Sequence and organization of the lactococcal prolate-headed bIL67 phage genome

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bIL67 is a broad-host-range prolate-headed phage that is active against *Lactococcus* cells. The complete phage genome sequence of 22 195 bp was established. Thirty-seven open reading frames (ORFs) organized in two clusters were identified. Functions were assigned to the putative products of six of the ORFs on the basis of comparison of the deduced amino acid sequences to known proteins, analysis of structural features of the proteins and search for conserved motifs. These were a DNA polymerase, a protein involved in recombination, a lysin, a terminase subunit, a structural protein and a holin.

Keywords: bacteriophage, DNA sequence, lactic acid bacteria

INTRODUCTION

Lactococcal bacteriophages remain a major problem in the dairy industry, since they delay or completely prevent acidification by the starter culture. Many virulent lactococcal bacteriophages have been isolated from unsuccessful fermentations and are classified into 12 species according to morphology and DNA homology (Jarvis *et al.*, 1991). They have a double-stranded DNA genome ranging from 18 to 54 kb and carry either cohesive or redundant ends.

Information about genome organization is scarce and concerns only the most prevailing species of lactococcal phages. These species are usually designated according to their morphology and life cycle, and referred to as prolate-headed phages, which are always virulent, and small-isometric-headed phages, which are either virulent or temperate. The genome of virulent small-isometric-headed phages has most probably a modular organization. Such an organization, originally described for lambdoid phages (Campbell & Botstein, 1983), was suggested by Loof & Teuber (1986) and Jarvis & Meyer (1986) from heteroduplex studies that revealed homologous regions covering from 81 to 94% of the genome and distinct regions of non-homology. Certain regions of small-isometric-headed virulent bacteriophage genomes have been characterized, such as the lysin gene (Platteeuw & de Vos, 1992), the *mcp* gene for the major coat protein (Chung *et al.*, 1991), three genes encoding minor structural proteins (Kim & Batt, 1991), the *llaIM* gene encoding a

type-II methyltransferase (Hill *et al.*, 1991), the *bpi* gene encoding a putative regulator (Lakshmidhevi *et al.*, 1990), the *per* locus presumed to be an origin of replication (Hill *et al.*, 1990), the region surrounding the *cos* site (Chandry *et al.*, 1994; E. Bidnenko, S. D. Ehrlich & M.-C. Chopin, unpublished) and a cluster of genes encoding structural proteins (R. Parreira & others, unpublished). Certain genes from temperate small-isometric-headed phages have also been identified: the *int* gene encoding the integrase (Lillehaug & Birkeland, 1993), the repressor (van de Guchte *et al.*, 1994), the lysin gene and genes encoding structural proteins (Arendt *et al.*, 1994). In the case of virulent prolate-headed phages less information is available, since only the lysin genes from three related phages (Shearman *et al.*, 1989; Ward *et al.*, 1993; Geis, 1992) and the N-terminal part of certain structural proteins (Schouler *et al.*, 1992) have been characterized.

A better understanding of the bacteriophage life cycle is required for designing strategies to prevent phage infection. As a step towards this goal, the whole genome (22 195 bp) of the prolate-headed phage bIL67 has been sequenced.

METHODS

Bacterial strains and media. *Escherichia coli* JJC128F', *araD139* Δ (*ara-leu*)7696 *galE15 galK16* Δ (*lac*)X74 *hsdr⁻ hsdm⁺* Str^R F' [*lacI^q* Δ (*lacZ*)M15 *traD36*], was grown at 37 °C in LB broth (Miller, 1972). *Lactococcus lactis* IL1403 (Chopin *et al.*, 1984) was grown at 30 °C in M17 broth (Terzaghi & Sandine, 1975) in which lactose was replaced by glucose.

Phage bIL67 is from our collection and was propagated on *L. lactis* IL1403. It belongs to the c2 phage group (Jarvis *et al.*,

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1991). Phage DNA was extracted as described for phage λ (Sambrook *et al.*, 1989). Double-stranded DNA of phage M13mp18 (Yanisch-Perron *et al.*, 1985) was purchased from Boehringer.

Molecular cloning and DNA sequencing. *E. coli* was transformed by electroporation (Dower *et al.*, 1988). Digestion by restriction enzymes, alkaline phosphatase treatment, ligation, luminescent labelling, electrophoresis and hybridization were carried out according to the suppliers' protocols.

