International Dairy Journal 19 (2009) 476–480

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Streptococcus thermophilus phage monitoring in a cheese factory: Phage characteristics and starter sensitivity

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ARTICLE INFO

Article history:
Received 18 September 2008
Received in revised form
13 February 2009
Accepted 18 February 2009

ABSTRACT

Nineteen *Streptococcus thermophilus* phages isolated from an Argentinean cheese plant were studied regarding their host range, morphology, packaging mechanism and nucleotide sequence of the variable region VR2 (one of the genetic determinants of host specificity). According to their features, they were morphologically classified into *Siphoviridae* family, morphotype B1. Among the phages tested, 13 distinct restriction patterns were found, and only one phage showed a *pac*-type mechanism. The classification based on the VR2 sequence proved to be very useful, since phages with very different VR2 sequences showed clearly different host ranges.

1. Introduction

Commercial fermentative dairy processes are vulnerable to virulent bacteriophages (ubiquitous in the environment) that can lyse lactic acid bacteria, thereby delaying lactic acid production and even stopping the fermentation process (Moineau and Lévesque, 2005). Since large quantities of milk are used in dairy fermentation processes, phage infection can lead to substantial economic losses. Despite the development of different countermeasures (culture rotation, improved sanitization programs, use of bacteriophage-resistant strains, etc.), phage contamination continues to be the most significant cause of failed and delayed fermentations. This problem proved to be more serious in the last decade because of the use of defined strain cultures, which establish a continuous reservoir of hosts that helps in maintaining phage contamination (Bruttin et al., 1997).

Streptococcus thermophilus is one of the most important bacteria used as a component of starter cultures for the production of fermented milk products and a large variety of cheeses (Reinheimer et al., 1997). As many other lactic acid bacteria, it is susceptible to the lethal action of bacteriophages. Taking into account the extensive use of *S. thermophilus* in Argentinian dairy processes and the significant number of episodes linked to phage infections for this species (Binetti et al., 2005; Quiberoni et al., 2006; Suárez et al., 2002), it becomes indispensable to improve control measures to keep phage particles at low levels. One of the most applied strategies used to minimize phage problems is the traditional non-phage-related strains rotation. A proper application of rotation programs needs to be accompanied by a deep knowledge of the interaction phage/sensitive strain, as well as the ecological phage diversity within a certain dairy plant. This should include not only host range, but additional studies based on genetic characteristics of phages that help for selecting the most adequate dairy strain(s) by considering each particular industrial situation.

Nowadays, the knowledge of eight complete genomic sequences for *S. thermophilus* phages (four *cos*- and four *pac*-type) has allowed the development of different molecular methods to detect, identify, characterize and quantify them, facilitating the classification of *S. thermophilus* bacteriophages according to diverse criteria (Binetti et al., 2005; Brüssow et al., 1994; del Río et al., 2007, 2008; Duplessis and Moineau, 2001; Quiberoni et al., 2006). Moreover, some studies have addressed the biological function of phage gene products. In this context, at least three structural proteins were demonstrated to be involved in the phage-host interactions in *S. thermophilus* (Duplessis and Moineau, 2001; Duplessis et al., 2006). A PCR method to amplify a variable region belonging to one of these genetic determinants was designed in order to detect and classify *S. thermophilus* phages (Binetti et al., 2005).

In the present work, the phage diversity of one Argentinean cheese plant was studied. The final purpose was to guide this factory in the selection of adequate strains in order to minimize phage infection problems. The selection criteria were based on the characterization of phages found in this industrial environment by means of molecular tools previously developed that complement the traditional microbiological methodologies.

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doi:10.1016/j.idairyj.2009.02.009

2. Materials and methods

2.1. Bacterial strains and culture conditions

All samples for phage monitoring were accompanied by a sample of the commercial lactic starter used in the respective cheese manufacture. *S. thermophilus* strains were cultivated in Elliker broth (Biokar, Beauvois, France) at 42 °C, and kept as frozen (−80 °C) stocks in Elliker broth with added glycerol (15% v/v) in the INLAIN (Instituto de Lactología Industrial, Santa Fe, Argentina) collection.

