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# The Complete Genome Sequence of PM2, the First Lipid-Containing Bacterial Virus To Be Isolated[1]

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Bacteriophage PM2 was isolated from the Pacific Ocean off the coast of Chile in the late 1960s. It was a new virus type, later classified as *Corticoviridae,* and also the first bacterial virus for which it was demonstrated that lipids are part of the virion structure. Here we report the determination and analysis of the 10,079-bp circular dsDNA genome sequence. Noteworthy discoveries are the replication initiation system, which is related to the rolling circle mechanism described for phages such as $\phi$X174 and P2, and a 1.2-kb sequence that is similar to the maintenance region of a plasmid found in a marine *Pseudoalteromonas* sp. strain A28. © 1999 Academic Press

*Key Words:* PM2; nucleotide sequence; structural proteins; replication initiation protein; plasmid maintenance region.

## INTRODUCTION

Phage PM2 was isolated from the Pacific Ocean off the coast of Vina del Mar, Chile (Espejo and Canelo, 1968a). It represented a new virus type later classified as *Corticoviridae.* PM2 was also the first phage for which the presence of lipids as part of the virion structure was rigorously demonstrated (Camerini-Otero and Franklin, 1972). The icosahedral virion has a diameter of about 60 nm (Franklin, 1974; Harrison *et al.,* 1971; Silbert *et al.,* 1969). A circular dsDNA genome is surrounded by a lipid bilayer that is enclosed inside a protein shell (Harrison *et al.,* 1971). The original host was a gram-negative marine bacterium, *Pseudoalteromonas espejiana* BAL-31 (Espejo and Canelo, 1968b; Chan *et al.,* 1978; Gauthier *et al.,* 1995). Recently an alternative host, *Pseudoalteromonas* sp. ER72M2, was described (see Kivelä *et al.,* 1999). PM2 was the subject of intensive study from the time of its discovery until the mid 1980s (some 400 references) and several literature reviews have also been published (Franklin, 1974; Franklin *et al.,* 1976, 1977; Brewer, 1980). However, studies on the virion structure and reconstitution have yielded somewhat conflicting results (Kivelä *et al.,* 1999; Satake *et al.,* 1980; Schäfer and Franklin, 1975, 1978a; Schäfer *et al.,* 1974).

The PM2 genome is a highly supercoiled circular dsDNA molecule (Espejo *et al.,* 1969). The length has been determined, using restriction endonuclease fragments, to be about 10 kb (Streeck and Gebhardt, 1979) and about 25% of the PM2 genome sequence has been published (Miller *et al.,* 1984; Sheflin and Kowalski, 1984). The GC content has been estimated to be 42–43% (Espejo *et al.,* 1969). The genome also contains regions that adopt the Z-conformation under physiological conditions (Miller *et al.,* 1983; Stockton *et al.,* 1983).

There have been only a few reports concerning the replication of PM2 DNA. These studies suggested that the replication takes place at a point of contact with the host plasma membrane (Brewer, 1978). Electron microscopy revealed replication intermediates consisting of double-stranded circular molecules with growing tails no longer than the length of the genome (Espejo *et al.,* 1971). Based on this result, the replication has been proposed to utilize a rolling circle mechanism (Canelo *et al.,* 1985). Rolling circle replication begins by the binding of the viral initiation protein to a site within its own coding sequence followed by the introduction of a nick. The newly created 3′ end serves as a primer for new leading strand DNA synthesis (Espinosa *et al.,* 1995; Kornberg and Baker, 1992a; Novick, 1998).

PM2 transcription is carried out by the host RNA polymerase holoenzyme (Zimmer and Millette, 1975a). Active transcription initiation requires the presence of a supercoiled DNA template (Richardson, 1974; Zimmer and Millette, 1975b) and to our knowledge PM2 DNA has the highest number of negative supercoils (51) detected in a naturally occurring molecule (Gray *et al.,* 1971). Promoter mapping has been carried out by binding *Escherichia coli* and *P. espejiana* RNA polymerase (RNAP) molecules to PM2 DNA and assaying for inhibition of restriction endonuclease cleavage (Streeck and Gebhardt, 1979; Bull *et al.,* 1988). Two and nine protected sites were

[1] Nucleotide sequence data for PM2 have been deposited with the GenBank Data Library under Accession No. AF155037.