The random library of phage genome fragments was established as follows. bIL67 DNA was partially digested with DNaseI (Anderson, 1981). For this purpose, 500 ng of phage DNA, in 100 μ l 50 mM Tris/HCl (pH 7.5)/1 mM $MnCl_2$, was digested with 2×10^{-3} or 2×10^{-4} units of enzyme for 5–30 min. The reaction was arrested by addition of EDTA to 50 mM followed by a phenol/ $CHCl_3$ (1:1, v/v) extraction. Fragments obtained were made blunt-ended by treatment with T4 DNA polymerase (Boehringer) and were separated in 1.2% (w/v) agarose gel. The fragments ranging from 300 to 500 bp, 500 to 700 bp and 700 to 900 bp were excised from the gel and recovered by the Freeze-Squeeze procedure using Spin-X tubes as described by the manufacturer (Costar). The isolated fragments were then cloned in M13mp18 which was cut with *Sma*I and dephosphorylated.

Single-stranded DNA from recombinant phages grown in *E. coli* JJC128F' was isolated by the perchlorate method (Zimmermann *et al.*, 1989) as adapted for the Beckman Biomek 1000 Laboratory Workstation (Sorokin *et al.*, 1993). Single-stranded DNA was sequenced by using *Taq* polymerase (Applied Biosystems) on CATALYST (Applied Biosystems), or by the Sequenase (Applied Biosystems) procedure, manually. The dye-labelled standard sequencing primers of Applied Biosystems and Applied Biosystems Sequencer ABI-373 were used. Complementary DNA strands of M13 were obtained according to the protocol supplied with Dynabeads (strappedin-coated magnetic beads) template preparation kit (Dyna).

Unclonable regions were synthesized by PCR. Sequencing of PCR products was performed essentially according to the protocol supplied with the Applied Biosystems PCR sequencing kit, on the DNA Thermal Cycler from Perkin-Elmer.

Data handling and computer analysis. DNA sequence assembly was performed using Staden's programmes (Staden, 1982). ORF predictions were performed with Genmark software (Borodovsky & McIninch, 1993). Sequence data were analysed using the University of Wisconsin Genetics Computer Group (uwgCG) package (Devereux *et al.*, 1984). The search for sequence homology was carried out using FASTA (Pearson & Lipman, 1988) (in Swissprot release 27) and BLAST (Altschul *et al.*, 1990) (in GenBank release 80). Search for blocks of homology was done using MACAW software (Schuler *et al.*, 1991).

PCR. Reactions were performed using the Gene ATAQ Controller (Pharmacia-LKB) or the DNA Thermal Cycler 9600 (Perkin Elmer). The standard *Taq* polymerase buffer of Promega, 0.2 mM of each dNTP, 0.1 μ M primers and 2.5 units *Taq* polymerase were used. The final volume of the reactions was 100 μ l. Phage bIL67 DNA (50 ng) or phage M13 supernatant (2 μ l) was used as starting material. The conditions of a cycle were: 94 °C for 1 min, 50 °C for 2 min, 72 °C for 3 min. In total, 30 cycles of amplification were run. After the PCR reactions were completed, DNA was loaded in 1% low-gelling agarose gel and purified using the Freeze-Squeeze procedure (Costar).

RESULTS AND DISCUSSION

Determination of the bIL67 DNA sequence

The DNA sequencing strategy used follows closely that developed for sequencing of the entire yeast artificial chromosome, within the context of the *Bacillus subtilis* genome sequencing project (P. Serror, A. Sorokin & S. D. Ehrlich, unpublished). Briefly, a random library of 960 clones in M13mp18, covering the phage genome 27 times, was established. Seventy-five per cent of the bIL67 sequence was determined by shotgun sequencing of 149 clones. This sequence was organized in 17 non-overlapping clusters of a size ranging from 270 to 2700 bp. To fill the gaps between the clusters, PCR-synthesized inserts of M13 phages corresponding to the ends of each cluster were hybridized with the random library. The positive M13 clones were sequenced. Close to 96% of the sequence was thus determined. This sequence, determined on only one strand in some regions, was completed by synthesis and sequencing of the complementary strand of appropriate clones (see Methods). The remaining 4% of the genome was apparently absent from the random library, probably because of toxicity and/or of structural instability in the cloning system used. The sequence of this last part was determined directly from PCR products spanning the gaps. Thus, a linear bIL67 DNA sequence of 22195 bp was established, with an average redundancy of 6.2, each region being sequenced at least once on both strands. Restriction sites determined from the sequence analysis were in agreement with those found by endonuclease digestions. The total G + C content of the bIL67 phage genome is 36%, which is similar to that of the lactococcal hosts (Kilpper-Bälz *et al.*, 1982).

