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Purification and Protein Composition of PM2, the First Lipid-Containing Bacterial Virus To Be Isolated[1]

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The marine, icosahedral bacteriophage PM2 was isolated in the late 1960s. It was the first phage for which lipids were firmly demonstrated to be part of the virion structure and it has been classified as the type organism of the *Corticoviridae* family. The host, *Pseudoalteromonas espejiana* BAL-31, belongs to a common group of marine bacteria. We developed a purification method producing virions with specific infectivity approximately as high as that of the lipid-containing phages PRD1 and ϕ6. The sensitivity of the virus to normally used purification media such as those containing sucrose is demonstrated. We also present an alternative host, a pseudoalteromonad, that allows enhanced purification of the virus under reduced salt conditions. We show, using N-terminal amino acid sequencing and comparison with the genomic sequence, that there are at least eight structural proteins in the infectious virus. © 1999 Academic Press

Key Words: PM2 bacteriophage; purification; infectivity; structural proteins.

INTRODUCTION

Although a common structural feature of animal viruses is the presence of a membrane, there was no firm demonstration of lipids in bacteriophages until the late 1960s. This and other characteristics found in bacteriophages had created visions that viruses infecting prokaryotic hosts are distinct from those infecting eukaryotes. In recent years, based in particular on studies of lipid-containing bacteriophages, considerable structural and functional similarities between viruses infecting prokaryotic and eukaryotic hosts have been revealed. The enveloped dsRNA bacteriophage ϕ6 contains an internal polymerase particle that is very much like that found in *Reoviridae* (Butcher *et al.,* 1997; Juuti *et al.,* 1997; Mindich and Bamford, 1988). Bacteriophage PRD1 genome organization, replication strategy, and virion structure resemble those of adenoviruses (Bamford *et al.,* 1995; Benson *et al.,* 1999; Butcher *et al.,* 1995; Grahn *et al.,* 1999; Rydman *et al.,* 1999).

There have been several early suggestions that lipids are structural components of phage virions. However, isolation of a pseudomonad and its phage off the coast of Vina del Mar in Chile was the first demonstration of a phage with nucleic acid, protein, and lipid constituents (Camerini-Otero and Franklin, 1972; Espejo and Canelo, 1968a,b). The phage was designated PM2 and the host was recently defined to be a member of the *Pseudoalteromonas* genera (*Pseudoalteromonas espejiana* BAL-31), common, marine, gram-negative bacteria (Gauthier *et al.,* 1995). The original isolation name was *Pseudomonas* BAL-31 and the bacterium is the source of the common DNA exonuclease *Bal*-31.

Two characteristics drew attention to the phage. In addition to the lipids, the genome was a highly supercoiled circular dsDNA molecule (Espejo and Canelo, 1969; Espejo *et al.,* 1969). The PM2 literature (some 400 entities) is divided into two roughly equal parts. The virological and structural features of the phage were analyzed in one part and the DNA was examined in topological studies in the other. There are also several literature reviews (Brewer, 1980; Franklin, 1974, 1977; Franklin *et al.,* 1976). The virion composition is approximately 14% nucleic acid, 14% lipid, and 72% protein (Camerini-Otero and Franklin, 1972). The sedimentation coefficient and densities of the particle in sucrose and CsCl are determined to be $s_{20°,W}$ 293S, 1.24 g/cm^3, and 1.29 g/cm^3, respectively (Camerini-Otero and Franklin, 1975), but see also our results below. Electron microscopy and low-angle X-ray and neutron diffraction studies revealed that the particle is icosahedral with a diameter of about 60 nm (Espejo and Canelo, 1968a; Franklin, 1974; Harrison *et al.,* 1971; Silbert *et al.,* 1969). The fivefold vertices have clear extensions. The outermost layer is composed of protein and the lipid bilayer is located internally (Harrison *et al.,* 1971). The consensus view from the literature is that there are four structural pro-

[1] Sequence data from this article have been deposited with the GenBank Data Library under Accession No. AF155038.

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teins (Brewer, 1976; Datta *et al.,* 1971; Schäfter *et al.,* 1974a) although indications of a higher number have also been published (Brewer and Singer, 1974).

The genome contains about 10 kb of circular dsDNA as determined from restriction endonuclease fragments (Streeck and Gebhardt, 1979). It contains 51 negative supercoils when packaged into the virion (Espejo *et al.,* 1971; Gray *et al.,* 1971; Ostrander and Gray, 1974). This is to our knowledge the highest number detected in a natural molecule. Previously about 2500 bp of a portion of the genome sequence has been published (Miller *et al.,* 1984; Sheflin and Kowalski, 1984; M26134, M32693, M32694) and stretches of amino acid sequences and amino acid compositions of several proteins have been determined (Brewer and Singer, 1974; Hinnen *et al.,* 1976). The phage life cycle takes approximately 60 min at 25°C (Cota-Robles *et al.,* 1968) and an infected cell liberates about 50 infectious progeny particles according to one-step growth experiments and some 600 according to single-burst experiments (Franklin *et al.,* 1969). Thin-section electron micrographs revealed virus-size membrane vesicles lining the cytoplasmic membrane and, late in infection, packaged virions at the same location. The viruses are liberated by cell lysis (Cota-Robles *et al.,* 1968; Dahlberg and Franklin, 1970).

The particle structure has been proposed to consist of two protein shells with a lipid bilayer in between (Schäfer *et al.,* 1974a). Considerable efforts have been invested in biochemical *in vitro* reconstitution experiments where disrupted phage particles have been assembled to form infectious virions (Hinnen *et al.,* 1974; Schäfer and Franklin, 1975; Schäfter *et al.,* 1978; Tsukagoshi *et al.,* 1977b). In particular, the role of lipids in the *in vitro* assembly experiments was investigated to learn about the biogenesis of a viral membrane and membranes in general. Finally, a complete reconstruction of an infectious virion from its DNA, protein, and lipid constituents was published (Schäfer and Franklin, 1978a). In later experiments, however, Satake *et al.* (1980) indicated that lipid-free subparticles used in the reconstruction could not be isolated. They also criticized the two-protein-shell model of the virion structure.

