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ORIGINAL ARTICLE

Molecular characterization of a new lytic bacteriophage isolated from cheese whey

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Abstract In this study, we isolated and characterized a lytic *Lactococcus lactis* bacteriophage from the sera of a failed fermentation. The phage was isolated and cultured in *L. lactis* subsp. *cremoris* in M17 medium. The isolated bacteriophage was characterized by multiplex PCR, pulsed-field electrophoresis, DNA restriction digestion, analysis of the N-terminal sequence of the phage major structural protein, transmission electron microscopy and sequencing and analysis of a conserved fragment of its genome. Analysis of the viral genome indicates that its genome is composed of a DNA strand of approximately 48 kb in length, and PCR and microscopy confirmed that IL-P1 belongs to the group of 936-type phages in the family *Siphoviridae*, which is the most abundant type of lactococcal virus in dairy products worldwide. To our knowledge, this is the first report of a virus within this family that has a presumptive genome larger than 40 kb.

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Introduction

In the dairy industry, the majority of the starter cultures that are used for cheese production contain various strains of *Lactococcus lactis* bacteria. During cheese production, approximately 1 %-10 % of the fermentations are slow or fail to produce lactic acid and to reach the desired pH, which is required for product formation. Often, this suboptimal performance is due to bacterial infection with lytic bacteriophages, which compromises the product quality or may even prevent product formation [1]. Brazil is a major producer and consumer of dairy products worldwide, and the state of Minas Gerais accounts for approximately 26 % of the national production, 21 % of which is used as raw material for cheese production. Previous estimates have suggested that fermentation failure may occur as often as every two months within the same factory (unpublished data).

A large number of phages isolated from milk serum have been characterized by several techniques, including electron microscopy [2–4], total protein profile analysis [2, 5], serology [6], DNA-DNA hybridization [7], restriction digestion [4, 6, 8], PCR [4, 9, 10], and genomic sequencing [2, 11, 12]. Bacteriophages that infect lactic acid bacteria belong to the order *Caudovirales* and are typically characterized by an isometric or elongated head and a tail. The majority of the *L. lactis* phages studied thus far belong to the family *Siphoviridae* and can be subdivided into three main groups based on their structural and molecular characteristics. The 936-type phages, which account for approximately half of all the lactococcal bacteriophages that have been isolated, are virulent and have a small isometric head, whereas the c2-type phages, which are also virulent, have an elongated head; finally, the P335-type contains both virulent and non-virulent phages that are characterized by a small isometric head and a basal plate [13].

Studies regarding bacteriophage infection and replication are necessary to understand the impact of viral contamination on fermentation and dairy production. Knowledge regarding bacteriophage infection and propagation can allow researchers to design new strategies to prevent contamination, thereby increasing product yields. Currently, there is only one published report that describes the isolation and characterization of lactococcal phages in Brazil. More studies are needed to facilitate the development of new technologies that can reduce viral contamination and prevent product loss.

Technological advances facilitate basic research regarding virus isolation and identification, which may lead to the development of virus-resistant bacterial strains that may be applied as starter cultures to dairy production [14]. Thus, our goal was to isolate and characterize a lactococcal lytic bacteriophage in cheese whey from a failed fermentation at a dairy manufacturer in Minas Gerais, Brazil. This valuable information will allow future researchers to develop methods to prevent viral infection or to develop bacterial strains that are resistant to infection.

Materials and methods

Phage isolation and propagation

Twelve percent reconstituted skimmed milk (LDR), M17 medium (HIMEDIA, USA), or M17 medium supplemented with 0.5 % glucose (GM17) was used to culture the bacteria. The cheese whey was centrifuged at 3,000*g* for 10 minutes, and the supernatant was filtered through a 0.45-μm membrane to eliminate the bacterial debris. The viral particles were precipitated with 10 % polyethylene glycol (PEG) 8000 and 1.0 M NaCl for 48 hours at 4 °C and were centrifuged at 11,000*g* for 20 minutes. The precipitate was resuspended in 2 mL of specific buffer [15], and an equal volume of chloroform was added. After sedimentation and centrifugation (4,000*g* for 10 minutes) at 4 °C, the aqueous phase containing the phage was transferred to a 1.5-mL microtube. This viral concentrate was used to infect cultures of *Lactococcus lactis* subsp. *lactis* (strain H16) and *Lactococcus lactis* subsp. *cremoris* (strain R14) during the bacterial exponential growth phase. The phages were isolated according to Hull [16].