2.2. Phage isolation, enumeration and morphology

For isolation of the phages, whey samples were centrifuged (3000 × *g*, 20 min at 4 °C) and the supernatants were filtered (0.45 μm pore diameter; Millipore, Indústria e Comércio Ltda., São Paulo, Brazil). The filtrates with and without thermal treatment (15 min, 90 °C) were used to investigate the presence of phages. For this aim, spot and turbidity tests were performed (Svensson and Christiansson, 1991). In those samples where an evident lysis was revealed, phages were counted using the double layer plaque titration method (Svensson and Christiansson, 1991). To isolate and purify the phages, lysis plaques were picked up and placed in 5 mL of Elliker-Ca (Elliker supplemented with 10 mM $CaCl_2$) broth. Tubes were kept 24 h at 4 °C and then inoculated with 200 μL of log phase indicator strains (OD_{560} 0.8). Incubation at 42 °C was carried out until the total lysis of the culture was reached. This procedure was carried out three consecutive times.

Once purified, micrographs of phage particles were taken according to Bolondi et al. (1995).

2.3. Host range

To determine the host range of bacteriophages, the microtitre plaque method (Zago et al., 2006) was initially used as screening test, and then confirmed using the traditional turbidity test (Svensson and Christiansson, 1991). In both cases, high-titre phage suspensions of $>10^8$ plaque forming units (pfu) mL^{-1} were used.

2.4. Isolation and analysis of phage DNA

Phages were propagated in 100 mL of Elliker-Ca broth, centrifuged (10 min, 10,000 × *g* at 4 °C), filtered (Millipore membranes, 0.45 μm) and treated (30 min, 37 °C) with 1 μg mL^{-1} DNAse I (Sigma–Aldrich, St. Louis, MO, USA) and 1 μg mL^{-1} RNAse A (Sigma–Aldrich). Phage DNAs were isolated as previously reported by Binetti et al. (2008). DNA pellets were washed with 70% (v/v) ethanol, dried, and resuspended in TE buffer (10 mM Tris–HCl, pH 8.0, containing 1 mM EDTA; Sambrook and Russell, 2001).

Purified phage DNAs were treated with *Eco*RV, *Pst*I and *Acc*I according to the specifications of the supplier (GE Healthcare, Little Chalfont, UK). Restriction patterns were visualized under UV light by ethidium bromide staining after electrophoresis in agarose gels (0.8%, w/v; Sambrook and Russell, 2001).

2.5. Genetic determinants of packaging mechanism (cos/pac)

To study the putative packaging determinants of *S. thermophilus* phages a multiplex PCR method was performed according to Quiberoni et al. (2006). Primers (Sigma–Genosys, The Woodlands, TX, USA) cos-FOR, cos-REV, pac-FOR and pac-REV were used (Table 1). PCR reactions were performed in a total volume of 25 μL containing 125 μM of each dNTP (GE Healthcare), 5 μM of each primer, 2.5 U *Taq* DNA polymerase (GE Healthcare) and 1 μL of diluted (1:50) phage DNA. Amplifications were carried out in a GeneAmp PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 53 °C for 45 s, and 72 °C for 1 min, and a final extension step at 72 °C for 5 min.

Table 1
Primers used in this study.

Primer	Sequence	Reference
cos-FOR	5′-GGTTCACGTGTTTATGAAAAATGG-3′	Quiberoni et al., 2006
cos-REV	5′-AGCAGAATCAGCAAGCAAGCTGTT-3′	Quiberoni et al., 2006
pac-FOR	5′-GAAGCTATGCGTATGCAAGT-3′	Quiberoni et al., 2006
pac-REV	5′-TTAGGGATAAGAGTCAAGTG-3′	Quiberoni et al., 2006
HOST1	5′-GAATGATACTGCTGGCAGTATTTCGGTTGG-3′	Binetti et al., 2005
HOST5	5′-CAGTCATGTAGCTATCGATGAAATTCCAACG-3′	Binetti et al., 2005

2.6. Characterization of the antireceptor variable region

Amplification of VR2 region was performed according to Binetti et al. (2005), using primers HOST1 and HOST5 (Table 1). PCR reactions were carried out as indicated, in a total volume of 50 μL with each primer at a concentration of 400 nM. PCR conditions included an initial denaturation step at 94 °C for 3 min, followed by 35 amplification cycles of 94 °C for 30 s, 50 °C for 1 min, and 72 °C for 1 min, and finally, an extension step at 72 °C for 7 min.

