Characterization of the prolate-headed lactococcal bacteriophage φvML3: location of the lysin gene and its DNA homology with other prolate-headed phages

C. A. SHEARMAN, 1* S. HERTWIG, 2 M. TEUBER 2 and M. J. GASSON 1

1 AFRC Institute of Food Research, Norwich Laboratory, Colney Lane, Norwich NR4 7UA, UK
2 Institut für Mikrobiologie, Bundesanstalte für Milchforschung, D-2300 Kiel 1, Federal Republic of Germany

(Received 5 September 1990, revised 20 December 1990; accepted 13 February 1991)

φvML3 is a virulent prolate-headed bacteriophage that attacks a genetically well-studied strain family including Lactococcus lactis subsp. lactis strains 712, C2 and ML3. A restriction map was constructed for φvML3 using a wide variety of restriction endonucleases. The DNA was highly refractory to in vitro restriction; this is a common feature of lytic lactococcal phages. Genome size was estimated as 23 kb, which is similar to the sizes of other phages of the same morphological group. The presence of heat-dissociable DNA fragments was noted in gel electrophoresis of restriction enzyme digests. Cohesive ends were confirmed by pretreating the DNA with T4 ligase, which rendered the composite fragment insensitive to heat. The phage φvML3 genome thus consists of linear and non-permuted double-stranded DNA with complementary cohesive ends. The lysin gene from this phage acts as a specific lysis agent against the lactic streptococci. This gene has been cloned and sequenced previously. Further specific subcloning of EcoRV fragments of φvML3 DNA has located the lysin gene in the central region of the genome, orientated from right to left. This location was confirmed by hybridization of a lysin gene probe to φvML3 DNA. The lysin gene probe showed homology to a number of other prolate-headed phages including P001. The lysin gene of P001 was shown to be located in the central region of its genome. However, the isometric phage P107 did not hybridize, in spite of encoding its own lysin gene.

Introduction

Lactic streptococci used in the manufacture of cultured milk products are susceptible to bacteriophage infection. Phages isolated from dairy fermentation nearly all have an isometric or prolate polyhedral head and a non-contractile tail, with or without a collar (Heap & Jarvis, 1980; Teuber & Lembke, 1983; Saxelin et al., 1986). Isometric phages are more common but prolate-headed phages are significant because of their wide host ranges (Heap & Jarvis, 1980). Analysis of isometric and prolate-headed phages at the genetic and molecular level has been described (Coveney et al., 1987; Jarvis, 1984; Lautier & Novel, 1987; Powell & Davidson, 1985; Powell et al., 1989; Relano et al., 1987; Teuber & Loof, 1987). Isometric and prolate-headed phages can be separated on the basis of DNA hybridization within each grouping, genome size and structural protein profiles. φvML3 is a prolate-headed phage that attacks a genetically well-studied strain family including Lactococcus lactis subsp. lactis strains 712, C2 and ML3 (Davies et al., 1981). The gene for bacteriophage lysin has been cloned and sequenced (Shearman et al., 1989).

This study was undertaken to further characterize phage φvML3 genome and locate the lysin gene. Evidence is presented of DNA hybridization with the lysin genes of other prolate-headed phages.

Methods

Bacterial strains, bacteriophages and cultural methods. The lactococcal strain used was L. lactis subsp. cremoris NCDO 1200. The Escherichia coli strains used were MC1022 (Casadaban & Cohen, 1980) and its recombinant derivatives (Table 1). The prolate-headed phages used were φvML3 (Reiter & Oram, 1963; Oram & Reiter, 1965), and P109, P029, P159, P167, P330, P177, P220 and P001 (Teuber & Lembke, 1983; Braun et al., 1989). The isometric phage used was P107 (Braun et al., 1989). Plasmids used and their derivations are listed in Table 1.