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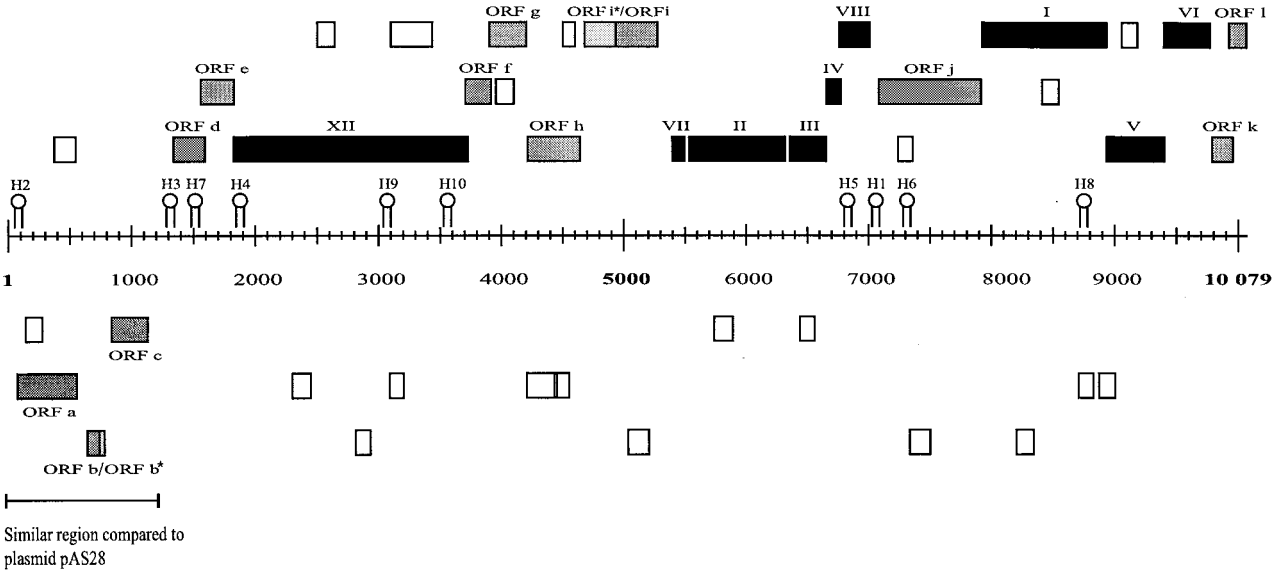



FIG. 1. A schematic presentation of the PM2 genome linearized from the unique *Eco*RII site. Open reading frames encoding polypeptides longer than 25 aa are shown. The ORFs beginning with GTG are marked with an asterisk. Identified genes are colored black and other potential protein-coding ORFs are colored gray. Hairpin structures (H1–H10) with $-\Delta G$ greater than 10 kcal/mol are also indicated. The region that is similar to the *Pseudoalteromonas* sp. strain A28 plasmid is indicated by a bar. The ORFs above the nucleotide scale bar are transcribed from left to right and those below are transcribed in the opposite direction. The three positions of the ORFs represent the three reading frames.

detected, respectively. The two *E. coli* RNAP binding sites overlapped with those detected with the *P. espejiana* RNAP. Additionally, the location of the late genes has been roughly mapped (Grzesiuk, 1983).

In earlier studies, the consensus for the number of structural proteins was 4 (Datta *et al.,* 1971b). This would provide a considerable genome capacity for the early functions. However, in the accompanying paper we report the presence of 8 structural proteins in the virion (Kivelä *et al.,* 1999). Studies of virus-induced protein production in infected cells revealed 13 membrane-associated protein products and 1 cytoplasmic protein product (Datta *et al.,* 1971a). Translation experiments of PM2 DNA *in vitro* revealed 12 gene products (Schäfer and Franklin, 1978b). Protein expression could be divided into early, starting some 10 min postinfection, and late, starting about 25 min postinfection (Brewer and Singh, 1982).

PM2 is the only characterized member of the *Corticoviridae* family (type organism) as well as the first lipid-containing phage to be isolated. As our laboratory has a history of studying lipid-containing bacterial viruses, we revisited this virus system. We have sequenced and analyzed the entire PM2 genome. Additionally, we have developed methods to isolate purified and infective virions and obtained information on the virion composition (Kivelä *et al.,* 1999, accompanying article).

## RESULTS AND DISCUSSION

### Sequencing and analysis of the genome

Each nucleotide in the circular dsDNA genome of PM2 was determined at least once from both strands by the automated primer walking sequencing method (see Materials and Methods). The genome was 10,079 bp long with a GC content of 42.2%. These results are in agreement with the previous estimates of the length (10,090 ± 60 bp; Streeck and Gebhardt, 1979) and the GC content (42–43%; Espejo *et al.,* 1969).