The phages were propagated with 1 mL of bacterial culture in the log phase. The sample was inoculated into 100 ml of GM17 medium containing 10 mM $CaCl_2$, and the culture was incubated until the bacteria reached an optical density (DO_{600}) of 0.6. The resulting cell suspension was infected with concentrated virus at a multiplicity of infection of 0.1-1 and was incubated at 30 °C for 30 hours. The suspension containing the propagated phage was centrifuged at 10,000*g* for 15 minutes, and the supernatant was filtered with a 0.22-μm membrane. The viral genome was extracted from the filtrate and analyzed; a second aliquot of the viral extract (50 mL) was purified with 10 % PEG 8000 and used for the protein analysis and electron microscopy studies.

Morphological analysis

Ten microliters of a 10X diluted viral suspension was added to a 200-mesh grid that was covered with Formvar® for 5 minutes. The excess liquid was removed with filter paper, and the reaction was covered with 10 μl of 2 % uranyl acetate for 20 seconds. The samples were analyzed with a transmission electron microscope (Zeiss EM 109 TEM) operating at 80 kV at the Nucleus of Microscopy and Microanalysis (NMM) at UFV. The data were analyzed, and the overall viral length, tail length, and capsid diameter were measured. When necessary, additional dilutions were performed to obtain higher-quality images.

Genome extraction

Genome extraction was performed as described previously [15]. Briefly, 50 μl of the isolated phage was added to an equal volume of lysis buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, and 0.5 % Tween 20) with 2 μl of proteinase K (20 mg/ml); the mixture was vortexed thoroughly and was incubated at 55 °C for 2 h. The samples were purified by standard phenol-chloroform extraction and ethanol precipitation procedures. The pellet was dissolved in 30 μl of distilled water, and the isolated nucleic acids were analyzed by electrophoresis in a 0.8 % agarose gel that was stained with ethidium bromide and analyzed under a UV light. To analyze the phage genome composition, 10 μl of genomic extract was digested with DNAse I (1 μg/mL) or RNase A (1 μg/mL) for 60 minutes at 37 °C. The digestion was analyzed via electrophoresis on a 1.0 % agarose gel.

Evaluation of the viral genome size

The length of the viral genome was estimated with pulsed-field electrophoresis using a Bio-Rad CHEF-DR® III Pulsed Field Electrophoresis Systems (Bio-Rad, USA). For this, 20 μL of the extracted genomic DNA was added to 20 μL of 2.0 % low-melt agarose (Bio-Rad, USA) according to the manufacture's recommendations. The samples were subjected to pulsed-field electrophoresis on a 1.0 % agarose gel at an angle of 120 degrees, with a switching time of 5-200 seconds at 14 °C. The gel was stained with ethidium bromide and analyzed under a UV light. The viral genome was digested with *EcoR* I, *Hind* III, *BamH* I (Fermentas, Canada) or *Hae* III (Invitrogen, USA)

enzyme according to the manufacturers' protocol. Approximately 1 μg of viral DNA was diluted in the manufacturer's recommended buffer. The dilution was incubated at 80 °C for 10 minutes and then at 37 °C for 10 minutes. Subsequently, 1 U of the respective enzyme was added, and the samples were digested for 3 hours at 37 °C. The digested samples were analyzed via electrophoresis on a 1 % agarose gel and stained with ethidium bromide.

Phage classification by multiplex PCR

The primers that were used to amplify specific segments of the viral genome were described previously by del Rio et al. [10]. The primers were originally designed to amplify conserved regions in the three major groups of lactococcal phages (Supplemental information). DNA amplification was performed with a mixture of 1 μg of DNA, 1X Special PCR Buffer containing 3.5 mM $MgCl_2$ (Phoneutria, Brazil), 0.5 mM dNTPs, 0.2 pmol of each primer, and 1.5 U of Taq DNA polymerase (Phoneutria, Brazil). PCR amplification was performed in a MasterCycler Personal (Eppendorf, Germany) thermocycler and the products were amplified with the following protocol: initial denaturation at 94 °C for 3 minutes, followed by 40 cycles of 94 °C for 30 seconds, 50 °C for one minute and 72 °C for one minute, and final extension at 72 °C for 7 minutes. The amplicons were analyzed by electrophoresis on a 1.2 % agarose gel stained with ethidium bromide and were analyzed under a UV light.

