM $MgCl_2$, 100 mM NH_4OAC, 20 mM NaCl, 5 mM KCl, 5 mM DTT, 0.1 mM Na_2EDTA, 1 mM ATP, 100 ng macaloid, 5 or 10% PEG 4000, and about 600 ng of [32p]UTP-labeled single-stranded ϕ8 RNA for each segment. Approximately 1 μg of procapsid was used per reaction. The samples were then treated with 10 units of RNase I (RNase One-Promega) (Meador et al., 1990) and incubated for 30 min at 28°C. Ten microliters stop solution (3× sample buffer (Studier, 1973) and 25 mM EDTA) was added and the samples were heated at 85°C for 5 min. The samples were then electrophoresed in 1.5 or 2% agarose gels.

Transfection assay

Procapsids were incubated with a mixture of plus-strand RNA from a transcription reaction using filled cores of ϕ8 prepared by Triton X-100 treatment of purified virions. Alternatively, the RNA could be derived from SP6 polymerase transcripts of cDNA plasmids. The reaction volume was 12.5 μl. After 90 min of incubation at 28°C the mixture was added to spheroplasts of *P. phaseolicola* strain LM2489 and incubation continued as described previously (Olkkonen et al., 1990), with the exception that protein P8 was not added to the procapsids and the nucleotide concentrations were 0.3 mM instead of 1 mM. The concentration of PEG 4000 was raised to 10% compared to the level of 5% used for ϕ6. The protoplasts were then plated on a lawn of LM2489 and plaques were assayed the next day. Spheroplasts were also transfected, as a control, with filled cores derived from purified ϕ8 virions by Triton X-100 treatment.

Construction of chimeric genomic segments

Plasmid pLM2736 produces a transcript that contains the genes of segment S and M with those of S at the 5′ end. It was prepared by ligating cDNA of M cut at the *Xhol* (N191) to the *Sacll* (N3124) site of S. The final construct has a T7 RNA polymerase promoter. Plasmid pLM2959 has the cDNA of segment S from the *Dral* (N160) site ligated to a *Bglll* site inserted into gene G of segment M at N4580. This construct has the genes of segment M at the 5′ end of the plus strand. We had previously shown that gene G could be deleted with no consequence (Onodera et al., 2001). Plasmid pLM3050 has an SP6 promoter that produces a transcript with the genes of segment M at the 5′ end and those of L at the 3′ end. Plasmid pLM3052 produces a transcript with the genes of segment L at the 5′ end and those of M at the 3′ end.

Preparation of protein P4

Gene 4 of ϕ8 was cut from plasmid pLM2622 with *Bglll* and *Sacl* and cloned into pT7T319U, which was then named pLM2769. This plasmid was transformed into JM109 and used for production of P4. The strain, LM3284, was grown at 28°C overnight with 1 mM IPTG induction. Cells were collected in 40 mM Tris–HCI, pH 8, with 500 mM NaCl and broken with a French press. The lysate was clarified by centrifugation at 35,000 rpm at 4°C for 2 h. The supernatant liquid was fractionated with ammonium sulfate precipitation and the fraction precipitating at 57.5% saturation was dissolved in buffer and applied to a sucrose gradient and spun for 18 h at 35,000 rpm. The peak was applied to a HiTrap QHP 1-ml column and eluted with 175 mM NaCl, 10 mM Tris–HCl, pH 8. This material was precipitated with ammonium sulfate and dissolved in 100 μl of 60 mM NaCl, 10 mM Tris–HCl, pH 8. The concentration was determined to be 3.8 mg/ml.

NTPase activity of P4 and procapsids

Two methods were used. The first was the coupled assay with pyruvate kinase and lactic dehydrogenase (Pullman et al., 1960). The second involved thin-layer chromatography of radioactive ATP or GTP. Reactions were performed in 10 μl 50 mM Tris acetate, pH 7.4, with 2 mM ATP, 10 μCi per reaction. One microliter of each reaction was applied to sheets of PEl–cellulose and developed with formic acid and lithium chloride (Sadis and Hightower, 1992). Quantitation was done with a Molecular Dynamics phosphoimager.

Protein sequencing

The N-terminal amino acid sequence of the low-molecular-weight procapsid protein was determined by the Protein Core Facility at Columbia University.

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