The nucleotides were numbered starting from the unique *Eco*RII (′CCTGG) restriction site and proceeding in the direction in which most of the open reading frames (ORFs) are transcribed. Two previously sequenced regions, encompassing nucleotides 499 to 971 and 973 to 1353 (M32693, M32694; Sheflin and Kowalski, 1984), and current data differed at 10 positions, namely one mismatch and nine gaps of 1 or 2 nucleotides. The other published sequence (M26134; Miller *et al.,* 1984) matched the region 6485–8270 in the current data. This sequence differed more dramatically from ours, there being several longer gaps and insertions as well as an unclear region where many of the adenine residues have previously been determined as guanines.

The PM2 sequence was analyzed for ORFs by considering ATG and GTG as possible initiation codons. Forty-two ORFs, possibly coding for peptides longer than 25 amino acids, were found (Fig. 1). Twenty-one of these ORFs exhibited a putative ribosome binding site sequence in front of them. Sequences of these ribosome-binding sites were compared to the complementary sequence of the 3′ end of the ribosomal 16S RNA of the host *Pseudoalteromonas* sp. ER72M2 (TAAGGAGTGATC; Kivelä *et al.,* 1999). This sequence was found to be identical to that of *E. coli.* For the proper binding of the ribosome the average complementarity should be at

TABLE 1

Genes and Potentially Protein Coding Open Reading Frames (ORFs) of Bacteriophage PM2

| Strand and ORF/gene[a] | Ribosome binding site sequence[b] *Pseudoalteromonas* sp. ER72M2 *TAAGGAGGTGATC* | Nucleotide coordinates[c] | Protein size (aa) | Molecular mass (kDa) | Specific features[d] |
|---|---|---|---|---|---|
| Lower | | | | | |
| ORF a | A**AAGGA**TTAAT*ATG* | 550–77 | 157 | 18.1 | Amino acid similarity to plasmid pAS28, leucine-zipper pattern |
| ORF b | **TT**AC**GA**TT**T**ATATT*ATG* | 755–663 | 30 | 3.5 | Amino acid similarity to plasmid pAS28 |
| ORF b* | GT**AGG**CT**GT**TGA**C***GTG* | 773–663 | 36 | 4.2 | Amino acid similarity to plasmid pAS28 |
| ORF c | **TAA**C**GAG**TAC**A**AAACTT*ATG* | 1128–850 | 92 | 10.3 | Amino acid similarity to plasmid pAS28 |
| Upper | | | | | |
| ORF d | A**AAGG**T**G**AAA**A**AAT*ATG* | 1359–1583 | 74 | 8.5 | |
| ORF e | **TA**GAAT**GGTGA**CGTT*ATG* | 1580–1822 | 80 | 8.9 | |
| Gene XII | GTGT**GAG**C**TTT**TGCTTG*ATG* | 1815–3719 | 634 | 72.1 | Replication initiation protein |
| ORF f | GAGA**GAG**CAT**A**GGGTT*ATG* | 3716–3910 | 64 | 7.2 | |
| ORF g | **T**GC**GGAGTTG**GTACAAA*ATG* | 3907–4212 | 101 | 11.0 | |
| ORF h | AGT**GGA**T**G**ATGAATA*ATG* | 4212–4643 | 143 | 15.7 | Transmembrane domain |
| ORF i | C**A**C**G**CT**GG**C**G**CG**C**CA*ATG* | 4936–5271 | 111 | 12.7 | |
| ORF i* | ACCTA**AT****GT**CCATCATTTA*GTG* | 4681–5271 | 196 | 22.1 | ATP/GTP-binding site motif A |
| Gene VII | AT**AGG**GCT**TT**A**G**C*CATG* | 5406–5510 | 34 | 3.6 | Structural protein, transmembrane domain |
| Gene II | CT**AA****GAGG**G**GTT**TTAA*ATG* | 5523–6332 | 269 | 30.2 | Major capsid protein |
| Gene III | **TAAGG**G**GGTG**CT*ATG* | 6345–6659 | 104 | 10.8 | Structural protein, transmembrane domain |
| Gene IV | ATGC**GAGGT**AACAAATA*ATG* | 6659–6781 | 40 | 4.4 | Structural protein, transmembrane domain |
| Gene VIII | A**AAGG**CTAAA**A**G**C**TA*ATG* | 6781–7008 | 75 | 7.3 | Structural protein, transmembrane domain |
| ORF j | **TT**AT**G**G**GGTG**AAAT*ATG* | 7079–7918 | 279 | 29.0 | Transmembrane domain |
| Gene I | A**AAGG**G**GTTAA**CTA*ATG* | 7918–8925 | 335 | 37.5 | Spike protein |
| Gene V | A**ATGGAG**CGA**A**CATA*ATG* | 8925–9407 | 160 | 17.9 | Structural protein, transmembrane domain |
| Gene VI | A**ATGG**G**GGTT**TT**T***ATG* | 9400–9783 | 126 | 14.3 | Structural protein, transmembrane domain |
| ORF k | A**A**GC**GG****GGT**CGAGCT*ATG* | 9780–9941 | 53 | 6.0 | Transmembrane domain |
| ORF l | CT**AGG**G**G**AAA**A**G**C***ATG* | 9922–10077 | 51 | 5.7 | |