The methods used to prepare infectious virions have not been critically evaluated (see Results), making it difficult to interpret the biochemical and biophysical characteristics of the infectious particles and the reconstitution experiments. During the past 16 years there have been no primary publications on PM2 virology. PM2 is the only characterized member of the *Corticoviridae* family (type organism) as well as the first lipid-containing phage to be isolated. It is of importance to put the PM2 knowledge on a reliable basis. As our laboratory has a history of studying lipid-containing bacterial viruses, we initiated a basic characterization of this virus system. On the one hand, we determined and analyzed the entire PM2 genome sequence (Männistö *et al.,* 1999, accompanying paper) and on the other hand, we report here methods to obtain infectious purified virions as well as show that the virion contains at least eight structural proteins instead of four.

RESULTS

An alternative host for PM2

Bacteriophage PM2 and its original host *P. espejiana* BAL-31 were isolated from the Pacific Ocean. Due to the high salt dependence (~400 mM NaCl minimum) of BAL-31 we investigated an alternative host (ER72M2) isolated from the East River, New York City, by Dr. Leonard Mindich. The growth conditions and requirements of ER72M2 were quite similar to those of BAL-31 except that this new host grows in less salt (200 mM NaCl). Growth was inhibited over approximately 35°C and $MgSO_4$ was required for both hosts. To identify ER72M2 we determined its 16S rRNA gene sequence (GenBank Accession No. AF155038). It was almost identical to other pseudoalteromonads (Table 1) and 99.5% similar to BAL-31. Thus ER72M2 is also a *Pseudoalteromonas* species. The plating efficiency of PM2 on BAL-31 and ER72M2 was practically the same regardless of the host used to grow the virus. We also tested the propagation of PM2 (plaque assay) with the other bacterial species listed in Table 1. It appeared that PM2 did not infect any of the other species tested.

The PM2 plaques on BAL-31 were heterogeneous, 1–5 mm in diameter but rather uniform on ER72M2 (1–2 mm). The virus stock (~1×10^{12} PFU/ml) prepared in SB medium (containing 0.5 M NaCl) lost about 50% of its infectivity in a week at 4°C. When PM2 was plated on ER72M2 at lower salt concentration (100 or 200 mM) the plaque count was reduced to around 50%. We optimized the growth of PM2 in liquid SB medium using both hosts. The best results were obtained by infecting the cells at a density of 6×10^8 CFU/ml using a multiplicity of infection (m.o.i.) of 10 at 28°C. The maximal lysate titer obtained was about 2×10^{11} PFU/ml. Approximately 300 new virus particles were released from each infected ER72M2 cell about 70–90 min after infection according to the one-step growth experiment. A one-step growth curve using strain ER72M2 is shown in Fig. 1.

Purification of PM2

We used the published procedures (Hinnen *et al.,* 1974; Salditt *et al.,* 1972; Silbert *et al.,* 1969) to produce PEG-concentrated and rate zonal (sucrose gradient) purified virus particles under the high-salt conditions reported previously. However, only a low yield of infectious virus was obtained (~4×10^{12} PFU/liter of ER72M2 lysate; ~2×10^{11} PFU/liter of BAL-31 lysate). Using this virus material (and later material from the optimized virus purification method) we tested different ion concentra-

TABLE 1

Bacterial Strains

Name	Strain[a]	Growth T (°C)	Broth[b]	Comparison of 16S rRNA sequence with ER72M2 strain: GenBank number	Sequence similarity (%)
Pseudoalteromonas sp.	ER72M2	28	SB	AF155038	100
Pseudoalteromonas nigrifaciens	DSM[c] 8810	20	SB	X82146	99.8
Pseudoalteromonas atlantica	DSM 6839	20	SW	X82134	99.7
Pseudoalteromonas carrageenovora	DSM 6820	20	SW	X82136	99.5
Pseudoalteromonas espejiana	BAL-31; ATCC[d] 27025	28	SB	X82143	99.5
Pseudoalteromonas undina	DSM 6065	20	SB	X82140	99.2
Pseudoalteromonas haloplanktis subsp. *haloplanktis*	DSM 6060	20	SB	X67024	99.1
Pseudoalteromonas haloplanktis subsp. *tetraodonis*	DSM 9166	20	SB	X82139	98.9
Pseudoalteromonas luteoviolacea	DSM 6061	20	SW	X82144	95.5
Alteromonas macleodii	DSM 6062	20	SB	X82145	89.0
Salmonella typhimurium	LT2	28	SB	Z49264	88.8
Escherichia coli	K12	28	SB	J01859	88.1
Pseudomonas syringae	pv. Phaseolicola	28	SB	Z76669	86.4

[a] Bacterial strain or/and source of the strain used in this study.
[b] SB broth (Espejo and Canelo, 1968a); SW broth (modified medium 123 of DSMZ), see Materials and Methods.
[c] German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany.
[d] American Type Culture Collection, Manassas, Virginia.

tions in the virus storage buffer starting from the published buffer B (20 mM Tris–HCl, pH 7.2, 1 M NaCl, and 10 mM $CaCl_2$; Hinnen *et al.,* 1974). It appeared (Fig. 2) that the lowest limits for the virus stabilization were 10 mM NaCl and 2.5 mM $CaCl_2$ (note that about 50% reduction of infectivity occurred in a week also in SB broth). Calcium ions could not be replaced by magnesium ions (not shown). Based on this information a new standard buffer, PM2 buffer, was designed (20 mM Tris–HCl, pH 7.2, 100 mM NaCl, and 5 mM $CaCl_2$). The effect of the divalent cation chelators EDTA and EGTA on PM2 infectivity in buffer B was also tested. Tenfold molar excess of EDTA