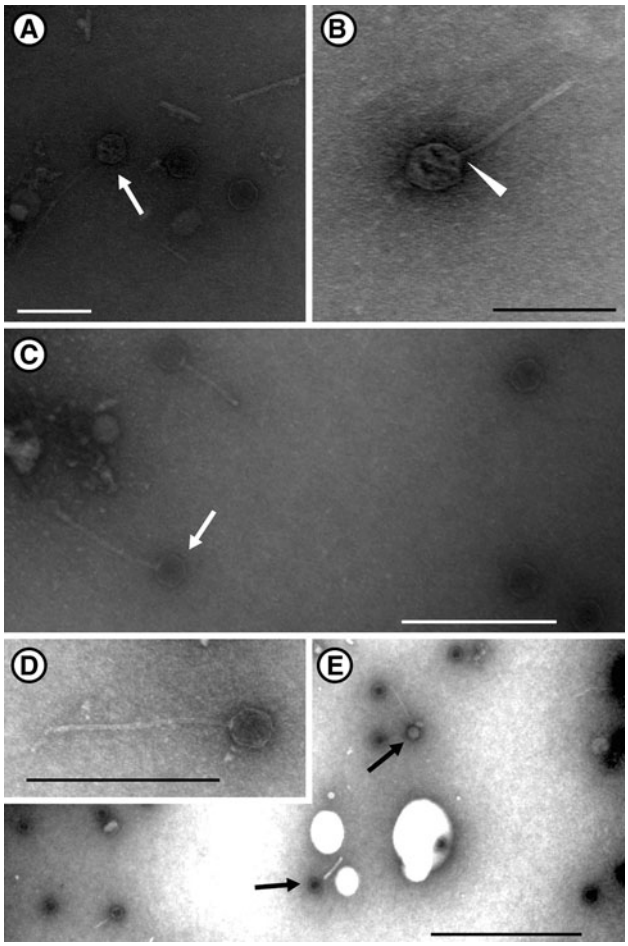


Fig. 1 Transmission electron microscopy of IL-P1. Diluted viral suspensions were placed onto 200-mesh grids covered with Formvar® and 2 % uranyl acetate and analyzed using a transmission electron microscope. Intact viral particles are indicated by arrows. Phage IL-P1 has an average size of 230 nm, a 180-nm-long tail, an isometric head approximately 50 nm in diameter, and does not have a neck (white arrow head on **B**) or basal plate. Note in Figures **A**, **C** and **E** the presence of particles without a tail (not intact) generated by the purification method used. Scale bars: **A** = 100 nm; **B** = 100 nm; **C** = 200 nm; **D** = 200 nm; **E** = 500 nm

Sequencing and analysis of PCR products

The PCR products were cloned into pGEM-T Easy Vector (PROMEGA, USA) according to the manufacturer's recommendations. Competent *Escherichia coli* DH5α cells