Genome organization

The sequence was translated in all six frames and the criterion chosen for ORFs was the presence of a start codon (AUG, UUG or GUG) preceded by a ribosome binding site (RBS) complementary to the 3' end of the 16S rRNA of *Lactococcus lactis* (3' UCUUCCUCCA 5') (Ludwig *et al.*, 1985). When no length limitation was set, 37 ORFs could be identified, 11 of which were shorter than 300 bp. The protein-coding regions were checked all along the sequence, using a programme developed for gene recognition in newly sequenced DNA (Borodovsky & McIninch, 1993), which is based on non-homogeneous Markov chains. The presence of the 37 ORFs was thus confirmed. The ORFs were organized in two divergent clusters (Fig. 1). It is tempting to correlate this organization with that deduced from transcriptional studies of the prolate-headed phage c2 (Beresford *et al.*, 1993), where early and late regions were identified. As discussed below, putative genes encoding lysin and a structural protein, which were expected to be expressed late, are present in the leftward orientated cluster (Fig. 1). We therefore speculate that this cluster corresponds to the late transcribed region and, accordingly, the other cluster to the early transcribed region (Fig. 1).

Early and late regions are separated by two intergenic regions, the structure of which is detailed below.

Fig. 1. Genetic organization of the bIL67 genome. The 22195 bp linear genome of bIL67 is represented as an open bar with vertical 1 kb markers. ORFs are shown in boxes either above or below the genome. They are at three different heights, depending on which of the three frames on each strand is used. Direction of transcription is indicated by arrows. Gene functions are indicated when assigned.

Upstream of the early and the late regions, putative promoters were identified, having an extended -10 consensus box (TATGCTATAAT and TTTTGTGATATATT, respectively), and no significant resemblance to the -35 consensus box. Promoters of this type (A/TntnTGa/tTATAAT, lacking a clear -35 sequence) are known to allow σ^{70} -dependent transcriptional initiation in *E. coli* (Kumar *et al.*, 1993). One such promoter was recently shown to be functional in another lactococcal phage, bIL66 (E. Bidnenko, S. D. Ehrlich & M.-C. Chopin, unpublished). Fourteen of the 21 early genes overlap by 1–8 bp and might, therefore, be transcriptionally coupled. A putative terminator sequence (CCACTCAATCAAGAGTGGTTTTTGTGTT; underlined sequences can form a stem structure) is present 30 bp downstream of the last early gene. A striking feature of the early genes is their small average size, about 350 bp. Late genes are, on average, longer and eight of them overlap by 1–50 bp. Translational signals of the 37 identified genes are compiled in Table 1.

Intergenic regions

The cohesive ends (*cos* site) of the prolate-headed phage P001 have the sequence 5' TCAAGCCTAACT 3' (Billard *et al.*, 1992). Since the same sequence is present in the intergenic region of bIL67, in which transcription of early and late genes converge, we propose that it corresponds to the *cos* site of this phage. This *cos* site contains a single-stranded 3' overhang, as already observed for several phages active on Gram-positive bacteria. Six different types of direct repeats and one inverted repeat have been identified in the vicinity of the *cos* site (Fig. 2). The repeats denoted R2 present a dyad symmetry, evocative of those

found at the *cos* site of phage λ . Some of the structural features near the cohesive ends could be involved in recognition by terminase and host factors.

In the intergenic region upstream of the early and late genes (Fig. 1), which is 680 bp long, several short direct (7 bp) and inverted repeats are present. The role of these repeats is not known, but similar structures are often found in the DNA replication origin. If bi-directional replication were initiated in this region, it would be co-directional with phage transcription. Such co-directionality is observed, for instance, in the chromosome of *E. coli* and *B. subtilis* (Burland *et al.*, 1993; Ogasawara *et al.*, 1994; Sorokin *et al.*, 1993).