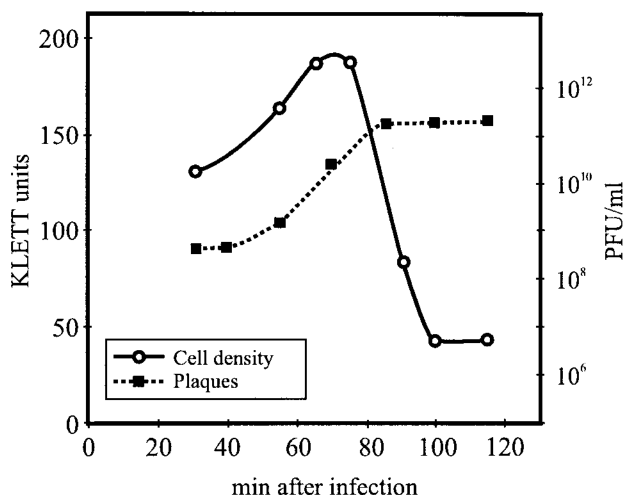


FIG. 1. One-step growth curve of bacteriophage PM2 on *Pseudoalteromonas* sp. ER72M2. Cells were grown in SB broth at 28°C and infected at a m.o.i. of 10 at time zero. 15 min after infection, cells were collected (5000 rpm, 10 min, 28°C) and resuspended in fresh medium at 28°C to remove nonadsorbed phage particles. Turbidity (cell density) was measured using a Klett-Summerson colorimeter (A_{540}).

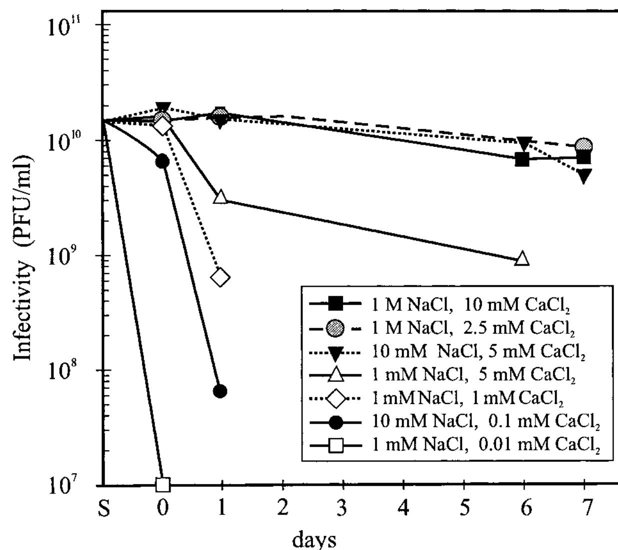


FIG. 2. Effect of $CaCl_2$ and NaCl concentrations on the infectivity of bacteriophage PM2. The number of infectious phage particles was measured after different incubation times at 4°C. Time 0 infectivities were measured after a 10-min incubation period. The initial infectivity of the virus in buffer B (20 mM Tris–HCl, pH 7.2, 1 M NaCl, and 10 mM $CaCl_2$) is indicated on the *x*-axis (S).

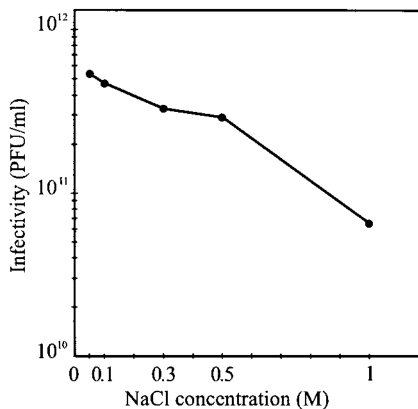


FIG. 3. Effect of NaCl concentration in a linear 5–20% (w/v) sucrose gradient on the infectivity of polyethylene glycol-concentrated bacteriophage PM2 (in 20 mM Tris–HCl, pH 7.2, and 5 mM $CaCl_2$). The number of infectious particles was determined in the virus zone.

had no measurable effect on phage titer overnight at 4°C but the corresponding EGTA concentration reduced the titer by about 50%. This indicates that the calcium ions, absolutely necessary for the virion integrity, are very tightly bound.

Next we examined the purification methods, using both hosts, to reveal the cause(s) for the low yield. Using the published PEG precipitation method (8% PEG, 8000 rpm, 10 min of centrifugation; Hinnen *et al.,* 1974) for viruses grown on BAL-31 the recovery of infectivity was acceptable but variable (5–27%) compared to infectivity in the fresh lysate (100%). However, in the case of ER72M2 cells the conditions had to be changed (10% PEG, 8000 rpm, 40 min) to obtain 30–50% yields. Less than 5% of the infectivity remained in the supernatant after collection of the PEG concentrated viruses. Also, DNase had to be added to the ER72M2-grown virus lysate to decrease the viscosity of the PEG-concentrated virus preparation. The surprise came when we tested the virus infectivity dependence on the salt concentration in a 5–20% (w/v) sucrose gradient. When the published buffer B was used in the gradient (1 M NaCl; Hinnen *et al.,* 1974) only a few percent of the infectivity was recovered but lowering the salt concentration dramatically improved the yield (Fig. 3). Analyzing the effect of high NaCl concentration (1–4.5 M) without sucrose, it appeared that at up to 2.5 M NaCl the virus maintained its infectivity and about 50% reduction was observed in 4.5 M NaCl (overnight storage at 4°C). Based on this, the sucrose gradient buffer was changed to PM2 buffer.