Standard media and methods of cultivation of L. lactis subsp. cremoris were as described by Terzaghi & Sandine (1975). Cultural conditions for E. coli were as described by Mamatis et al. (1982). All E. coli strains containing pUC19 (Vieira & Messing, 1982; Norrander et al., 1983) and its recombinant derivatives were selected on L agar.
Table 1. E. coli strains

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Plasmid</th>
<th>Derivation or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5765</td>
<td>pUC19</td>
<td>Vieira &amp; Messing (1982)</td>
</tr>
<tr>
<td>5945</td>
<td>pTG262</td>
<td></td>
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<tr>
<td>5781</td>
<td>pFI106</td>
<td>Shearman et al. (1989)</td>
</tr>
<tr>
<td>5886</td>
<td>pFI140</td>
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<tr>
<td>5996</td>
<td>pFI160</td>
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<tr>
<td>5998</td>
<td>pFI162</td>
<td></td>
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<tr>
<td>6064</td>
<td>pFI192</td>
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</tr>
<tr>
<td>6066</td>
<td>pFI194</td>
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* The background strain is E. coli MC1022.

DNA isolation, manipulation and characterization. Restriction endonucleases and T4 DNA ligase (BRL or Boehringer Mannheim) were used according to the manufacturer’s recommendations. Restriction digests were heated at 70°C for 10 minutes and held on ice before loading on an agarose gel unless stated otherwise. Plasmid DNA isolations, restriction enzyme digests, DNA ligations and agarose gel electrophoresis were performed on caesium chloride/ethidium bromide density gradients.

Transformation. E. coli was transformed by the calcium chloride method described by Maniatis et al. (1982).

Preparation of bacteriophage φvML3 DNA. High titre phage lysates were prepared and concentrated by polyethylene glycol precipitation and purified on caesium chloride stepped gradients (Bachrach & Friedman, 1971). Phage DNA was extracted by dialysis against 50% (v/v) formamide in TE buffer (0·1 M Tris/HCl, 0·01 M-EDTA, pH 8·5). Further purification of the phage DNA was performed on caesium chloride/ethidium bromide density gradients.

Detection of bacteriophage lysis. E. coli cultures carrying cloned phage φvML3 DNA were tested for lysis production by centrifuging 10 ml overnight cultures and resuspending the cell pellets in 1 ml SM buffer (100 mM-NaCl, 10 mM-MgSO4, 7H2O, 50 mM-Tris/HCl, pH 7·5, 0·01% gelatin). After 5 min incubation at room temperature to lyse the cells, debris was removed by centrifugation and the supernatant was placed in a well cut into a plate already seeded with L. lactis subsp. cremoris NCDO 1200 cells. Zones of lysis developed around wells containing lysis-positive cell extracts after incubation at 30°C (Shearman et al., 1989).

DNA-DNA hybridization. The Southern blot technique (Southern, 1975) was used to transfer restriction endonuclease digests of φvML3 DNA separated by electrophoresis onto Hybond (Amersham) nitrocellulose filters (0·45 μm porosity). The filter was air-dried and UV crosslinked by exposure to a transilluminator for 3 min. The 0·49 kb HincII-EcoRI fragment from pFI140 was used as a probe. This contains a partial lysin gene (see Fig. 1). The probe was labelled and detected using a labelling and detection kit provided by Boehringer Mannheim which utilizes digoxigenin-11-DUTP.

The other phage DNA fragments (P109, P029, P159, P167, P330, P177, P220, P001, P107) were transferred to Zeta-Probe membranes (Bio-Rad) and hybridized under stringent conditions (50% v/v, formamide, 42°C) with chemically labelled (Chemiprobe kit, Hameln) phage DNA. Antigenic sulphone groups were coupled to the cytosine moieties of the DNA. After hybridization, the membranes were washed for 30 min at 68°C in 2 × SSC (1 × SSC is 0·15 M-NaCl, 0·015 M-sodium citrate) plus 0·1% (w/v) SDS and for 30 min at room temperature in 0·1 × SSC plus 0·1% SDS. The labelled DNA was visualized by a specific monoclonal antibody reaction and washed in 0·5 M-NaCl plus 0·5% (v/v) Brij 35T for 3 × 20 min at room temperature. An alkaline phosphatase anti-immunoglobulin antibody conjugate was added and the membrane was washed in 0·5 M-NaCl plus 0·3% Brij 35T for 3 × 20 min at room temperature followed by the addition of chromogenic substrate.