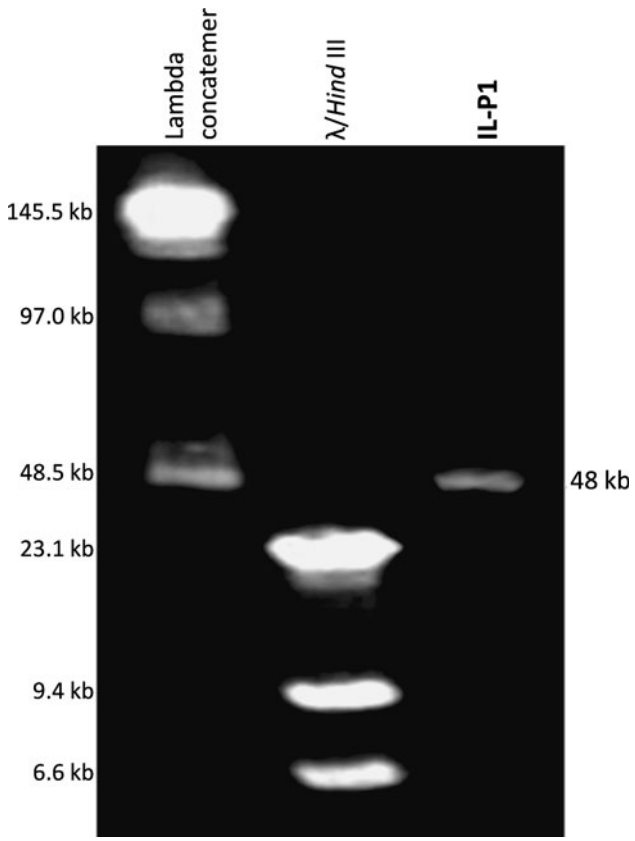


Fig. 2 Pulsed-field gel electrophoresis of the viral genome. The IL-P1 genome was extracted with phenol-chloroform and was subjected to pulsed-field agarose gel electrophoresis to determine the approximate size of the genome. The gel was stained with ethidium bromide and viewed under an ultraviolet light, which revealed a band that was approximately 48 kb in length

were transformed by heat shock at 42 °C for 90 seconds. The transformed bacteria were recovered in LB medium at 37 °C for 2 hours with shaking at 180 rpm, and the bacteria were plated on selective medium containing 50 g/mL ampicillin. Colony PCR with sequence-specific primers was used to verify the presence of the insert. An aliquot of the positive colonies was stored at −80 °C, whereas a second aliquot was used for plasmid purification using a Wizard® Plus SV Miniprep (PROMEGA, USA) kit according to the manufacturer's instructions. The plasmid DNA was sequenced by the Genomic Sequencing Laboratory of the Federal University of Viçosa. The sequences were aligned with other viral sequences from the GenBank database using the BLAST software. The evolutionary distance was analyzed using the neighbor-joining and bootstrap configurations in the CLUSTALW software.

Analysis of the phage major structural protein

The total phage protein content was analyzed by electrophoresis on a 15 % polyacrylamide gel under denaturing conditions (SDS-PAGE) [17]. Phage protein (15 µl) was loaded and electrophoresed at 190 V for 1 h. The Broad Range SDS-PAGE Standard was used as a molecular size marker, and the gel was stained with 3 % ammoniacal silver. Phage proteins were transferred to a PVDF membrane (200 V, 1.40 h), stained with Coomassie brilliant blue solution for ten minutes, and revealed by treatment with 50 % methanol. The chosen band was excised from the membrane and inserted into a PPSQ33A Shimadzu sequencer at the Nucleus of Biomolecules (NuBio) at Federal University of Viçosa.

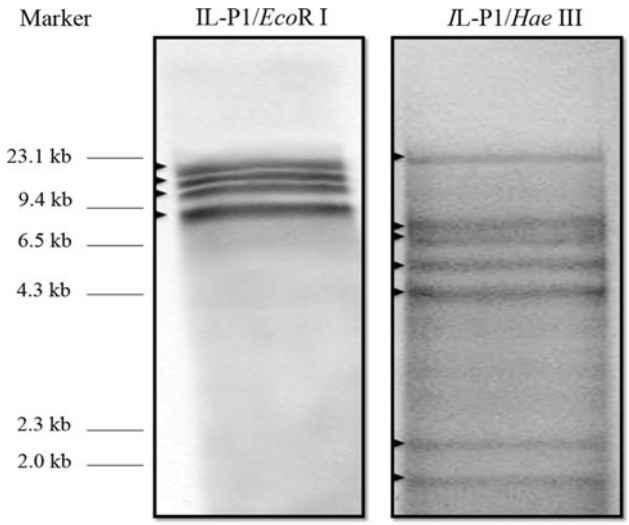


Fig. 3 Restriction digestion of the IL-P1 genome with EcoR I and Hae III. The phage genome was digested with EcoR I, Hind III, BamH I, or Hae III for 3 hours at 37 °C, electrophoresed on a 1 % agarose gel, and stained with ethidium bromide. Digestion of the IL-P1 genome with EcoR I generated four fragments of approximately 19.0, 12.0, 9.0, and 7.5 kb (small triangles in the left column), whereas Hae III digestion produced seven fragments of about 20.0, 7.5, 6.5, 5.0, 4.5, 2.5 and 2.1 kb (small triangles in the right column)