Putative function of the ORFs

Using the BLAST (Altschul *et al.*, 1990) and FASTA (Pearson & Lipman, 1988) programs, similarities between proteins specified by bIL67 ORFs and protein sequences present in databases were searched for. Significant homology was detected for translation products of three ORFs, encoding a recombinase, a lysin and a structural protein. The putative function of three additional proteins can be deduced from the presence of conserved domains and structural features in common with known proteins (a DNA polymerase, a terminase subunit and a holin). In the following sections the relevant ORFs are discussed.

ORFs 3, 4, 5 and 6

Five domains typical of DNA polymerases have been identified in the protein specified by ORF3 (Fig. 3). Although the overall similarities among distantly related DNA polymerases are weak, three exonuclease domains

Table 1. General features of the bIL67 ORFs

Gene designation	Start*	Stop*	Product size (aa)	Similarities	Translation start†
Early 1	15 132(AUG)	15 278(UGA)	48	DNA pol	AAAGAGGUuuuuuu AUG UUU AUU
2	15 379(AUG)	15 579(UAA)	66		GGAGGUuuuuucaa AUG ACA AUA
3	15 581(AUG)	16 480(UAA)	299		AAAGGgGUugauuuuu AUG CAG GAC
4	16 550(AUG)	16 738(UGA)	62		GAGGUuaccga AUG GAU AAA
5	16 735(AUG)	17 115(UAA)	126		AAAGGAacuaaaca AUG AAA AUU
6	17 115(AUG)	17 483(UGA)	122		GAGGUaucaauua AUG GCG AUU
7	17 562(AUG)	17 735(UAA)	57		GAGGgaacaaaa AUG AUU AAA
8	17 735(AUG)	18 085(UAA)	116		GGAGGUuuuuagaua AUG GCU AAA
9	18 138(AUG)	18 263(UAA)	41		GAGGau AUG GCG AAA
10	18 295(AUG)	18 480(UAA)	61		AGAAuaucauaaa AUG CUA GGG
11	18 480(AUG)	18 683(UAA)	67	Erf	GGAGGUuuuuuuuu AUG GCA UUA
12	18 751(AUG)	18 903(UAA)	50		AAGGAGGauuuuuu AUG UUG ACU
13	18 903(AUG)	19 442(UAA)	179		AAAGGAGugagccuua AUG GAA AGC
14	19 508(AUG)	19 879(UAA)	123		GAGGgaacacaaaa AUG AAA AUU
15	19 892(AUG)	20 056(UGA)	54		AAAGGAGuucagga AUG GGAUUA
16	20 053(AUG)	20 532(UAG)	159		GAAAGGauacauacaacu AUG AAA AUC
17	20 554(AUG)	20 887(UAA)	107		AAAGGgaacgaa AUG UUU AAC
18	20 870(AUG)	21 172(UAA)	100		GAGGUuacuacu AUG AUU AAU
19	21 194(AUG)	21 568(UAA)	124		GAGGaacuaaaaa AUG AAA CUU
20	21 573(AUG)	21 779(UAA)	68		GAGGUguaaaaaa AUG GAA UUA
21	21 780(AUG)	21 953(UAG)	57	abi-105 target Lysin	GAGGUguaa AUG GUU UAU
Late 22	14 448(AUG)	14 299(UAA)	49		GGAGGUauaagc AUG GCA GAA
23	14 288(AUG)	13 803(UAA)	161		GAGGauagaa AUG AAG AAA
24	13 757(AUG)	13 077(UAG)	226		GGgGGUgguuuu AUC AAA AUA
25	13 067(AUG)	12 228(UGA)	279		AGGAGGaguga AUG AGC UUA
26	12 277(UUG)	10 832(UAA)	481		AAAGGAGGaaaaa UUG AAA GUA
27	10 801(AUG)	10 097(UAG)	234		AGAAAGGgaguuuuu AUG GCU GAA
28	10 079(AUG)	9 462(UAA)	205		AGGAGGauaaaaauu AUG GCA UAU
29	9 390(AUG)	9 136(UAA)	84		AGGAGauuagaaaa AUG AAG AAA
30	8 948(AUG)	8 544(UAA)	134		GGAGGUaaaauu AUG GCA AAC
31	8 551(AUG)	6 689(UGA)	620	Tail protein Terminase subunit	AAGGAGuauaacaaca AUG GCU AAA
32	6 390(AUG)	4 834(UAA)	518		AAGGAGauuuuuuuu AUG AGUUUA
33	4 834(AUG)	4 535(UAA)	99		GAGGacuauuuuuuuu AUG GCU AUG
34	4 542(UUG)	2 656(UAA)	628		GGAGGgcuuggcga UUG AAU AAG
35	2 663(AUG)	1 725(UAG)	312		AGAAAGGAuuu AUG AAU AAA
36	1 670(AUG)	477(UAA)	397	Holin	GAGGUuuuuaaaa AUG GAU AGU
37	458(AUG)	165(UAG)	97		AAAGGAG aagagag AUG AUG AUU

16S rRNA 3' UCUUCCUCCA 5'

*Numbers refer to position in the sequence; start and stop codons are shown in parentheses.