The second surprise came when we continued to purify the virus using equilibrium density centrifugation in sucrose (in PM2 buffer). Practically no infectivity was recovered in the virus zone. We interpreted this to be the effect of sucrose. We next tested the virus infectivity in different density gradient media (Fig. 4). It appeared that the virus was sensitive to normally used media such as sucrose, CsCl, and glycerol but stable in iodixanol (OptiPrep). Examination of the sucrose inactivation curve revealed that the effective concentration in the rate zonal centrifugation (15–17% (w/v) in the position of the virus zone) does not yet inactivate the virus. However, the equilibrium concentration (about 1.26 g/cm^3, 55% w/v) dropped the infectivity almost two orders of magnitude (specific infectivity of 2×10^{10} PFU/mg protein in the equilibrated virus zone). Virus equilibration in CsCl at a density of 1.28 g/cm^3 did not inactivate the phage (specific infectivity 4–6 $\times 10^{12}$ PFU/mg protein) but the yield was about one-fourth compared to iodixanol. The drop of the total infectivity in CsCl (in Fig. 4) is due to the reduced yield. The equilibrium position of the particle in iodixanol was about 30% (w/v) corresponding to 1.16 g/cm^3. For these reasons the purification procedure was changed to contain a rate zonal sucrose gradient step in low salt and an equilibrium centrifugation step using iodixanol. The results of one such PM2 purification are shown in Table 2. The yield was approximately 3.5 mg protein of 1× purified and 1 mg protein of 2× purified virus per liter of cell lysate. When the two hosts were compared it appeared that considerably fewer impurity protein bands were detected during the purification, and the recovery of the infectivity was considerably higher when ER72M2 was used as the host. For these reasons strain ER72M2 was selected as the new standard host and used subsequently.

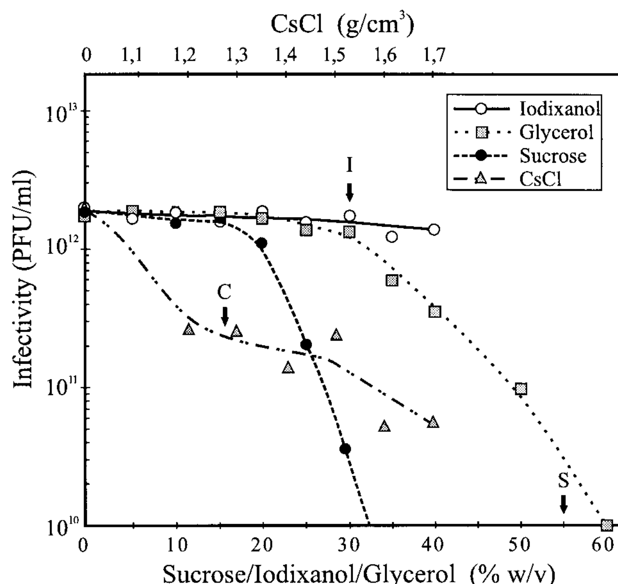


FIG. 4. Infectivity of PM2 in different concentrations of a variety of gradient materials. The virus preparation in PM2 buffer (20 mM Tris–HCl, pH 7.2, 100 mM NaCl, and 5 mM $CaCl_2$) was used as the control to which the gradient materials were added. The equilibrium positions of the virus (arrows) were measured in sucrose (S), cesium chloride (C), and iodixanol (I).

TABLE 2

Purification of Bacteriophage PM2

	Cell lysate	PEG precipitate[a]	1× virus[b]	2× virus[c]
Specific infectivity (PFU/ mg protein)	—	5.8×10^{11}	5.0×10^{12}	8.8×10^{12}
Recovery of infectivity (PFU/ liter of lysate)	1.2×10^{14}	4.7×10^{13}	2.0×10^{13}	1.4×10^{13}
Recovery of infectivity (%)	100	39	17	12

[a] Particles precipitated from the cell lysate with polyethylene glycol.
[b] After purification using 5–20% sucrose rate zonal centrifugation.
[c] After further purification using iodixanol isopycnic centrifugation.

Structural proteins of PM2

The PM2 protein pattern at the different stages of the purification process and the Western blot pattern of purified virus proteins obtained using anti-PM2 serum are shown in Fig. 5A. The protein bands detected in the 2× purified virus lane were subjected to N-terminal amino acid sequence analysis. The sequences obtained were compared to the PM2 genome sequence (Männistö *et al.,* 1999, accompanying paper) and to the SwissProt protein database. The conclusion is that there are at least eight structural proteins in the infectious PM2 virion (Fig. 5B). Below protein P3 there is an area of diffuse staining (vertical line in Fig. 5A). This whole area was divided into six equal slices and each of them was subjected to N-terminal amino acid sequence analysis. This yielded three different PM2-derived protein sequences (P4, P7, and P8). The positions of these proteins were determined on the basis of the slices giving the strongest signal. The genome sequence revealed an additional open reading frame (ORF j) among the genes coding for structural proteins (Fig. 7). We screened the area corresponding to this size protein (overlapping with P2) by N-terminal amino acid sequencing but did not find a signal matching the ORF j sequence.

The major coat protein P2 sequence appeared in three protein bands migrating differently in the protein gel: the major band, a minor band below the major one (vertical line in Fig. 5A), and a band with an apparent molecular mass of a dimer (P2*). Analysis of the linear 5–20% rate zonal sucrose gradient (Fig. 6) revealed a large band in the position of an approximately 60-kDa protein that migrated as the dimer of P2 (30 kDa) in tricine–SDS–PAGE. We determined the N-terminal amino acid sequence of the large band at the top of the gradient (arrow in Fig. 6). Instead of P2, it turned out to be a Hsp60 homologue. The N-terminal amino acid sequence was determined for this protein resulting in AAKEVLFAGDARAKMLTGVNILANA . . . not encoded by the viral genome (Männistö *et al.,* 1999). The closest match (76%) to this sequence was that of *Caulobacter crescentus* HSP60-type chaperone. The ER72M2 HSP60 was also recognized by polyclonal antiserum against *Escherichia coli* hsp60. Using this antiserum we showed that HSP60 expression was not induced in the host bacterium by PM2 infection.