Results

Viral structure

Transmission electron microscopy indicated that phage IL-P1 has an average size of 230 nm, a 180-nm-long tail, and an isometric head that is 50 nm in diameter. Interestingly, no basal plate was observed (Fig. 1). These characteristics are consistent with the 936-type phages that belong to the family *Siphoviridae*, order *Caudovirales*. In general, this group is characterized by an isometric head that is 45-65 nm in diameter, a collar, a tail that is 100-200 nm in length, and the absence of a basal plate [18, 19]. Different

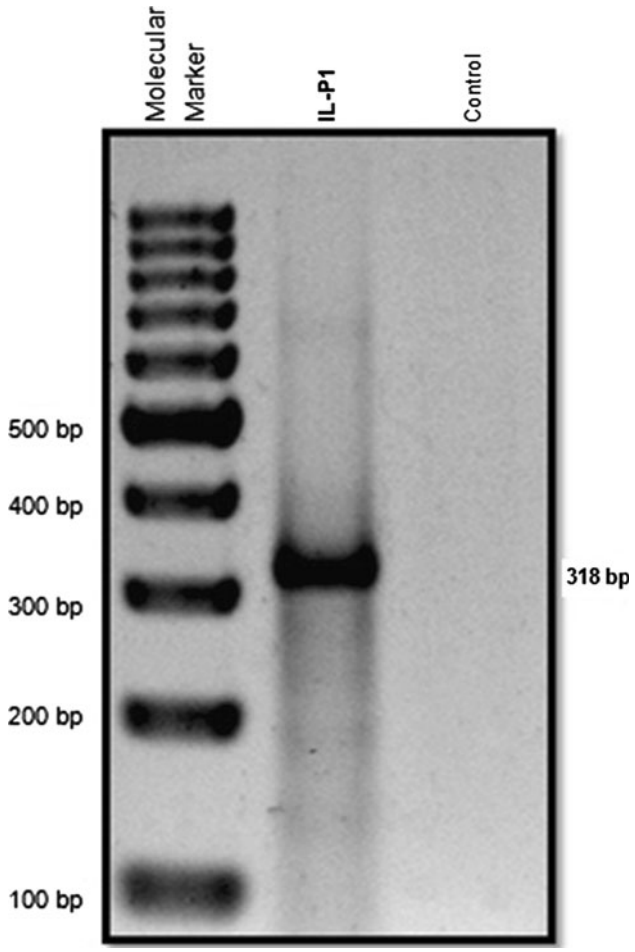
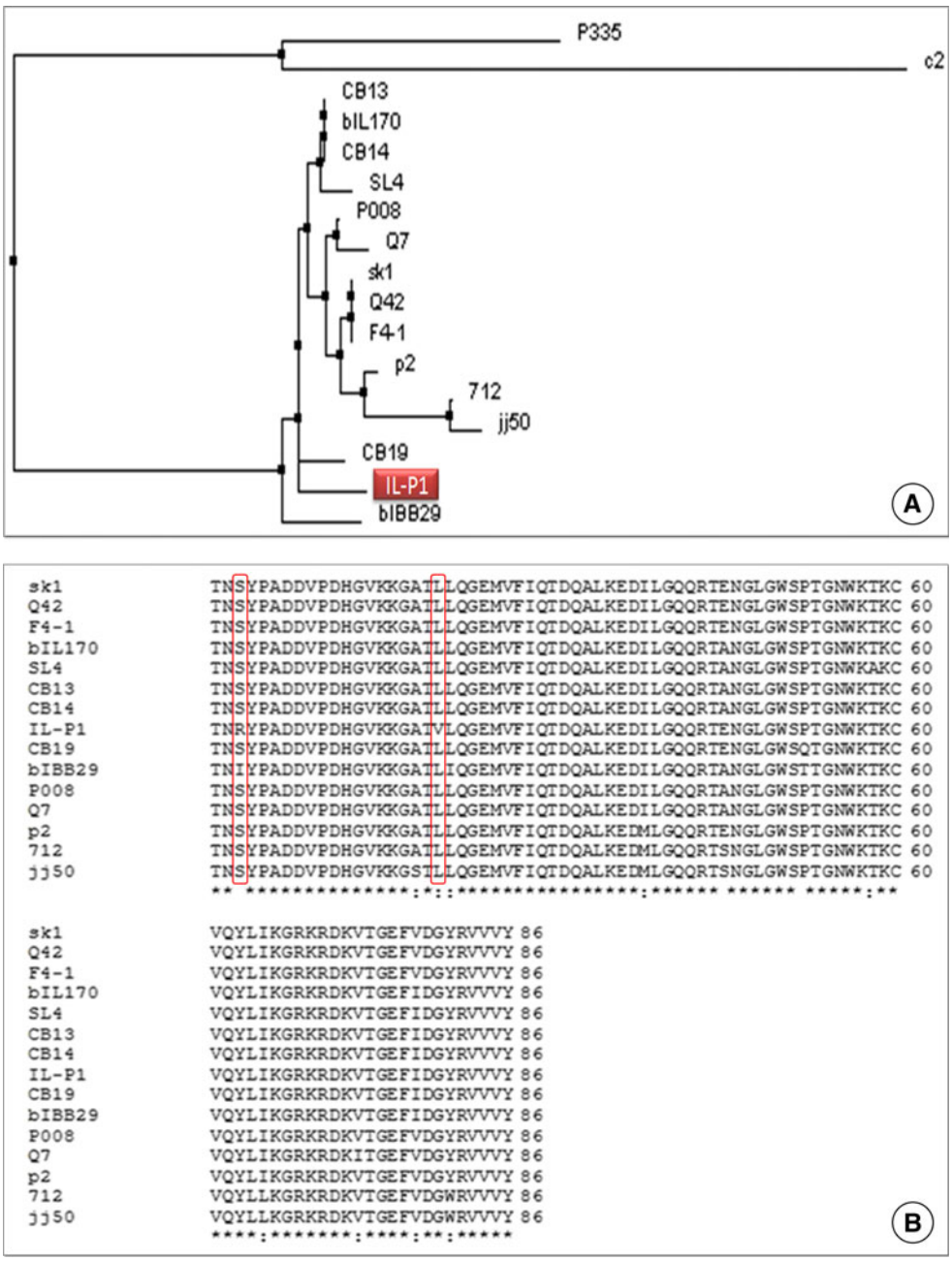