[a] The upper strand is transcribed from left to right in the PM2 genome linearized from the unique *Eco*RII site (see Fig. 1). The lower strand is transcribed to the opposite direction. Open reading frames starting with the GTG codon are marked with an asterisk.

[b] The DNA sequence preceeding the 5′ ends of the genes and ORFs. Complementary nucleotides to the 3′ end of *Escherichia coli* and *Pseudoalteromonas* sp. ER72M2 16S rRNA are shown in bold and the initiation codon is shown in italics.

[c] Nucleotide numbering starts from the unique *Eco*RII site and the coordinates indicate the first and last nucleotides of the gene/ORF including the termination codon.

[d] Protein functions, homologous regions found from GenBank, putative transmembrane domains, leucine zipper pattern, and ATP/GTP binding site motif suggested by Prosite program are indicated.

least 3 but preferably 5 bases. Additionally the average spacing between the complementary nucleotides and the initiation codon should be 7 nucleotides (Stormo *et al.,* 1982). We used the following criteria: the exact match contained at least 3 bases and the distance of these bases from the initiation codon was no more than 10 nucleotides. The PM2 ORFs with putative ribosomal binding sequences are listed in Table 1. All three stop codons, TAA, TGA, and TAG, are used in the PM2 sequence, with TAA being the most frequent.

Potential transcription termination hairpin loops with high free energies ($-\Delta G > 10$ kcal/mol) were localized by computer analysis (Pc/Gene Release 6.70; Hairpin loops prediction program version 2.40). Ten hairpin structures with $-\Delta G$ values of 25.6, 19, 17.2, 16.6, 13.8, 12.8, 12.4, 10.6, 10.4, and 10.4 kcal/mol that were centered at nucleotide positions 7049, 59, 1290, 1866, 6822, 7282, 1507, 8762, 3023, and 3551, respectively, were detected (H1–H10, Fig. 1).

## Identification of genes and ORFs

The previous literature has described only four structural proteins in the PM2 particle (Datta *et al.,* 1971b) although there have also been observations of several other structural protein species (Brewer and Singer, 1974). Using highly purified virus material, we determined the N-terminal amino acid sequences of the structural

proteins separated by SDS–PAGE (Kivelä *et al.,* 1999, accompanying paper) and compared them with the PM2 genomic sequence. We found 10 different peptide sequences, 8 of which matched the N-termini of potential gene products. These proteins were named P1–P8 and the corresponding genes were designated with Roman numerals I–VIII, respectively (Fig. 1). The previously identified proteins I–IV (Brewer and Singer, 1974; Datta *et al.,* 1971b; Marcoli *et al.,* 1979; Satake *et al.,* 1981; Schäfer *et al.,* 1974) were given corresponding designations P1–P4. Correlation with the previous data was done by comparing the protein sizes and amino acid compositions, and in the case of proteins II and IV partial amino acid sequences were also available (Brewer and Singer, 1974; Hinnen *et al.,* 1976). The rest of the structural proteins were numbered P5–P8 (and their genes V–VIII) according to their mobility in SDS–PAGE (Kivelä *et al.,* 1999).

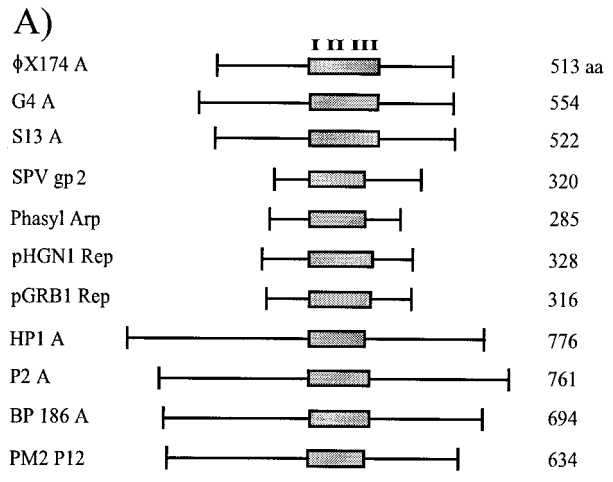
To further identify potential genes coding for proteins that are not part of the virion structure, we searched the amino acid sequences derived from the ORFs against the GenBank library. Interestingly, the longest ORF, coding for a 72.1-kDa polypeptide, exhibited significant sequence similarity to the replication initiation A proteins of temperate dsDNA phages P2 (Q06419), 186 (P41064), and HP1 (P51711) and the retron phage EC67 (P21312/P21313) (Liu *et al.,* 1993; Sivaprasad *et al.,* 1990; Esposito *et al.,* 1996; Hsu *et al.,* 1990) (Fig. 2). This ORF was designated gene XII and the corresponding protein P12.