†The RBS and the beginning of the ORFs are shown in capital letters.

Fig. 2. Region of cohesive ends. The direct repeats are represented by boxes. The inverted repeats are indicated as arrows below the horizontal bar representing the phage DNA. Ends of ORF 21 and ORF 37 are represented as arrows on the horizontal line. Scale, sequence of the repeats and the cos region are shown below the figure. Cohesive ends are boxed.

Fig. 3. Comparison of the organization of the conserved regions involved in the catalytic activity between $\phi 29$ DNA polymerase and bIL67-encoded products by ORF 3 to ORF 6. Proteins are represented with the N terminus at the left. Thick vertical lines delimit proteins. Open squares indicate the conserved regions between DNA-dependent DNA polymerases (Blanco *et al.*, 1991). DR refers to direct repeat (see text for sequence).

Fig. 4. Highly conserved regions within DNA-dependent DNA polymerases and homology with phage bIL67. One representative polymerase from each group is presented. (a) Poll-like DNA polymerases represented by BsIII for the exopolymerase domains and by T7 for the polymerization domains (not characterized for BsIII), (b) viral α -like DNA polymerases, (c) cellular α -like DNA polymerases, and (d) protein-primed α -like DNA polymerases. Numbers in parentheses indicate the amino acid position relative to the N-terminal end of each DNA polymerase. Relevant amino acid similarity among the different groups is indicated in white letters. The following conservative amino acid substitutions were considered: S and T; A and G; K, R and H; D, E, Q and N; L, I, M, C, V, Y, and F (Blanco *et al.*, 1991, 1992). Asterisks indicate specially conserved residues and/or Poll residues involved in catalytic activity.

located in the N-terminal region and six domains involved in the polymerization function, located in the C-terminal region, have been identified in 27 different enzymes (Blanco *et al.*, 1991). Domains corresponding to 3'-5' exonuclease activity (I, II and III) were found in ORF 3 (Fig. 4). The Y residue in domain III, which plays an important role in catalytic activity, is replaced by an L residue in ORF 3, as previously observed for the *B. subtilis* DNA polymerase BsIII (Blanco *et al.*, 1992). Two domains (region 2a and region 2b) out of the six involved in the polymerization function were also found in ORF 3. Remarkably, two additional polymerase domains, known as region 4 and region 5, are present downstream of ORF

3 in ORF 4 and ORF 5, respectively. Moreover, proteins encoded by ORFs 4 and 5 are basic (pI 10.43 and pI 10.20, respectively) and have one and two helix-turn-helix motifs, respectively, which is indicative of DNA binding capacity. In contrast, polymerase regions 1 and 3 were not identified. It is possible that the proteins encoded by ORFs 4 and 5 are subunits of a DNA polymerase. Interestingly, the cluster composed of ORFs 4, 5 and 6 is flanked by direct repeats of 25 bp (AATTAAGTCATCAAGTTAAGAGAGG), which include a ribosome binding site. This organization is evocative of evolution by exchange of DNA modules, via a Campbell type recombination between two circular molecules, a

process proposed for lambdoid phages (Campbell & Botstein, 1983).