The upper protein band in purified virus gave two different N-terminal amino acid sequences. One was that of P2*. The other sequence was not of PM2 origin (the upper arrow in Fig. 5A, ISVNTXVTI . . .). No match to this sequence was found in the protein databank. Based on analysis by electron microscopy of negative-stained, purified, infectious viruses (not shown) and the sedimentation behavior of the virus and this protein complex, we determined it to be the flagellin protein of ER72M2. Another protein that was not encoded by the PM2 genome (the lower arrow in Fig. 5A, MRTTTKQQE . . .) was not related to any of the proteins in the databank.

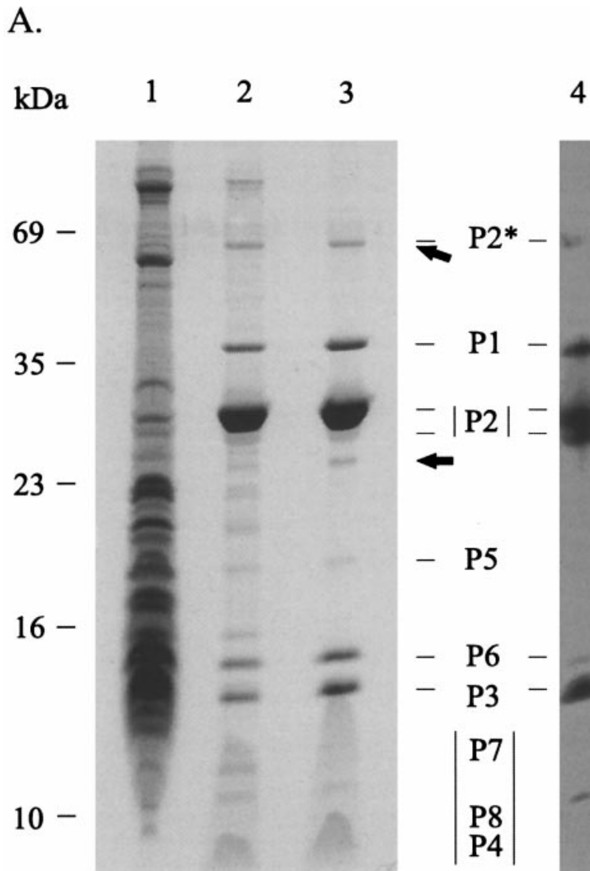
Additional observations

We also examined infected cells (BAL-31 and ER72M2) at 5, 10, 20, 35, 50, and 60 min postinfection (p.i.) using thin-section electron microscopy (not shown). Early in infection (5 and 10 min p.i.) both filled and empty particles were seen in direct contact with the cell surface. The first intracellular particles were visible about 50 min p.i., always lined up with the host membrane. These results are identical with those previously published for BAL-31 infected cells (Cota-Robles *et al.,* 1968; Dahlberg and Franklin, 1970).

We also tested the ability of the polyclonal anti-PM2 serum to inactivate or precipitate the virus. To our surprise neither inactivation nor precipitation was detected although the antiserum detected almost all the phage structural proteins in the Western blot (Fig. 5A). For this reason the antiserum could not be used for inactivation, so we removed the unadsorbed phages in the one-step growth experiment by washing the cells.

DISCUSSION

The new host, ER72M2, has several advantages over BAL-31: the lower salt concentration tolerance and superior behavior of the ER72M2-grown PM2 during purification. However, the lysate titers were approximately the same with both hosts and the same as reported previously for BAL-31 grown virus by Espejo and Canelo (1968a) as well as by Franklin *et al.* (1969). For these reasons we selected ER72M2 as the standard host for PM2. It is somewhat surprising that PM2 did not grow on any of the other pseudoalteromonads although, as judged by 16S rRNA sequences, some of them were more closely related to BAL-31 or ER72M2 than these two hosts were to each other. This indicates that PM2



B.

Protein	N-terminal amino acid sequence	Molecular mass	
		Sequence[1]	Tricine-SDS-PAGE[2]
P2*	MRSFLNLNSI	30,2 kDa	62 kDa
P1	MIVKKKLAAG	37,5 kDa	37 kDa
P2	MRSFLNLNSI	30,2 kDa	28 kDa
P5	MKKAHMFLAT	17,9 kDa	18 kDa
P6	ANFLTKNFVW	14,3 kDa	13 kDa
P3	MNTSVPTSVP	10,8 kDa	12 kDa
P7	MINKTTIKTV	3,7 kDa	-
P8	MLGALMGVAG	7,3 kDa	-
P4	MQKPSGKGLK	4,4 kDa	-

[1]Molecular masses of the proteins were determined from the genomic sequence (Männistö *et al.*, 1999).
[2]Molecular masses of the proteins were determined from the tricine-SDS-polyacrylamide gel using structural proteins of bacteriophage ϕ6 as standards.

FIG. 5. (A) Structural proteins of bacteriophage PM2 from different purification stages (Coomassie blue stained tricine–SDS–PAGE) and structural proteins detected by immunoblotting using the polyclonal PM2 antiserum. Lane 1, polyethylene glycol concentrated viruses from the lysate. Lane 2, virus zone after the rate zonal sucrose gradient centrifugation (1× purified virus). Lane 3, purified virus after the isopycnic centrifugation in iodixanol (2× purified virus). Lane 4, immunological detection of virus proteins (2× purified virus) was performed with the polyclonal anti-PM2 serum. Numbers on the left indicate the molecular mass of standard proteins (kDa). The structural proteins of PM2 are indicated between lanes 3 and 4. P2* indicates the major coat protein migrating as a dimer (see also the text). Precise positions of the small proteins (P4, P7, and P8) were difficult to determine in the Coomassie blue-stained gel. Their positions were estimated applying the data from the determination of the N-terminal amino acids of the structural proteins (see also the text). (B) Proteins detected in lane 3 (Fig. 5A) were transferred to PVDF membrane, which was stained with Coomassie blue. N-terminal amino acids were sequenced from visible bands and the entire area below the P3 protein band. The arrows in A indicate the positions of those proteins for which corresponding genes were not found in the virus genome (see also the text).