The rest of the ORFs that have not yet been confirmed to code for a protein are named with lowercase letters in alphabetical order from left to right in the linearized genome sequence (Fig. 1).

## Structural proteins

The molecular masses of the eight identified structural proteins range from 3.6 to 37.5 kDa as described in Table 1. Computer analysis predicts hydrophobic transmembrane helices for six of these proteins. This suggests that most of the PM2 structural proteins are associated with the viral membrane. The same phenomenon can also be observed in another lipid-containing bacteriophage, PRD1, which has a number of structural proteins associated with the internal membrane (Bamford *et al.,* 1995). The only structural proteins in PM2 that do not contain transmembrane domains are proteins P1 and P2. Protein P1 has been identified as a spike protein located on the vertices of the icosahedral virus particle and protein P2 is the major capsid protein. Protein P3 has been suggested to be connected to the lipid bilayer and P4 both to the lipid bilayer and to the DNA (Hinnen *et al.,* 1974; Schäfer *et al.,* 1974).

The particle integrity is strongly dependent on calcium ions (Kivelä *et al.,* 1999; Snipes *et al.,* 1974). We searched for putative calcium binding sites in the amino acid sequences with the Prosite consensus motif program.




B)

| Replication initiation protein | Motif I futlt | Motif II gxuHUHuxuu | Motif III ugxYuakYuxk ugxYugrYuxk | Acc. number |
|---|---|---|---|---|
| ϕX174 A | FdTLT | GRLHFHaVhF | VGFYVAKYVNK | P03631 |
| G4 A | FdTLT | GRLHFHaVhF | VGFYVAKYVNK | P25243 |
| S13 A | FdTLT | GRLHFHaVhF | VGFYVAKYVNK | P07928 |
| SPV4 gp2 | FVTLT | mRpHYHICFF | -aNYtARYtTK | P11334 |
| Phasyl Arp | FLTLT | GRIHYHLLVa | IGRYVGKYISK | P19071 |
| pHGN1 Rep | mVTLT | GYaHIHLGVF | LGAYLAaYMAg | S06780 |
| pGRB1 Rep | mVTLT | GYVHIHLGVF | LGAYLAaYMAg | S10152 |
| HP1 A | FYTLT | GTpHWHaLaY | ta-YIAKYIAK | P51711 |
| P2 A | FITLT | GTpHWHMMLF | -G-YIAKYISK | Q06419 |
| 186 A | FYTLT | GTpHWHMLMF | tG-YVAKYISK | P41064 |
| PM2 P12 | FYTiT | aTpHWHMLLF | VG-YIAKYISK | AADA43543 |

FIG. 2. (A) Comparison of PM2 protein P12 to some replication initiation proteins belonging to superfamily I. The region containing the three consensus motifs (I–III) is shown in gray. (B) Alignment of the three conserved sequence motifs (I–III) among superfamily I replication initiation proteins (Ilyina and Koonin, 1992; Koonin and Ilyina, 1993). The capital letters indicate the exact matches and the lowercase letters indicate differing amino acids from the consensus sequences. SwissProt or PIR accession numbers are indicated for each amino acid sequence.

Putative binding sites were found in the gene products of ORF h, ORF j, and gene VI.

## Origin of replication

We identified a gene coding for the possible replication initiation protein (P12) of PM2 on the basis of the similarity to the other bacteriophage A proteins and related cyanobacterial and archaebacterial plasmid Rep proteins (Fig. 2, see above). Replication initiation proteins of coliphage P2 and its lambdoid relatives 186 and HP1 (Bertani and Six, 1988) as well as the A protein of the isometric ssDNA bacteriophage $\phi$X174 belong to the group I superfamily of replication initiation proteins. The $\phi$X174 replication mechanism is well characterized and serves as a prototype for the entire superfamily I (Baas and Jansz, 1988; Hayashi *et al.,* 1988; Kornberg and Baker, 1992b). Three consensus motifs, I–III (Fig. 2), are