Fig. 4 Phage classification via multiplex PCR. A portion of the IL-P1 genome was amplified via multiplex PCR with primers that are specific for conserved genomic regions in the three major groups of lactococcal phages. PCR amplification produced an amplicon that was approximately 320 bp in size



members of this group have slight variations in these morphological characteristics, although these variations are not determinants of virus classification. The only Brazilian report that describes a morphological analysis of lactococcal bacteriophages was published by Oliveira et al. [8]. In that study, the authors isolated ten different phages that had isometric heads approximately 43 nm in diameter and 142-nm-long tails.

Genome analysis

The phage, which we termed "Immunovirology Laboratory-Phage 1" (IL-P1), was isolated and propagated in *Lactococcus lactis* subsp. *cremoris*. The viruses were filtered with a 0.22-μm membrane, and the viral genome was extracted. Aliquots of the viral genome were digested with DNase I or RNase A. While DNAse I digestion degraded the viral genome, RNase A digestion had no effect, indicating that the viral genome is composed of DNA. Pulsed-field gel electrophoresis studies indicated that the IL-P1 genome is approximately 48 kb in length (Fig. 2).

Digestion of the IL-P1 genome with *Eco*R I and *Hae* III produced four (19.0, 12.0, 9.0, 7.5 kb) and seven (20.0, 7.5, 6.5, 5.0, 4.5, 2.5, 2.1 kb) DNA fragments, respectively, whereas digestion with *Hind* III and *Bam*H I produced only one fragment (Fig. 3). All of the reactions were repeated at

Table 1 Phage sequences that are related to IL-P1 and were aligned using the BLAST software