2.7. Nucleotide sequence analysis

PCR products were purified using MicroSpin Columns (GE Healthcare) and their nucleotide sequences were determined by primer extension at the DNA Sequencing Service of Macrogen Inc. (Seoul, Korea). Sequence data were assembled and compared using ClustalW Tools (http://www.ebi.ac.uk/Tools/clustalw/), BioEdit Sequence Alingment Editor (Hall, 1999), and Pregap and Gap4 from Staden Package (http://staden.sourceforge.net/) (Staden et al., 2003). A phylogenetic tree was constructed from the alignment using the MSVP 3.13d (1985–2002) software (Kovach Computing Services, Wales, UK) and the neighbour-joining method (Saitou and Nei, 1987).

3. Results and discussion

3.1. Host range

Nineteen phages were isolated from whey samples obtained during cheese-making with an abnormal fermentation pattern in one Argentinean cheese plant between 2001 and 2007. Fourteen commercial *Streptococcus thermophilus* strains were isolated from the starter cultures used in the monitored cheese plant, and identified as phage-sensitive (Table 2). The isolated phages were tested against the 14 strains. Results are shown in Table 3. Some phages revealed a very limited host range, being capable to infect only one (ϕNL10 and ϕNL17) or two strains (ϕLy2, ϕLy3, ϕN2, ϕND2 and ϕNL16), while ϕNS1 was able to attack six strains. Phages ϕLy2 and ϕLy3 showed the same host range. Phages ϕNS1, ϕNS2, ϕNF2 and ϕNL18 shared a broad range, infecting four similar strains (ST NL1, ST TNL, ST NL13 and ST NL18). Most of strains showed to be sensitive to several of the phages tested.

All phages revealed similar morphology, showing isometric capsids ranging from 66 to 70 nm diameter, flexible, non-contractile long tails ranging from 285 to 310 nm in length, and from 7.9 to 10.1 nm in width (Table 2). These features suggested to include them into *Siphoviridae* family and B1 morphotype (Ackermann, 2001), as all *S. thermophilus* phages previously studied (Binetti

Table 2
Streptococcus thermophilus bacteriophages used in this study.

Phage	Source[a]	Host	Dimensions (nm)[b]	Genome size (kb)[c]
ϕNS1	Cremoso cheese, October 2001 (1.2×10^6)	ST NL1	$67 \times 267 \times 8.5$	35.1
ϕNS2	Cremoso cheese, October 2001 (5×10^4)	ST NL1	$65 \times 305 \times 9$	34
ϕLy-1	Cuartirolo cheese, July 2004 (5×10^4)	ST Ly1	$67 \times 302 \times 10$	32.9
ϕLy-2	Cuartirolo cheese, July 2004 (1×10^7)	ST Ly1	$71 \times 288 \times 9.5$	34.3
ϕLy-3	Cuartirolo cheese, July 2004 (1.2×10^7)	ST Ly3	$66 \times 275 \times 9$	34.3
ϕN2	Cremoso cheese, June 2005 (4.1×10^3)	ST N2	$69 \times 295 \times 7.5$	33.4
ϕTNL	Barra and Cremoso cheese, June 2006 (1.5×10^4)	ST TNL	$68 \times 310 \times 10.1$	32.4
ϕNL10	Cremoso cheese, August 2006 (9×10^5)	ST NL10	$68 \times 285 \times 7.8$	30.5
ϕNC1	Cremoso cheese, October 2006 (1.8×10^4)	ST NL12	$66 \times 266 \times 9.5$	29
ϕNB1	Barra cheese, October 2006 (3.7×10^5)	ST NL12	$72 \times 270 \times 10.1$	29
ϕNF1	Semi-direct starter culture, October 2006 (3×10^1)	ST NL12	$68 \times 275 \times 10$	29
ϕNC2	Cremoso cheese, November 2006 (3×10^1)	ST NL13	$70 \times 272 \times 9.8$	34.6
ϕNF2	Semi-direct starter culture, November 2006 (5×10^1)	ST NL13	$65 \times 293 \times 8.5$	35.1
ϕND1	Barra cheese, February 2007 (6.4×10^5)	ST ND1	$67 \times 290 \times 10$	31.6
ϕND2	Barra cheese, February 2007 (3×10^1)	ST ND2	$67 \times 302 \times 9$	32
ϕNL-15	Barra cheese, March 2007 (2×10^1)	ST NL15	$68 \times 288 \times 9.5$	31.1
ϕNL-16	Cremoso cheese, March 2007 (1×10^6)	ST NL16	$67 \times 276 \times 8.8$	32.1
ϕNL-17	Cremoso cheese, March 2007 (1×10^2)	ST NL17	$70 \times 270 \times 10$	34.6
ϕNL-18	Cremoso cheese, April 2007 (4×10^1)	ST NL18	$69 \times 300 \times 9.2$	35.1
ϕ0BJ[d]	Positive control for VR2	15-C		