Results

Restriction map of the φvML3 genome

A restriction map was constructed for φvML3 using a variety of restriction endonucleases (Fig. 1). The DNA was highly refractory to in vitro restriction. This appears to be a common feature of lytic lactococcal bacteriophages (Powell & Davidson, 1985, 1986; Powell et al., 1989).

A genome size of 23 kb was calculated by summation of the estimated fragment sizes. This size is similar to those of other lactococcal lytic phages of the same morphological type (21·8–23·4 kb: Jarvis, 1984; Jarvis & Meyer, 1986; Lyttle & Petersen, 1984; Coveney et al., 1987; Powell et al., 1989).

The cohesive ends of phage DNA

The presence of heat-dissociable DNA fragments was noted in gel electrophoresis of restriction enzyme digests. These bands appeared if the restriction digest was heated to 70°C for 10 min prior to loading the gel. The same effect was observed by concentration of the φvML3 phage DNA before digestion (data not shown). The presence of cohesive ends was confirmed by pretreating the DNA with T4 DNA ligase, which rendered the composite fragment insensitive to heat (Fig. 2). Therefore φvML3 bacteriophage DNA is linear and non-permutated double-stranded DNA with complementary cohesive ends.

Cloning of phage φvML3 DNA fragments

The lysin gene from φvML3 has been cloned and sequenced (Shearman et al., 1989). φvML3 DNA was partially digested with AluI and EcoRI linkers attached

(Lennox, 1955) containing 50 μg ampicillin ml−1, 20 μg isopropyl β-D-thiogalactopyranoside (IPTG) ml−1 and 20 μg 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside (X-Gal) ml−1. The E. coli-Lactococcus shuttle vector pTG262 and its pFI derivatives were selected in E. coli thiogalactopyranoside (IPTG) ml−1 and 20 μg chloramphenicol ml−1. Further purification of the phage DNA was performed on caesium chloride stepped gradients (Bachrach & Friedman, 1971). Phage DNA was extracted by dialysis against 50% (v/v) formamide in TE buffer (0·1 M Tris/HCl, 0·01 M-EDTA, pH 8·5). Further purification of the phage DNA was performed on caesium chloride/ethidium bromide density gradients.

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Characterization of φML3 bacteriophage

**Fig. 1.** Restriction map of the φML3 genome. The φML3 DNA was not cleaved by ApaI, BamHI, BglII, BglII, CiaI, HindIII, NdeI, PstI, PvuI, PvuII, SmaI, SphI or SstI. HaeIII and BstEII cut three sites, and BanI cut two sites, all of which are unmapped. A number of restriction enzymes cut multiple sites, including AccI, AluI, HincII and DraI. In cases where two enzymes will cut at the same sequence (e.g. BglII and Sau3A) only one is labelled. The calculation of the expected number of restriction sites is based on the base composition of the lysin gene sequence (Shearman et al., 1989) using the method described by Powell & Davidson (1986). Restriction fragments that hybridized with the lysin probe (black bar) are marked with cross-hatching, including the DraI fragment (see Fig. 3). Digestion with DraI yields 10 fragments which have not been mapped to the phage genome. One DraI site was located upstream of the lysin gene by sequencing. The 4.8 kb DraI fragment that hybridized has been drawn on the restriction map based on this information. The location and orientation of the lysin gene is shown with an arrow.

**Fig. 2.** Agarose gel electrophoresis of phage φML3 DNA restriction digests showing the effect of heat treatment (70 °C, 10 min) and pretreatment with T4 DNA ligase. Samples in lanes 2 and 4 were pretreated with T4 DNA ligase and those in lanes 1 and 3 were untreated. All samples were heated before loading. Lanes 1 and 2, BanI; lanes 3 and 4, HaeIII.

to the fragments. The cloned fragment that expressed lysin was restriction mapped but this was insufficient to locate the lysin gene on a restriction map of the complete bacteriophage genome (Fig. 1). A fragment derived from this clone was used as a probe to locate the lysin gene. Specific cloning of EcoRV fragments confirmed the hybridization results.

