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

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ϕ vML3 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 ϕ vML3 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 ϕ vML3 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 ϕ vML3 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 ϕ vML3 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. ϕ vML3 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 ϕ vML3 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 ϕ vML3 (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 Maniatis *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

Strain*	Plasmid	Derivation or reference
5765	pUC19	Vieira & Messing (1982)
5945	pTG262	0.6 kb <i>Hae</i> II <i>lac</i> fragment ex pUC18 in pCK17 (<i>Eco</i> RI, <i>Bam</i> HI); A. Mercenier, Transgene, Strasbourg, France
5781	pFI106	Shearman <i>et al.</i> (1989)
5886	pFI140	0.49 kb <i>Hinc</i> II- <i>Eco</i> RI fragment ex pFI106 in pUC19 (<i>Hinc</i> II, <i>Sma</i> I)
5996	pFI160	1.8 kb <i>Eco</i> RV fragment of phage ϕ vML3 in pTG262 (<i>Sma</i> I)
5998	pFI162	2.1 kb <i>Eco</i> RV fragment of phage ϕ vML3 in pTG262 (<i>Sma</i> I)
6064	pFI192	1.8 kb <i>Eco</i> RV fragment of phage ϕ vML3 in pUC19 (<i>Sma</i> I)
6066	pFI194	2.1 kb <i>Eco</i> RV fragment of phage ϕ vML3 in pUC19 (<i>Sma</i> I)

* The background strain is *E. coli* MC1022.

(Lennox, 1955) containing 50 μ g ampicillin ml⁻¹, 20 μ g isopropyl β -D-thiogalactopyranoside (IPTG) ml⁻¹ and 20 μ g 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* using 15 μ g chloramphenicol ml⁻¹.

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 by standard procedures (Maniatis *et al.*, 1982). Fragment isolation was by freeze-squeeze (Thuring *et al.*, 1975).

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

Preparation of bacteriophage ϕ vML3 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 ϕ vML3 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 ϕ vML3 DNA separated by electrophoresis onto Hybond (Amersham) nitrocellulose filters (0.45 μ m porosity). The filter was air-dried and UV crosslinked by exposure to a transilluminator for 3 min. The 0.49 kb *Hinc*II-*Eco*RI fragment from pFI140 was used as a probe. This contains a partial lysis 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 $\times$ SSC (1 $\times$ 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 $\times$ 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 $\times$ 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 $\times$ 20 min at room temperature followed by the addition of chromogenic substrate.

Results

Restriction map of the ϕ vML3 genome

A restriction map was constructed for ϕ vML3 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 was 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 ϕ vML3 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 ϕ vML3 bacteriophage DNA is linear and non-permuted double-stranded DNA with complementary cohesive ends.

Cloning of phage ϕ vML3 DNA fragments

The lysis gene from ϕ vML3 has been cloned and sequenced (Shearman *et al.*, 1989). ϕ vML3 DNA was partially digested with *Alu*I and *Eco*RI linkers attached

Fig. 1. Restriction map of the ϕ vML3 genome. The ϕ vML3 DNA was not cleaved by *ApaI*, *BamHI*, *BclI*, *BglI*, *ClaI*, *HindIII*, *NdeI*, *PstI*, *PvuI*, *PvuII*, *SalI*, *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 ϕ vML3 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.

ϕ vML3 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 ϕ vML3 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 ϕ vML3 DNA and pFI140, pFI160 and pFI192. (a) Agarose gel electrophoresis of restriction digests. (b) Hybridization pattern. Lane 1, λ DNA cut with *Hind*III; lane 2, ϕ vML3 *Hpa*I; lane 3, ϕ vML3 *Eco*RI; lane 4, ϕ vML3 *Mlu*I; lane 5, ϕ vML3 *Eco*RV; lane 6, ϕ vML3 *Dra*I; lane 7, ϕ vML3 *Hinc*II; lane 8, pFI192 *Eco*RI + *Hind*III; lane 9, pFI106 *Eco*RI + *Hind*III; lane 10, pFI140 *Eco*RI + *Hind*III.

Fig. 4. Hybridization of plasmid pFI140 with DNA fragments of prolate-headed phages and one (P107) isometric-headed phage after digestion with *Hpa*II. (a) Agarose gel electrophoresis patterns of *Hpa*II restriction digests of DNAs of the phages. (b) Hybridization of sulphonated pFI140 DNA with DNAs from phages. Lane 1, pFI140, digested with *Eco*RI and *Pst*I; 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.

genome (Fig. 1), confirming the *Eco*RV 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

Fig. 5. Hybridization of plasmid pF1140 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 pF1140 DNA with the digested DNA of phage P001. Lane 1, *Bst*EII; lane 2, *Eco*RI; lane 3, *Hae*III; lane 4, *Hha*I; lane 5, *Hind*III; lane 6, *Hpa*II. (c) Restriction map of the P001 phage genome. The region which hybridized with pF1140 is marked by a black bar. P001 DNA was not cleaved by *Apa*I, *Asp*718, *Ava*I, *Bam*HI, *Bcl*I, *Bgl*II, *Dpn*I, *Eco*RV, *Kpn*I, *Nae*I, *Pst*I, *Pvu*I, *Pvu*II, *Sac*I, *Sal*I or *Xho*I.

with *Hpa*II (Fig. 4a, lanes 3, 4 and 7 compared with lanes 5 and 8). Only the largest *Hpa*II fragment hybridized significantly with pF1140 (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 lysis, 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 lysis gene is located in a 1.7 kb fragment in the central region (Fig. 5).

Discussion

This study has shown that ϕ vML3 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 ϕ vML3 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) ϕ vML3 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 ϕ vML3 DNA has only a few restriction sites for many restriction enzymes (e.g. *Sau*3A cuts one site, expected number of sites 77); however, some enzymes cut frequently (e.g. *Hinc*II 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

(Jarvis & Meyer, 1986; Powell & Davidson, 1986; Coveney *et al.*, 1987; Relano *et al.*, 1987). Studies 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 (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*, *ScrFI* (Fitzgerald *et al.*, 1982). However isoschizomers, such as *BstNI* and *SmaI* 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 ϕ vML3 DNA. Comparison of the ϕ vML3 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 ϕ vML3 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 isometric phage P107 does not hybridize, in spite of encoding its own lysin gene. The lysin gene of ϕ vML3 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 ϕ vML3 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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