Phage	GenBank ID	Year	Country	Group	Identity
CB14	ACU46881.1	2009	Canada	936-like	96 %
CB13	ACU46826.1	2009	Canada	936-like	96 %
Q42	AAF85634.1	2000	Canada	936-like	96 %
F4-1	P26596.1	2009	United States	936-like	96 %
CB19	FJ848885.1	2009	Canada	936-like	95 %
bIL170	AF009630.1	2002	France	936-like	95 %
Q7	AAF85635.1	2000	Canada	936-like	94 %
SL4	FJ848881.1	2009	Canada	936-like	93 %
sk1	AF011378.1	1997	Australia	936-like	93 %
bIBB29	EU221285.1	2009	Poland	936-like	93 %
P008	AF011378.1	2006	Germany	936-like	91 %
712	ABB77578.1	2006	Ireland	936-like	91 %
p2	GQ979703.1	2010	Canada	936-like	90 %
jj50	ABB77632.1	2006	Ireland	936-like	90 %
P335	DQ838728.1	2008	France	P335-like	17 %
c2	L48605.1	1996	New Zealand	c2-like	10 %

least twice for each enzyme, and the results were reproducible.

A multiplex PCR was performed with primers that are specific for conserved regions of the genome in the three main groups of lactococcal bacteriophages (Supplemental information). A portion of the IL-P1 genome was amplified with these, which produced an amplicon that was approximately 320 bp (Fig. 4). Based upon the classification reported by del Rio et al. [10], this phage belongs to the large group of 936-type bacteriophages in the family *Siphoviridae*, order *Caudovirales*.

The nucleotide sequence was translated to its corresponding protein sequence, and an alignment was performed using the BLAST software. The fragment obtained in this work was very similar to that of the other 936-type phage sequences in the UniProt database (Fig. 5). Fourteen of the aligned sequences were selected to construct a distance-based tree using the CLUSTALW software (Table 1). IL-P1 shares a high degree of sequence identity with the major members of the 936-type phage group and is clustered with them. However, IL-P1 has two point mutations that phylogenetically separate it from the other related bacteriophages. Because of these mutations, IL-P1 is most closely related to the bIBB29 and CB19 isolates.

Protein analysis

We analyzed the total viral protein content by 15 % denaturing polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Fig. 6, the protein profile of phage

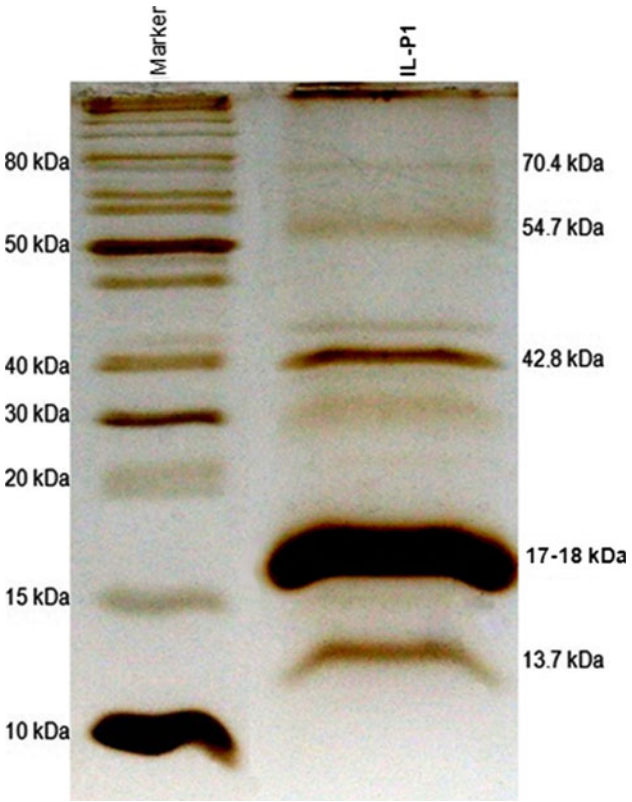


Fig. 6 Analysis of the IL-P1 protein content via SDS-PAGE. The phages were purified with polyethylene glycol 8000 and run on a 15 % polyacrylamide gel under denaturing conditions (SDS-PAGE). Staining with 3 % ammoniacal silver indicated three major protein bands approximately 13.5, 17, and 43 kDa in size, which is a pattern that is typical for the lactococcal phages. Minor bands corresponding to proteins 70.5, 55, 45, and 31.5 kDa in size were also observed

IL-P1 contains three major structural proteins and is similar to patterns observed for other lactococcal bacteriophages [20–22]. The most abundant protein band corresponds to a molecular mass of approximately 17 kDa, whereas the second-most abundant proteins correspond to approximately 43 and 13.5 kDa. The additional minor bands indicate the presence of proteins that are approximately 70.5, 55, 45 and 31.5 kDa. The major structural protein of 17 kDa was chosen to be N-terminally sequenced. The N-terminal sequence obtained has 13 amino acid residues and has 92 % query coverage and 100 % sequence identity with the C-terminal domain of the lactococcal bacteriophage P2 tail spike protein, Gpv [23–25] (Table 2).