[a] Phage source includes cheese type, isolation date and phage level (pfu mL^{-1}) in the sample tested.
[b] Capsid diameter × tail length × tail diameter.
[c] Calculated by adding the fragments obtained after restriction with *Eco*RV, *Pst*I and/or *Acc*I.
[d] Suárez et al. (2002).

et al., 2005; Brüssow et al., 1994; Bruttin et al., 1997; Quiberoni et al., 2006).

3.2. Restriction patterns

Among the 19 tested phages, there were found 13 distinct restriction patterns when *Eco*RV, *Pst*I and *Acc*I enzymes were used. The sizes of phage genomes were estimated by adding the size of each visible *Eco*RV (Fig. 1), *Pst*I and *Acc*I (data not shown) digested DNA fragments. They ranged from 28 to 35 kb (Table 2). Some phages with identical restriction profiles were isolated from diverse samples collected in the same day (e.g., ϕNC1, ϕNF1 and ϕNB1), suggesting that a same bacteriophage infected different vats of the dairy plant. On the contrary, some of them were isolated from samples collected in different moments (ϕNS1, ϕNF2 and ϕNL18, isolated in 2001, 2006 and 2007, respectively). These results indicated that the resident phage population detected in 2001 would persist in the dairy environment for at least 7 years, even when the concentration of phage particles were not enough to produce fermentation failures. These three bacteriophages shared identical restriction profiles, while they showed host ranges highly related but not the same. The differences in their infectivity patterns would obey to different possible causes as point mutations in genetic determinants involved in recognition of *S. thermophilus* host (Klaenhammer and Fitzgerald, 1994); prophage associated sequences in the host genome that protect it from becoming infected by additional phages, and mediate superinfection exclusion (Sun et al., 2006; Ventura et al., 2002); native phage resistance mechanisms (Sturino and Klaenhammer, 2006) or phage resistance associated to new spacers integrated within the clustered regularly interspaced short palindromic repeats (CRISPR) loci of *S. thermophilus* strains (Barrangou et al., 2007; Deveau et al., 2008). The remaining phages (ϕLy1, ϕN2, ϕTNL, ϕNL10, ϕND1, ϕND2, ϕNC2, ϕNL15, ϕNL16 and ϕNL17) showed unique DNA restriction profiles and host ranges.

3.3. Genetic determinants of packaging mechanism (cos/pac)

When the possible DNA packaging mechanisms were studied, 18 out of 19 tested phages revealed the presence of genetic determinants of *cos*-type packaging mechanism, since PCR products of the expected size for *cos*-type phages (170 bp) were observed. The exception was ϕNL10, which showed a PCR fragment of 427 bp, indicating a likely *pac*-type mechanism profile (data not shown).

Table 3
Host range of *Streptococcus thermophilus* phages isolated from whey samples.

Strain	Phage[a]																		
	ϕLy1	ϕLy2	ϕLy3	ϕN2	ϕNS1	ϕNS2	ϕTNL	ϕNL10	ϕND1	ϕND2	ϕNC1	ϕNB1	ϕNF1	ϕNC2	ϕNF2	ϕNL15	ϕNL16	ϕNL17	ϕNL18
ST Ly1	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
ST Ly3	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
ST N2	−	−	−	+	−	−	−	−	−	−	+	+	+	−	−	+	−	−	−
ST NL1	−	−	−	−	+	+	+	−	+	−	+	+	+	+	+	−	−	−	+
ST TNL	−	−	−	−	+	+	+	−	−	−	−	−	+	+	−	−	−	−	+
ST NL10	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−
ST ND1	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−
ST ND2	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	+	−	+
ST NL12	+	−	−	−	+	+	−	−	+	−	+	+	+	+	−	+	−	−	−
ST NL13	−	−	−	−	+	+	+	−	−	−	+	−	−	+	+	−	−	−	+
ST NL15	−	−	−	+	−	−	+	−	−	−	−	+	−	−	−	+	−	−	−
ST NL16	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	+	−	−
ST NL17	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
ST NL18	−	−	−	−	+	+	+	−	−	−	−	−	−	+	+	−	−	−	+

