Characterization of the prolate-headed lactococcal bacteriophage 4vML3: location of the lysin gene and its DNA homology with other prolate-headed phages

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4vML3 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 4vML3 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 4vML3 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 4vML3 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 4vML3 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.

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 4vML3 (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 Manatis 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>
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<tbody>
<tr>
<td>5765</td>
<td>pUC19</td>
<td>Vieira &amp; Messing (1982)</td>
</tr>
<tr>
<td>5945</td>
<td>pTG262</td>
<td>0.6 kb HaeIII fragment ex pUC18 in pCK17 (EcoRI, BamHI); A. Mercenier, Transgene, Strasbourg, France</td>
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<td>5781</td>
<td>pFI106</td>
<td>Shearman et al. (1989)</td>
</tr>
<tr>
<td>5886</td>
<td>pFI140</td>
<td>0.49 kb HincII-EcoRI fragment ex pFI106 in pUC19 (HincII, Smal)</td>
</tr>
<tr>
<td>5996</td>
<td>pFI160</td>
<td>1.8 kb EcoRV fragment of phage φML3 in pTG262 (Smal)</td>
</tr>
<tr>
<td>5998</td>
<td>pFI162</td>
<td>2.1 kb EcoRV fragment of phage φML3 in pTG262 (Smal)</td>
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<td>6064</td>
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<td>1.8 kb EcoRV fragment of phage φML3 in pUC19 (Smal)</td>
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* The background strain is *E. coli* MC1022.

**Results**

**Restriction map of the φML3 genome**

A restriction map was constructed for φML3 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 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 φML3 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 φML3 bacteriophage DNA is linear and non-permuted double-stranded DNA with complementary cohesive ends.

**Cloning of phase φML3 DNA fragments**

The lysis gene from φML3 has been cloned and sequenced (Shearman et al., 1989). φML3 DNA was partially digested with AluI and EcoRI linkers attached 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, Amersham) phage DNA. Antigenic sulphhydryl groups were coupled to the cysteine 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.

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**DNA isolation, manipulation and characterization.** Restriction endonucleases and T4 DNA ligase (BRL or Boehringer Mannheim) were used according to the manufacturer's recommendations. Restriction digestes 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. 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) plus 0.015 M-sodium citrate) 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.

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Characterization of φML3 bacteriophage

Expected no. of sites

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Sites</th>
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<tbody>
<tr>
<td>HpaI</td>
<td>28</td>
</tr>
<tr>
<td>KpnI</td>
<td>3</td>
</tr>
<tr>
<td>AatI</td>
<td>7</td>
</tr>
<tr>
<td>AarII</td>
<td>7</td>
</tr>
<tr>
<td>BglII</td>
<td>7</td>
</tr>
<tr>
<td>XbaI</td>
<td>7</td>
</tr>
<tr>
<td>NcoI</td>
<td>7</td>
</tr>
<tr>
<td>HaeIII</td>
<td>7</td>
</tr>
<tr>
<td>HpaI</td>
<td>7</td>
</tr>
<tr>
<td>EcoRI</td>
<td>7</td>
</tr>
<tr>
<td>MluI</td>
<td>3</td>
</tr>
<tr>
<td>DraI</td>
<td>3</td>
</tr>
<tr>
<td>EcoRV</td>
<td>7</td>
</tr>
</tbody>
</table>

Fig. 1. Restriction map of the φML3 genome. The φML3 DNA was not cleaved by ApaI, BamHI, BclI, BglII, Clai, HindIII, NdeI, PstI, PvuI, PvuII, SacI, 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.

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 genome. The 1.8 kb fragment (pFI160, pFI192, pFI193) showed lysin expression (Fig. 1).

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).
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).

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...
Characterization of φvML3 bacteriophage

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.
Comparing estimates of G + C content obtained from thermal denaturation and from CsCl buoyant density showed no evidence of atypical bases in the DNA of lactococcal prolate-headed phages (Nyiando, 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, ScrFI (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 φML3 DNA. Comparison of the φML3 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 φML3 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 φML3 has been cloned. Extracts of E. coli cultures expressing lysin are active against lactic streptococci (Shearman et al., 1989). This phenomenon of 'lysis from without' (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 φML3 and P001 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.

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References


Characterization of \( \phi vML3 \) bacteriophage