| Replication initiation protein | Recognition sequence Spacer Key binding region | Acc.nr. |
|---|---|---|
| ϕX174 A | CAACTTG*ATATTAATAACACTATAGACCAC[1] | V01128 |
| G4 A | CAACTTG*ATATTAATAACACTATAGACCAC[1] | V00657 |
| Phasyl Arp | TAACTGG*ACAACTCGATGTTACTCAATAAA[2] | X56069 |
| pHGN1Rep | TACGCGG*ACCAGCGAGACACGCAGATGGTC[2] | X52610 |
| pGRB1 Rep | TACGACG*ACCAGCGTGGAACGCAGATCGTC[2] | X81382 |
| pEE Rep | TGCTGCG*ATCGCACTGGTTGGAACCAATAA[2] | X16460 |
| P2 A | CGCCTCG*GAGTCCTGTCAATAACTGTGGAA[1] | Z11483 |
| 186 A | CCTCTCG*GAGTTCTGTCAATAACTGTACGG[2] | X53318 |
| PM2 P12 | AAGCTTG*CAGAGTTCACAATAAATCAAATA[3] | AF155037 |

FIG. 3. Alignment of nucleotide sequences of ORI regions of a number of phages or plasmids that have a superfamily I Rep protein and replicate via a rolling circle mechanism. The cleavage site of the replication initiation protein is indicated by an asterisk. The three functional domains of the ORI region of ϕX174 are indicated. Conserved bases found either near the cleavage site or in the binding region are shown in boldface type. The GenBank accession numbers for each sequence are also presented. [1] Experimentally determined ORI regions (Liu and Haggård-Ljungquist, 1996; van Mansfeld *et al.,* 1980). [2]Putative ORI regions determined previously (Koonin and Ilyina, 1993; Liu and Haggård-Ljungquist, 1994). [3] Putative PM2 ORI region indicated in this study.

common to these proteins from diverse subgroups but only the superfamily I members contain two active tyrosine residues within motif III, whereas the others contain only one active tyrosine residue. In the case of superfamily I, one of these tyrosines is involved in covalent linkage to the nicked DNA. Either of these tyrosine residues can be utilized in the cleavage–ligation reaction of ϕX174 A protein (Hanai and Wang, 1993; Roth *et al.,* 1984). In phage P2, the other tyrosine is dispensable in the cleavage reaction but both are essential for the proper function of the A protein (Liu and Haggård-Ljungquist, 1996). Two tyrosines are also found in PM2 protein P12. The finding that the replication of the circular PM2 genome most probably occurs via a rolling circle mechanism makes PM2 the first example of a lipid-containing bacteriophage using this replication strategy.

Replication is initiated when the initiation protein nicks the DNA at the ORI site. The protein stays covalently bound to the 5′end and the 3′end is extended by DNA polymerase. It has been possible to determine the replication origin regions for superfamily I members on the basis of conserved sequences in ssDNA phages ϕX174 and G4, temperate dsDNA coliphages P2 and 186, and plasmids pHGN1 and pGRB1 (Koonin and Ilyina, 1993; Liu and Haggård-Ljungquist, 1994). Based on this we predicted a putative replication origin inside gene XII in PM2. This sequence (nucleotide coordinates 2247–2276 in the genome) most closely resembles that of P2 and 186 (Fig. 3). In ϕX174 and its relative G4, the 30-nucleotide sequence surrounding the origin of replication is required for DNA synthesis and has three functional domains: the recognition sequence, the AT-rich spacer, and the key binding sequence (Fluit *et al.,* 1984; van Mansfeld *et al.,* 1980). In the region corresponding to the ϕX174 key binding domain there is a conserved CAATAA motif in P2, 186, Phasyl, and pEE as well as in the PM2 sequence (Fig. 3). In P2, 186, and PM2 these motifs are in identical positions. However, in PM2 an additional closely related CAAATA sequence is found at the same position as CAATAA in the Phasyl and the plasmid pEE (Fig. 3). The cleavage site in P2 and 186 differs from that of ϕX174, although there is one conserved G residue as well as a CT motif on the 5′ side of the cleavage site (Liu and Haggård-Ljungquist, 1994). In the PM2 genome this region is more similar to that of ϕX174 than to the dsDNA phages. After the cleavage site within the region corresponding to the ϕX174 spacer region a common GAGT(T/C)C motif can be found in P2, 186, and PM2 sequences (Fig. 3).