φML3 DNA was digested with EcoRV and the resulting mixture of fragments was ligated into the SmaI sites of pTG262 and pUC19. Clones of two fragments, the 2.1 kb fragment and the 1.8 kb fragment from the central portion of the genome (see Fig. 1), were isolated. E. coli cultures containing these cloned DNA fragments were tested for lysin expression and the 1.8 kb fragment (pFI160, pFI192, pFI193) showed lysin expression (Fig. 1).

**Hybridization of the lysin gene probe**

The 0.49 kb HincII–EcoRI fragment from pFI140 was used as the lysin gene probe. Different restriction digests of φML3 DNA were probed. Hybridization picked out the 14.5 kb HpaI fragment, 12.3 kb EcoRI fragment, 4.0 kb MluI fragment and DraI 4.8 kb fragment (Fig. 3). These fragments overlap in the central region of the
Fig. 3. Hybridization of the lysin probe with φvML3 DNA and pFI140, pFI160 and pFI192. (a) Agarose gel electrophoresis of restriction digests. (b) Hybridization pattern. Lane 1, λ DNA cut with HindIII; lane 2, φvML3 HpaI; lane 3, φvML3 EcoR1; lane 4, φvML3 MluI; lane 5, φvML3 EcoRV; lane 6, φvML3 DraI; lane 7, φvML3 HincII; lane 8, pFI192 EcoRI + HindIII; lane 9, pFI106 EcoRI + HindIII; lane 10, pFI140 EcoRI + HindIII.

Fig. 4. Hybridization of plasmid pFI140 with DNA fragments of prolate-headed phages and one (P107) isometric-headed phage after digestion with HpaII. (a) Agarose gel electrophoresis patterns of HpaII restriction digests of DNAs of the phages. (b) Hybridization of sulphonated pFI140 DNA with DNAs from phages. Lane 1, pFI140, digested with EcoRI and PstI; lane 2, P109; lane 3, P029; lane 4, P159; lane 5, P167; lane 6, P330; lane 7, P177; lane 8, P220; lane 9, c6A; lane 10, P107.

The lysin gene probe was also used against the genomes of a number of other prolate-headed phages (P109, P029, P159, P167, P330, P177, P220 and P001) and an isometric phage, P107. The DNA of all the prolate-headed phages showed strong hybridization signals with pFI140 (Fig. 4b, lanes 2–9). Some of these phage DNAs revealed similar restriction digest patterns.

genome (Fig. 1), confirming the EcoRV subcloning result. Comparison of restriction mapping data for this new lysin-expressing clone and the original fully sequenced clone (Shearman et al., 1989) allowed the orientation of the lysin gene on the bacteriophage genome map to be identified, running from right to left (Fig. 1, data not shown).
Characterization of ϕvML3 bacteriophage

Discussion

This study has shown that ϕvML3 appears to be a typical prolate-headed lactococcal phage. Its genome size is within the range of sizes previously reported (Jarvis, 1984; Jarvis & Meyer, 1986). The presence of cohesive ends was detected. Thus phage ϕvML3 DNA is linear and non-permuted double-stranded DNA with complementary cohesive ends. The specificity of the cohesion (opposite ends of the DNA associate but like ends do not) indicates that the single-stranded ends are complementary but not palindromic. Accessibility of the DNA to T4 DNA ligase indicates that unlike the small Bacillus phages (Yehle, 1978; Yoshikawa & Ito, 1981) ϕvML3 DNA does not have protein molecules covalently attached to its termini. The cohesive ends permit in vitro circularization so the possibility exists that replication and/or packaging of the DNA involves a circular or multimeric linear intermediate.