Discussion

In this study, we isolated and characterized a lytic bacteriophage (IL-P1) that caused cottage cheese fermentation failure at a manufacturing facility in Minas Gerais, Brazil.

Table 2 N-terminal sequence alignment of the major IL-P1 structural protein

Description	PDB ID:	Query coverage	Max ident	Alignment
C-terminal domain of the lactococcal bacteriophage P2 tail spike protein, Gpv	3AQJ	92 %	100 %	--AHHHHHHSGTDGS MHHHHHHHHSGDNPS ******** : *

Genomic and morphological analysis indicate that the bacteriophage IL-P1 contains a DNA genome that is approximately 48 kb in length, has a small isometric head that is approximately 50 nm in diameter, has a long tail that stretches 180 nm, and does not have a basal plate. PCR amplification of a portion of the genome indicated that IL-P1 belongs to the large group of 936-type phages. Generally speaking, this group is characterized by a small isometric head, a collar, a long non-contractile tail, and the absence of a basal plate. Moreover, the type-936 viral genome is comprised of linear, double-stranded DNA that is approximately 30 kb and contains cohesive ends. Representative phages within this group are the P008 phage, which has a 29.7-kb genome [25]; bLI170, which has a 31.8-kb genome [26]; bIBB29, with a 29.3-kb genome [12]; and sk1, with a 28.4-kb genome [27]. Thus, the bacteriophage isolated in this study is a 936-type phage that shares these common characteristics, such as protein content and general morphology, with other members of this group, but has a presumptive genome size of 48 kb.

When the IL-P1 genome was digested with either the *Eco*R I or *Hae* III restriction enzyme, four and seven fragments were produced, respectively. These results are consistent with the results published previously by Loof et al. [28] for the P008 phage genome, which is a member of the 936-type group. Loof and colleagues obtained four and six fragments when the P008 phage genome was digested with *Eco*R I or *Hae* III, respectively. Oliveira et al. [8] obtained similar results when they digested lactococcal phage genomes with *Eco*R I, which generated three to five DNA fragments. However, digestion of the IL-P1 genome with *Bam*H I and *Hind* III produced only one fragment, even though bioinformatic analysis predicts 10 fragments when a lactococcal phage genome is digested with *Hind* III [28]. Studies with other lactococcal phages have also shown that *Hind* III digestion produces only one fragment [8].

Phylogenetic analysis indicates that the bacteriophage isolated in this study is closely associated with the other 936-type phages. Detailed analysis indicates that IL-P1 is closely related to bIBB29, which may be due to a common mutation found in the amplified sequence, which belongs to a gene that encodes for a structural capsid protein. A high degree of identity was found between the IL-P1 major structural protein and a tail spike protein of the lactococcal phage P2, beyond other phage tail proteins. Moreover, Crutz-Le Coq et al. [19] created a three-dimensional model of the main tail structural protein for a 936-type lactococcal bacteriophage, which predicts a globular protein that is approximately 20 kDa. Since the predominant protein in IL-P1 is approximately 17 kDa, this confirms that the IL-P1 major structural protein is a tail protein.

Lactic acid bacteria (LAB) can be infected and lysed by an extensive range of bacteriophages, which can prevent or slow fermentation during dairy production. Contamination during fermentation can affect the quality of the dairy product and may even prevent product formation. In this work, we have isolated and characterized a new lactococcal 936-like phage (IL-P1) from a failed fermentation. IL-P1 has a presumptive 48-kb DNA genome, which is the largest known genome for members of this group. Moreover, IL-P1 possesses a long tail that is 180 nm in length and an isometric head that is 50 nm in diameter. Future studies regarding the mechanisms of viral infection and replication are needed to understand the impact of IL-P1 contamination on dairy fermentation. These results may allow the development of new strategies to prevent viral contamination or the development of a bacterial strain that is resistant to viral infection.

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