ORF 13

The product of ORF 13 shares 37.5% identity and 57.1% similarity to the Erf protein of *Salmonella typhimurium* phage P22 (Poteete, 1982). This protein is required for the circularization of the linear chromosome of the phage, by homologous recombination between its repetitive ends (Weaver & Levine, 1977). Since bIL67 has cohesive ends, the ORF 13 product probably plays a different role. In phage λ , the homologous recombination directed by the *red* system was shown to increase the amount of packageable DNA (Smith, 1983). The homologous recombination system of phage P22 and phage λ are structurally unrelated (Poteete *et al.*, 1991) but functionally interchangeable (Poteete & Fenton, 1984). We suggest that ORF 13 plays a role similar to λ *red*. Interestingly, ORF 12, located upstream of ORF 13, encodes a protein of 51 aa. The position of the gene and the size of the protein are evocative of the accessory recombination function (*arf*) gene of P22 (Poteete *et al.*, 1991). Nevertheless, no significant homology has been observed between ORF 12 and *arf* gene products. The ORF 12/13 cluster is flanked by two direct repeats of 31 bp with only two mismatches (in bold letters) (TTGACAAAAGAAAG**G**CAAACACGT TATAATTA and TTGACAAAAGAAA**A**TAAACACGTTATAATTA) which suggests that they may also correspond to an exchangeable module. Interestingly, a promoter consensus sequence (de Vos, 1987) is present in these repeats (underlined). No homologies with other components of the P22 or λ recombination systems have been observed in bIL67.

ORF 23

The ORF 23 product shares over 90% identity with the protein encoded by the gene located upstream of the lysin gene of the lactococcal prolate-headed phages ϕ VML3 (Shearman *et al.*, 1989) and c2 (Ward *et al.*, 1993), respectively. The function of this gene remains unknown in both cases. Moreover, the ORF 23 product shares 41% identity with the protein encoded by ORF 3 of the virulent isometric-headed bIL66 phage (E. Bidnenko, S. D. Ehrlich & M.-C. Chopin, unpublished). ORF 3 of bIL66 is located in the region which is a target of the abortive infection mechanism *abi*-105, active against isometric- and prolate-headed phages (including bIL67) (Gautier & Chopin, 1987). ORF 23 may thus be the target of *abi*-105. ORF 3, located near the cohesive ends of bIL66, is a middle-expressed gene, while ORF 23, located in the middle of the genome, might be one of the first late genes of bIL67.

ORF 24

The ORF 24 product shares very high identity (up to 95%) with the proteins encoded by the three lysin genes already sequenced from lactococcal prolate-headed

phages, ϕ VML3 (Shearman *et al.*, 1989), P001 (Geis, 1992) and c2 (Ward *et al.*, 1993). The published ϕ VML3 lysin gene sequence (Shearman *et al.*, 1989) has been recently corrected in the database (accession number X16178) and is in good agreement with the one from phage c2. However, in both cases, the position of the start codon of the gene was unclear. This question was recently answered by the report of the purification of the phage P001 lysin protein and the determination of its N-terminal amino acid sequence, MKVSQ (A. Geis & S. Hertwig, unpublished). Comparison between this sequence and the one deduced from the nucleotide sequence, revealed that the isoleucine codon, AUC, is used as a start codon (it is translated as the first methionine). Sequence comparisons revealed that the start codon is also AUC in phage bIL67 but AUA in phages c2 and ϕ VML3. AUA has already been found as a start codon *in vivo* in *E. coli* (Belin *et al.*, 1979) but the use of AUC as a start codon has never been reported before. Use of a very rare start codon could be a way to decrease translation of lysin during phage multiplication. The bIL67 lysin gene shares 40.3% and 49.3% homology with those of *B. subtilis* phages PZA (Paces *et al.*, 1986) and ϕ 29 (Vlcek & Paces, 1989), respectively, which were shown to exhibit muramidase activity.

ORF 31

The ORF 31 product shares 99% identity with the N-terminal part of protein POA17 from the prolate-headed phage ϕ 197 (Schouler *et al.*, 1992). However, antibodies raised against POA17 were shown to react with two minor structural proteins of molecular mass 45 and 46 kDa, respectively, while ORF 31 encodes a protein of 66.2 kDa. It is possible that this 66.2 kDa protein is processed to fragments of 45 and 46 kDa. These proteins were localized at the end of the tail of phage ϕ 197 by electro-immuno microscopy (H. Neve & P. Ritzenthaler, personal communication). A calcium-binding domain (Nakayama *et al.*, 1992), consisting of a 13 residue loop (DKNHDKVVSQDEM) flanked by α -helical domains, named EF-hand, was found from position 413 to 425. This is most probably related to the calcium requirement for the development of bIL67 and numerous other phages (Potter, 1970). However, it is still unknown if calcium plays a role in the lactococcal phage adsorption via a depolarization of the membrane, as observed for T4 (Letellier & Labedan, 1985), or participates in DNA injection, as observed for the PL-I *Lactobacillus* phage (Watanabe & Takesue, 1972).