## PM2 contains a 1.2-kb region homologous to plasmid pAS28 of Pseudoalteromonas sp. strain A28

When the nucleotide sequence of PM2 was compared to the GenBank sequences an additional major similarity was found. The left terminal 1.2 kb of the *Eco*RII-linearized PM2 sequence was about 66% similar to the maintenance region of plasmid pAS28, which is harbored by the algae-lysing *Pseudoalteromonas* sp. strain A28 (AB009311, Kato *et al.,* 1998). Within this region in the PM2 genome there are three potential protein-coding open reading frames: ORFs a, b, and c. Two of the ORFs, ORF a (157 aa) and ORF c (92 aa), are conserved at the amino acid level (over 70%) when compared to the corresponding ORFs of plasmid pAS28. The open reading frame corresponding to ORF b of PM2 is longer in pAS28. However, the organization of the open reading frames in plasmid pAS28 and in PM2 is strikingly similar. They are transcriptionally coupled and in both cases the orientation of the operon is opposite to that of the other operons in the genome (see Fig. 1 for PM2).

It is intriguing that the replication of the plasmid pAS28 occurs presumably via a rolling circle mechanism (Kato *et al.,* 1998) as we proposed for PM2. The plasmid possesses a large open reading frame coding for an 81-kDa protein that most probably is the replication initiation protein, since deletions of the gene prevent plasmid replication (Kato *et al.,* 1998). However, the protein does not share any sequence similarity with the PM2 replication initiation protein P12.

## Operon organization

We have analyzed the genomic sequence of PM2 and found 21 putative genes. The direction of transcription is presented in the genome map (Fig. 4). The eight identified structural genes (P1–P8) are clustered in the part of the genome that is obviously expressed late in the virus infection. On the other hand, the putative replication

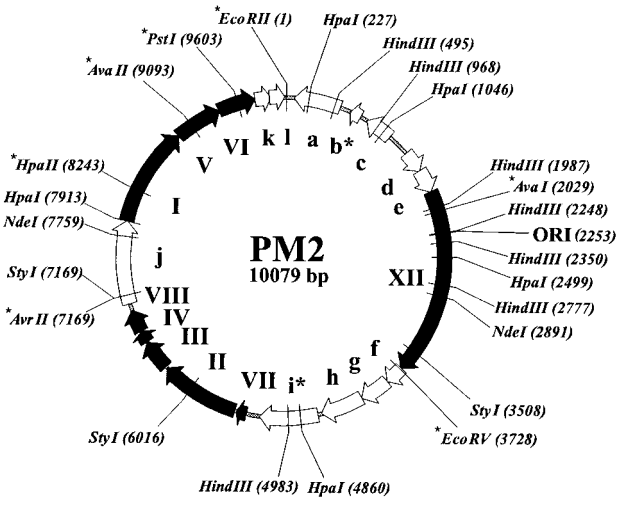



FIG. 4. Circular map of the PM2 genome. A number of restriction enzyme cleavage sites are shown. Unique cutting sites are denoted with an asterisk. *Hin*dIII, *Hpa*I, *Sty*I, and *Nde*I have seven, five, three and two sites, respectively. The position and the direction of transcription of the genes (black arrows) and open reading frames (white arrows) are also indicated. ORI indicates the replication initiation cleavage site (see Fig. 3). The numbers refer to the genome sequence numbering (see Fig. 1).

initiation protein, P12, must be expressed early in infection. Gene XII is likely to be transcriptionally coupled to the preceding ORFs, d and e. We also suggest that open reading frames a, b, and c code for early proteins, referring to their significant similarity to a region of plasmid pAS28 that is essential for plasmid maintenance. Since ORFs a, b, and c are transcribed in the opposite direction to the other early genes, at least two different promoters are needed for early gene expression. We suggest that the two promoters lie within a 200-bp region between ORFs c and d. Analysis revealed several promoter consensus sequences in this region (−35 region TTGACA and −10 region TATAAT; Busby and Ebright, 1994). Earlier studies have shown that there are two *E. coli* (Streeck and Gebhardt, 1979) and five *P. espejiana* BAL-31 (Bull *et al.,* 1988) RNA polymerase binding sites within this region. The putative transcription termination hairpin structure (see above) that has the second highest free energy is located downstream of ORF a, potentially enhancing the expression of the upstream early operon. The remainder of the genes could be structural components of the phage or nonstructural proteins involved in morphogenesis or host cell lysis. They are most probably organized into two operons, the first containing ORFs f–i and the second the remainder of the genes (Fig. 4). This suggestion is in agreement with the previous observation that the late PM2 transcripts originate from *Hin*dIII fragments 1 and 2 (nucleotide coordinates 4982–494 and 2776–4982, respectively, on the circular sequence map; Grzesiuk, 1983). This result was obtained by hybridizing the viral mRNA, which was pulse-labeled at 42 to 44 min postinfection, with the cloned genomic fragments. Also, the short *Hin*dIII fragment 4 (494–967) was concluded to express late proteins, but this is not obvious from our data. However, the exact location of the promoters awaits further experiments. There is also a possibility that the last two ORFs (ORF k and l) are early genes, although this seems unlikely. The expression of the capsid protein P2 and the structural proteins P7, P3, and P4 seems to be enhanced by three transcriptional termination signals H5, H1, and H6 (Fig.1). In another lipid-containing bacteriophage, PRD1, expression of the abundant capsid protein is also regulated by efficient transcriptional termination with a strong hairpin loop ($-\Delta G$ of 25 kcal/mol) (Bamford *et al.,* 1991; Grahn *et al.,* 1994), which is comparable to that of H1 (25.6 kcal/mol).