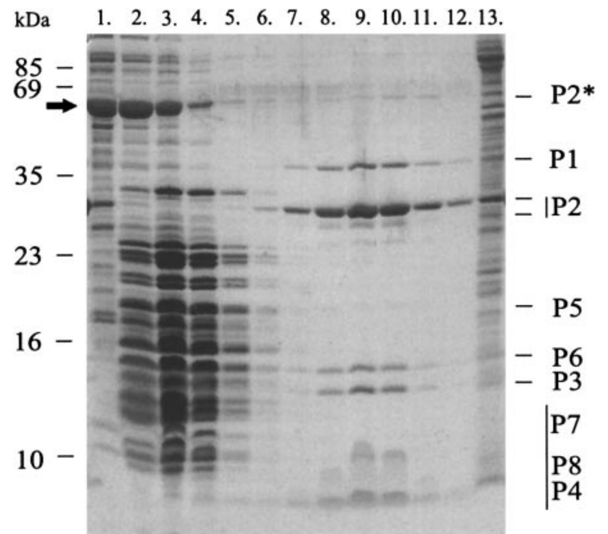


FIG. 6. Purification of PM2 by rate zonal centrifugation. Polyethylene glycol-precipitated particles were purified using a 5–20% (w/v) sucrose gradient in PM2 buffer. The fractions were collected and analyzed in a tricine–SDS–polyacrylamide gel. Fraction 13 contains the pellet. The virus zone was located in fractions 8–10. A protein complex corresponding to the hsp60-type chaperone (arrow) was seen at the top of the gradient (fractions 1–3; see also Results). Numbers on the left indicate the molecular masses of marker proteins (kDa) and on the right the structural proteins of PM2 are indicated (see Fig. 5).

recognizes a cell surface structure that is not common for all pseudoalteromonad isolates.

In our hands, the published PM2 purification methods yielded virus particles but their specific infectivity was low (~8×10^{10} PFU/mg protein and ~1×10^{11} PFU/mg protein for 1× purified viruses grown on BAL-31 and ER72M2, respectively, and ~2×10^{10} PFU/mg protein for 2× purified viruses grown on ER72M2). The method described here produces viruses with specific infectivity up to 1×10^{13} PFU/mg protein. This is very close to values obtained for the other two lipid-containing phages studied in our laboratory (PRD1, ~1×10^{13} PFU/mg protein, Bamford and Bamford, 1991; Walin *et al.,* 1994; ϕ6, ~9–15 × 10^{11} PFU/A_{260}, Vidaver *et al.,* 1973; ~1×10^{13} PFU/mg protein, Olkkonen and Bamford, 1989).

Infectivity of the virion was strongly dependent on $CaCl_2$ (Fig. 2). Snipes *et al.* (1974) have also shown that

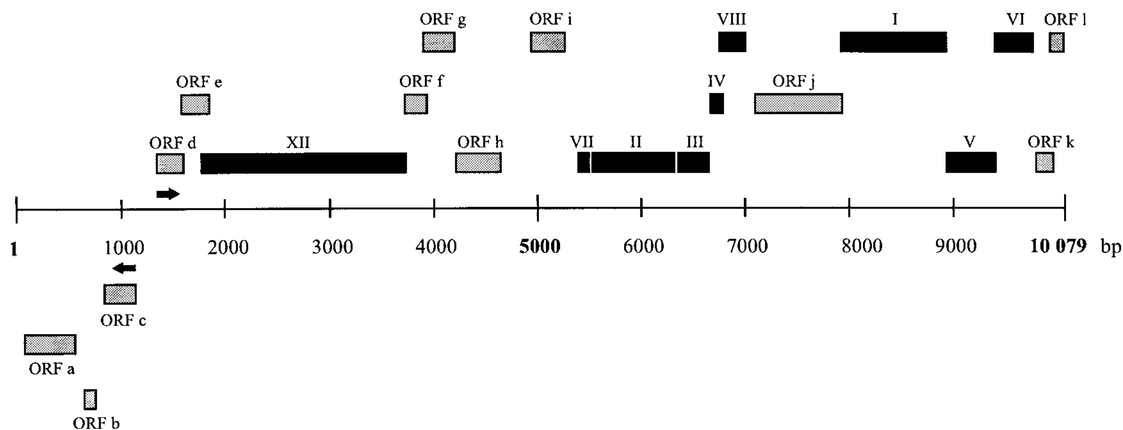


FIG.7. Organization of the circular dsDNA genome of bacteriophage PM2 linearized at the unique *Eco*RII site. The roman numerals (I to VIII and XII; black) are the identified genes (encoding structural proteins P1–P8 and replication initiation protein P12, respectively). For gene and protein nomenclature see Männistö *et al.* (1999). ORFs (gray) that are possibly coding for PM2 proteins are designated ORF a–ORF l. The ORFs in the different DNA strands are separated by the nucleotide scale bar. The genes and ORFs in different reading frames are shown. Arrows indicate the transcription direction.

calcium ion concentrations over 1.5 mM are needed to preserve PM2 infectivity. High ionic strength does not seem to be needed to stabilize the virion although it is a requirement for host viability. The virion sensitivity to sucrose or enhanced sensitivity to combined sucrose and NaCl is intriguing, as high NaCl concentrations (up to 2.5 M) alone had no effect on PM2 infectivity. This cannot be interpreted as an osmolarity effect only since the salt concentration used has considerably higher osmolarity than that of the inactivating sucrose concentration.