Phage ϕvML3 DNA has only a few restriction sites for many restriction enzymes (e.g. Sau3A cuts one site, expected number of sites 77); however, some enzymes cut frequently (e.g. HincII cuts 13 sites, expected number 19). A wide variety of restriction enzymes do not cut the DNA (Fig. 1). Resistance to in vitro restriction is a common feature of lytic lactococcal bacteriophages

Fig. 5. Hybridization of plasmid pFI140 with the DNA of phage P001 digested with several restriction endonucleases. (a) Agarose gel electrophoresis patterns of restriction digests of the DNA of phage P001. (b) Hybridization of sulphonated pFI140 DNA with the digested DNA of phage P001. Lane 1, BsrEII; lane 2, EcoRI; lane 3, HaeIII; lane 4, HhaI; lane 5, HindIII; lane 6, HpaII. (c) Restriction map of the P001 phage genome. The region which hybridized with pFI140 is marked by a black bar. P001 DNA was not cleaved by ApaI, Asp718, AvaI, BamHI, BclI, BglII, DpnI, EcoRV, KpnI, NaeI, PstI, PvuI, PvuII, SacI, Sal or XhoI.

with HpaII (Fig. 4a, lanes 3, 4 and 7 compared with lanes 5 and 8). Only the largest HpaII fragment hybridized significantly with pFI140 (Fig. 4b, lanes 3, 4 and 7 compared with lanes 5 and 8). No hybridization signal was found with P107 (Fig. 4b, lane 10). This isometric phage induces a phage lysin, but belongs to a phage group which is unrelated to the prolate-headed phages.

Probing further restriction endonuclease digests of the P001 genome showed that the lysin gene is located in a 1.7 kb fragment in the central region (Fig. 5).
showed no evidence of atypical bases in the DNA of lactococcal prolate-headed phages (Nyiendo, 1975). The lack of restriction is not due to the presence of methylated bases, as shown by the lack of sites for DpnI (Coveney et al., 1987). In some Bacillus phages it has been proposed that the lack of restriction sites for specific enzymes is linked to their presence in the bacterial host (Kruger & Bickle, 1983). There is only one characterized type II restriction enzyme in Lactococcus, ScrF1 (Fitzgerald et al., 1982). However isoschizomers, such as BstNI and Smal which do not cut the lactococcal phage c6A (Powell & Davidson, 1986; Powell et al., 1989), have been found in species of non-lactic streptococci, Staphylococcus and Bacillus (Kessler et al., 1985). These genera are closely related (Stackebrandt & Woese, 1981) so similar specificities could be present in the lactic streptococci. It is unlikely that this explains the general scarcity of restriction endonuclease sites on \( \phi ML3 \) DNA. Comparison of the \( \phi ML3 \) restriction map (Fig. 1) with those obtained for other prolate-headed phages (Powell & Davidson, 1985; Braun et al., 1989; Powell et al., 1989) shows no striking similarities. Comparison of prolate-headed phages has previously shown no correlation between homology based on DNA hybridization and restriction maps (Powell et al., 1989).

The lysin gene from phage \( \phi ML3 \) has been located on the restriction map of the genome by subcloning and hybridization (Fig. 1). The genomes of a number of prolate-headed phages showed hybridization with the lysin gene probe and thus would appear to be homologous. However, the isomorphic phage P107 does not hybridize, in spite of encoding its own lysin gene. The lysin gene of \( \phi ML3 \) has been cloned. Extracts of \( E. coli \) cultures expressing lysin are active against lactococcal streptococci (Shearman et al., 1989). This phenomenon of ‘lysis from within’ (Weidel, 1951, 1958; Delbrück, 1940) enables phages to lyse strains which they do not infect. The prolate-headed phages appear to possess an homologous lysin gene which is therefore not involved in their differences of host range. The lysin genes of \( \phi ML3 \) and PO01 have both been located in the central region of their genomes. More detailed characterization of lactococcal phages will show whether they are suitable as phage vector systems and may indicate ways of combating phage infection in dairy fermentations.

This research was supported by contracts BAP 0009-UK and BAP 0409-UK from the Biotechnology Action Programme of the Commission of the European Community.

References


Characterization of \( \phi v \text{ML3} \) bacteriophage