## CONCLUSIONS

The PM2 genome is tightly packed with information as is customary for viral genomes. The few exceptions are the short regions separating the inverted sequence related to the plasmid pAS28. The sequence information gives strong evidence for a rolling circle replication mechanism and defines the replication initiation site. The discovery of a plasmid maintenance region within the PM2 genome is intriguing. Could PM2 DNA be maintained as a plasmid? Sequence information of the structural proteins parallels that of another lipid-containing phage, PRD1. In both viruses, most of the structural proteins have signatures of membrane proteins. It is very likely that the overall structure of PM2 resembles that of PRD1, where the lipid bilayer is surrounded by only one protein shell.

## MATERIALS AND METHODS

### Strains

The host bacterium *Pseudoalteromonas* sp. ER72M2 was isolated and kindly provided by Dr. Leonard Mindich (Public Health Research Institute, New York; see also Kivelä *et al.,* 1999). Bacteriophage PM2 was obtained from ATCC (ATCC 27025-B1).

### Isolation of bacteriophage PM2 DNA

Bacteriophage PM2 was purified as described in the accompanying paper (Kivelä *et al.,* 1999). DNA was isolated from the purified virus (protein concentration 0.5 mg/ml, Bradford assay using BSA as standard; Bradford, 1976) by adding SDS to a final concentration of 2%. The mixture was incubated for 45 min at 37°C and the DNA was extracted twice with phenol and three times with ether. DNA was precipitated by adding 1/10 of the volume of 3 M NaCl and 3 vol of 94% EtOH. Precipitated DNA was collected by centrifugation and the pellet was washed with 75% EtOH, dried under vacuum, and resuspended in 10 mM Tris–HCl, pH 8.0, 1 mM EDTA.

### Determination of the DNA sequence

The sequence of both strands of the PM2 DNA was determined using an automated sequencer (Perkin–Elmer ABI Prism 377XL) and BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Sequencing was done by a primer-walking technique using 34 specific primers. The first four oligonucleotides were designed based on the previously published PM2 sequence (Miller *et al.,* 1984). Oligonucleotide primers were synthesized using the Applied Biosystems 394 DNA/RNA Synthesizer. Each sequencing reaction was performed in 10 $\mu$l containing 750 ng of PM2 DNA, 3.2 pmol of specific primer, and 4 $\mu$l of the BigDye terminator mixture. The cyclic sequencing reaction was incubated for 25 cycles in an automated temperature cycling reactor (Peltier Thermal PTC-200 Cycler, MJ Research) under cycling conditions of 95°C for 10 s, 55°C for 10 s, and 60°C for 2 min. The DNA was ethanol-precipitated and prepared for electrophoresis as described by the manufacturer (Perkin–Elmer). The sequences obtained were assembled and the consensus sequence was edited using the XGAP sequence assembly program and other programs that are included in the Staden Package (Bonfield *et al.,* 1995).

### Analysis of the sequence

The nucleotide sequence was analyzed for open reading frames using the EMBL GeneSkipper 1.2 program. Nucleotide and amino acid sequence similarities were searched using National Center for Biotechnology Information BLAST programs (Altschul *et al.,* 1997). Amino acid sequences were scanned for known active site motifs and protein family signatures using the PROSITE 14.0 program (Bairoch *et al.,* 1997) and analyzed for transmembrane regions using both TMPRED (Hofmann and Stoffel, 1993) and DAS (Cserzo *et al.,* 1994; Rost *et al.,* 1996; von Heijne, 1992) programs. The primary structures and hydrophobicity of amino acid sequences were analyzed using PROTSCALE (Argos and Rao, 1986; Deleage and Roux, 1987; Kyte and Doolittle, 1982) and secondary structure predictions were performed using NNPREDICT (Kneller *et al.,* 1990), PREDATOR (Frishman and Argos, 1996), DOUBLE PREDICTION (Deleage and Roux, 1987), and GIBRAT (Gibrat *et al.,* 1987) programs.

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