The improved purification methods combined with N-terminal amino acid sequence analysis revealed eight structural proteins in infectious virions. We named the proteins (and the genes) so that the earlier published proteins I–IV retained their protein numbers (P1–P4; see also Männistö *et al.,* 1999, accompanying paper). The new proteins (P5–P8 and the respective genes) were named according to their mobility in tricine–SDS–PAGE (P5 slowest and P8 fastest). P1 has been shown to form the vertex spike structure and P2 is the major coat protein. Protein P3 has been suggested to be an internal component of the particle together with protein P4, which has been proposed to interact with the DNA (Brewer, 1976; Brewer and Singer, 1974; Datta *et al.,* 1971; Hinnen *et al.,* 1974, 1976; Marcoli *et al.,* 1979; Satake *et al.,* 1980, 1981; Schäfer *et al.,* 1974a,b). In addition, an endolysin activity has been associated with P3 (Schäfer and Franklin, 1978b; Tsukagoshi *et al.,* 1977a).

We observed that the major coat protein forms both a major and a minor monomeric species, as is the case, for example, in phage PRD1 (Bamford and Bamford, 1990). In addition we detected small amounts of this protein in the apparent position of a dimer although the protein gel samples were boiled under reducing conditions in 1% SDS. This phenomenon could be related to the auto-cross-linking of the coat protein found, for example, in HK97 phage (Duda, 1998; Popa *et al.,* 1991).

All of the PM2 structural proteins except P1 and P2 (the spike and the major coat protein) showed signatures of membrane proteins (Männistö *et al.,* 1999, accompanying paper). This indicates that the PM2 structure resembles that of PRD1 where the DNA is enclosed in a membrane vesicle that is surrounded by a protein capsid (Butcher *et al.,* 1995). One major difference is the genome, which is linear in PRD1 and circular in PM2. The assembly processes also seem to differ as no mature PM2 virions are detected in the cell interior but are always lined up along the plasma membrane.

MATERIALS AND METHODS

Virus and bacterial strains

Wild-type bacteriophage PM2 was obtained from ATCC (ATCC 27025-B1). *Pseudoalteromonas espejiana* BAL-31 (ATCC 27025; previously *Alteromonas espejiana* BAL-31; Chan *et al.,* 1978; Espejo and Canelo, 1968b; Gauthier *et al.,* 1995) and *Pseudoalteromonas* sp. ER72M2 were used as host cells for PM2. ER72M2 was isolated and kindly provided by Dr. Leonard Mindich (Public Health Research Institute, New York). The other bacterial strains and their origins are listed in Table 1.

Media

SB broth (Espejo and Canelo, 1968a) contained 8 g nutrient broth (Difco), 26 g NaCl, 12 g $MgSO_4 \cdot 7H_2O$, 1.5 g $CaCl_2 \cdot 6H_2O$, and 0.7 g KCl per liter of water. SW broth (modified medium 123 of DSMZ, German Collection of Microorganisms and Cell Cultures) contained 5 g Bacto peptone (Difco), 1 g Bacto yeast extract (Difco), 26 g NaCl, 12 g $MgSO_4 \cdot 7H_2O$, 2 g $CaCl_2 \cdot 6H_2O$, 0.7 g KCl,

0.1 g KBr, 0.03 g H_3BO_3, 5 mg $Na_2Si_3O_7$, 0.04 g $SrCl_2 \cdot 6H_2O$, 3 mg NaF, 2 mg NH_4NO_3, and 1 mg $Fe_3PO_4 \cdot 4H_2O$ per liter of water. $MgSO_4$ and $CaCl_2$ were autoclaved separately and added to the sterilized and cooled medium. Plates contained 15 g and soft agar contained 4 g of agar per liter of broth.

Growth of PM2

Virus stocks were obtained by collecting the soft agar layer from semiconfluent SB plates and adding SB broth (4 ml/plate) followed by incubation for 4 h at 28°C with aeration. The debris was removed by centrifugation. Due to thermosensitivity, the soft agar was cooled to 47°C before addition of the host and virus. The virus dilutions were done in 150 mM NaCl and 5 mM $CaCl_2$. Growth of the virus in liquid SB medium (28°C) was carried out by infecting aerated cultures of ER72M2 or BAL-31 at a cell density of 6×10^8 CFU/ml with a m.o.i. of 10. For ER72M2 the lysate was treated with DNase I for 30 min at 28°C (50 μg/ml; Sigma).

Virus purification

We set out to purify the virus using published methods (Hinnen *et al.,* 1974; Salditt *et al.,* 1972; Silbert *et al.,* 1969) to produce 1× purified viruses. The following parameters differed from the optimized method (see below). The virus was concentrated from the lysate with 8% (w/v) polyethylene glycol (PEG 6000; Sorvall GS3 rotor, 8000 rpm, 20 min, 5°C) and resuspended in buffer B (20 mM Tris–HCl, pH 7.2, 1 M NaCl, and 10 mM $CaCl_2$; Hinnen *et al.,* 1974). Purification of the phage preparation was done in a linear 5–20% (w/v) sucrose gradient in buffer B (Sorvall AH629 rotor, 24 000 rpm, 1 h 35 min, 15°C). To produce 2× purified viruses according to Hinnen *et al.* (1974) we equilibrated the particles in a linear 20–70% (w/v) sucrose gradient (PM2-buffer; Sorvall AH629 rotor, 24 000 rpm, 16 h, 15°C).

The optimized purification and storage method

After the removal of bacterial debris from the lysate (Sorvall GS3 rotor, 8000 rpm, 20 min, 5°C), solid PEG 6000 was added and dissolved for 20 min at 4°C by magnetic stirring (note that SB contains nearly 0.5 M NaCl). Virus precipitate was collected (Sorvall GS3 rotor, 8000 rpm, 40 min, 5°C) and resuspended on ice in PM2 buffer (20 mM Tris–HCl, pH 7.2, 100 mM NaCl, and 5 mM $CaCl_2$; 12 ml/liter of lysate). Aggregates were separated from the virus concentrate (Sorvall SS34 rotor, 7000 rpm, 10 min, 5°C) before layering on top of a linear 5–20% (w/v) sucrose gradient (PM2 buffer). Purification by rate zonal centrifugation was carried out (Sorvall AH629 rotor, 24 000 rpm, 1 h 10 min, 15°C) and the light-scattering virus zones (1× purified) were collected and concentrated by differential centrifugation (Sorvall T647.5 rotor, 32 000 rpm, 3 h 30 min, 5°C). The viruses were resuspended on ice in PM2 buffer (700 μl/liter of lysate). The 1× virus was purified further with isopycnic centrifugation using a preformed 5–40% (w/v) iodixanol (Opti-Prep, Nycomed Pharma) gradient in PM2 buffer (Sorvall TH660 rotor, 45 000 rpm, 16 h, 10°C). The 2× purified virus zones were collected as above and resuspended in PM2 buffer (500 μl/liter of lysate).