ORF 32

A search for conserved motifs suggests that the ORF 32 gene product could be a DNA binding protein with ATPase activity. Two ATP binding domains and a helix-turn-helix motif were found in the product of ORF 32. The ATP/GTP-binding site motif B (Walker *et al.*, 1982) was found at position 178–190 (TLDGGREQLVIID). The conserved domain which could be involved both in ATP binding and in phosphorylation of the substrate (Brenner, 1987), was found at position 459–482. This domain is observed in a

Fig. 5. Primary sequence of protein 37. For comparison, the primary sequence of ϕ 29 protein 14 is shown above the protein 37 sequence. Identical amino acids are represented by vertical bars and homologous amino acids by colons. The charged side chains are indicated by + or - above the sequence. Potential transmembrane domains, defined as regions of 20 generally hydrophobic residues with no net charge, are underlined. Potential β -turn regions as predicted by the Chou-Fasman prediction algorithm are indicated by 't'. The putative dual start motifs are indicated by arrows.

number of enzymes conferring antibiotic resistance, such as phosphotransferases and protein kinases (Brenner, 1987). The common pair of aspartic residues, presumably involved in the binding through Mg^{2+} of the phosphate groups of ATP (Brenner, 1987), were found at positions 462 and 479. Several other residues, less conserved, were also found in this region (data not shown).

Up to now, in all double-stranded DNA bacteriophages studied, the cutting and packaging of the phage chromosome is mediated by the phage-encoded enzyme, terminase. Terminases are ATP-binding and hydrolysing proteins that assemble to form multimeric packaging complexes (Black, 1988). As the product encoded by ORF 32 is the only one among all proteins encoded by the phage having an ATP-binding site, it could be a subunit of the terminase.

ORF 37

Endolysin activity accumulates in the cytoplasm during the vegetative cycle of many phages (Young, 1992). To gain access to its substrate, the cell wall, the lysin should be released from the cell. The release is thought to require a small (60–130 aa residues) phage-encoded protein, holin, which accumulates throughout the late gene expression period and has been shown, in the case of phage λ , to make pores in the membrane (Bläsi *et al.*, 1990). Prototype holin genes in phages λ , 21, T7 and ϕ 29 share no sequence homology but the distribution of charged and hydrophobic residues is conserved among the different proteins (Young, 1992). The proteins have two membrane-spanning α -helical domains separated by a short loop containing a turn, a short hydrophilic N terminus and a highly-charged C terminus. This suggests a common topology in the membrane, with both N- and C-terminal domains located in the cytoplasm (Young, 1992). Usually, the holin proteins exhibit a dual translational start motif (Young, 1992). All these characteristics were found for the protein encoded by ORF 37 (Fig. 5). Using software MACAW (Schuler *et al.*, 1991), a block of homology (26.7% identity and 53% similarity) was found between the 60 C-terminal amino acids of the proteins specified by ORF 37 and gene 14 of *B. subtilis* phage ϕ 29 (Steiner *et al.*, 1993). These results suggest that the ORF 37-encoded protein is a holin. The location of the bIL67 holin gene, 12.6 kb downstream of the lysin gene resembles the organization of T7, in which the holin gene (designated 17.5), is

separated from the lysin gene (designated 3.5) by more than 25 kb (Dunn & Studier, 1983), while most of the other holin genes map immediately upstream of the lysin gene.

CONCLUSIONS

bIL67 is the first prolate-headed phage from *L. lactis* for which the complete genomic sequence has been determined. Its genome is small and very compact since non-coding regions represent only 5% of the chromosome. It is organized in two transcriptional units, the early region and the late region, which represent 31% and 64% of the genome, respectively. Searches for protein homology allowed assignment of putative functions to products of three genes among the 37 identified. However, only one of these proteins shows homology with the product of a heterologous phage (*erf* gene of *S. typhimurium* phage P22). The three other assignments of function were based on analysis of protein structure or on the search for conserved domains. Phage proteins commonly show little or no amino acid homology with proteins from databases. Even the proteins which have the same function on different phages show little sequence conservation, and the similarities are mainly observed at the structural level, as illustrated by the case of the holin (Young, 1992) or the connector (Donate *et al.*, 1993).

The presence of several different direct repeats (two discussed above and a third one flanking ORF 24) suggests that, like other phages (Campbell & Botstein, 1983), lactococcal phages evolve by exchange of DNA modules. Moreover, these exchanges seem to involve essential functions such as DNA replication and recombination.

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