The stability of the purified virus was tested under different mono- and divalent ion conditions by incubating the virus preparation at 4°C for the desired period followed by the determination of the number of infectious particles. The inactivation ability of gradient materials was analyzed by incubating the virus under different conditions at 4°C overnight prior to determination of infectivity. We also determined the conditions for virus storage at −80°C and in liquid nitrogen, analyzing the loss of the infectivity after one cycle of freezing and thawing. Virus preparations in PM2 buffer were supplemented with 20% (v/v) glycerol, 1 mg/ml bovine serum albumin (BSA), or 10% (v/v) DMSO. The best results were obtained with 20% glycerol in liquid nitrogen (30–40% infectivity remaining after overnight storage and some 25% after 2 months). With the other supplements one to two orders of magnitude of inactivation occurred under similar conditions (overnight storage).

Production of polyclonal anti-PM2 serum

Polyclonal antibodies against bacteriophage PM2 were raised by immunizing a rabbit four times subcutaneously at 21-day intervals. Before the immunization a preimmune blood sample was taken. For the primary immunization, the 2× purified virus (250 μg, specific infectivity 9×10^{12} PFU/mg protein) emulsified with complete Freund's adjuvant was used. Incomplete adjuvant and 320 μg of 2× virus were used in subsequent boosters. Two weeks after the last immunization, serum was collected. Specificities of the preimmune serum and the anti-PM2 serum were determined by Western blotting (1:30,000–1:50,000 dilutions) using anti-rabbit IgGs (DAKO) as secondary antibodies and ECL detection reagents (Amersham). To test neutralization, approximately 200 plaque-forming units of 1× purified PM2 in PM2 buffer were treated with different dilutions of the antiserum (1:5–1:10,000) for 2 h at 22°C and plated on *Pseudoalteromonas* sp. ER72M2 cells. Virus aggregation was tested by incubating 250 μg (2×10^{12} PFU) of 1× PM2 with different antibody dilutions (1:10–1:10,000) in PM2 buffer for 1.5 h at 22°C. Aggregation was analyzed by rate zonal centrifugation using a linear 5–20% (w/v) sucrose gradient in PM2 buffer (Sorvall TH641 rotor, 24 000 rpm, 1 h 5 min, 15°C) followed by determination of the viral material in the gradient fractions and in the pellet.

Determination of the 16S rRNA gene sequence of ER72M2

The small-subunit rRNA gene and the following ITS (16S–23S rRNA intergenic spacer) region were sequenced. Bacteria were resuspended in 30 μl of sterilized water and heated at 92°C for 10 min. Three microliters of suspension was used in PCR to amplify the region. The amplification was done using specific primers (25 pmol) according to Edwards *et al.* (1989), Dynazyme (1 U/50 μl of reaction; Finnzymes), and 1.25 mM dNTP mixture. After amplification, PCR products were purified (MicroSpin S-400 HR columns, Pharmacia Biotech) and sequenced using the specific primers (Edwards *et al.,* 1989). The 16S rRNA gene sequence of ER72M2 was compared against GenBank using BLASTN 2.0.3 software (Altschul *et al.,* 1997). Sequence assembly and phylogenetic analysis were done with XGAP software of the Staden Package (Bonfield *et al.,* 1995).

Analytical methods

Protein concentration was measured by the Coomassie blue method of Bradford (1976) using BSA as a standard. Specific infectivity of the virus sample was determined by measuring the number of plaque-forming units in proportion to 1 mg protein. The proteins were separated using modified tricine–SDS–PAGE (devoid of the spacer gel) according to Schägger and von Jagow (1987) having a stacking gel and 14% (w/v) acrylamide concentration in the separation gel. The N-terminal amino acid sequencing was performed by transferring the proteins from the tricine–SDS–polyacrylamide gel to a Millipore PVDF membrane that was stained with Coomassie blue prior to the protein bands being cut for Edman degradation. The Edman degradation was carried out using a Procise 494A protein sequencer (Perkin–Elmer/Applied Biosystems, Foster City, CA). N-terminal amino acid sequences were compared with the amino acid sequences derived from the virus genome sequence (Männistö *et al.,* 1999) and with the SwissProt database using BLASTP 2.0.3. software (Altschul *et al.,* 1997). Densities of the particles were determined in a linear 20–70% (w/v) sucrose gradient (PM2 buffer, Sorvall AH629 rotor, 24 000 rpm, 16 h, 15°C), in a CsCl gradient (20 mM Tris–HCl, pH 7.2, and 5 mM $CaCl_2$) with an average density of 1.3 g/cm^3 (Sorvall TH641 rotor, 30 000 rpm, 19 h, 10°C) and in a preformed 5–40% (w/v) iodixanol gradient (Sorvall TH660 rotor, 45 000 rpm, 16 h, 10°C). Thin-section electron microscopy was carried out as previously described (Bamford and Mindich, 1980) except that the cells were resuspended in 10 mM potassium phosphate, pH 6.5, 0.5 M NaCl, 10 mM $CaCl_2$, 50 mM $MgSO_4$, and 10 mM KCl. Negative staining of the viruses in low salt (20 mM Tris–HCl, pH 7.2, 5 mM $CaCl_2$, and 10 mM NaCl) was performed using 1% potassium phosphotungstate, pH 6.5. The micrographs were taken with a JEOL 1200EX electron microscope operating at 60 kV.

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