[a] +, indicates that the strain was sensitive to the phage; −, not sensitive to the phage.

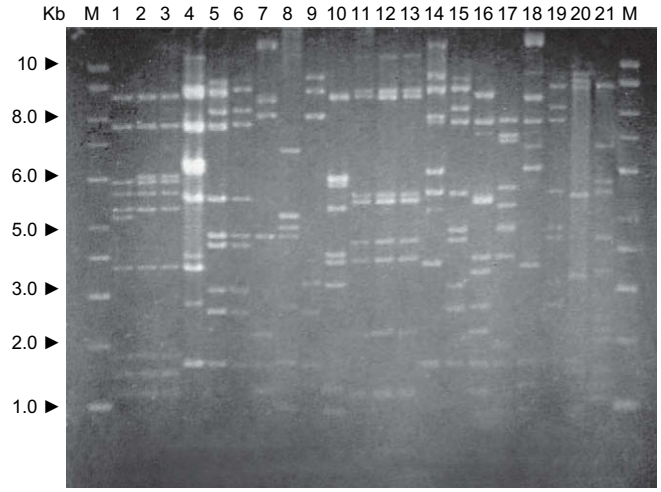

Fig. 1. Agarose gel electrophoresis of the *EcoRV*-generated DNA fragments of *S. thermophilus* phages. Lane M, 1 kb DNA ladder (Amersham Biosciences); 1, ϕLy1; 2, ϕLy2; 3, ϕLy3; 4, ϕN2; 5, ϕNS1; 6, ϕNS2; 7, ϕTNL; 8, ϕNL10; 9, ϕND1; 10, ϕND2; 11, ϕNC1; 12, ϕNB1; 13, ϕNF1 14, ϕNC2; 15, ϕNF2; 16, ϕNL15; 17, ϕNL16; 18, ϕNL17; 19, ϕNL18; 20, ϕP13.2; 21, ϕAbc2.

The high preponderance of phages that seems to belong to the *cos*-type group is not surprising, since previous studies based on *S. thermophilus* phage diversity reported similar findings (Le Marrec et al., 1997; Quiberoni et al., 2003). Another remarkable observation is that the potential *pac*-containing phage was only capable to attack one host strain (ST NL10), although this strain was also sensitive to a *cos*-type phage. Lévesque et al. (2005) suggested that a given phage-sensitive *S. thermophilus* strain was rarely infected by both *cos*- and *pac*-type phages. Quiberoni et al. (2006) reported that five of the tested *S. thermophilus* strains were sensitive only to *cos*-type phages, whereas the two others were sensitive to both groups of phages. As it is shown in our results, it is likely that this behaviour can be verified in most cases, but not always.

3.4. Comparison of host range and antireceptor variable region (VR2)

When primers HOST1 and HOST5 were used to amplify a fragment of VR2 region, it was possible to obtain PCR products of approximately 700–800 pb from 17 of the isolated phages, while in the remaining phages (ϕLy1 and ϕNF1) no PCR amplification was observed (data not shown). Similar restriction profiles do not necessarily imply identical VR2 sequences. This could be the explanation for the absence of amplicons for phage ϕNF1 in contrast to ϕNC1 and ϕNB1. When DNA of ϕLy2, ϕNL15, ϕNC1, ϕNB1 and ϕTNL were used as template, weak PCR signals were obtained.

In the case of the remaining 12 phages, the sequences of the purified PCR products were compared with VR2 sequences of phage 0BJ (Binetti et al., 2005), and all the *S. thermophilus* phages in GenBank database: DT1 (AF085222), DT2 (AF348739), DT4 (AF348738), MD1 (AF348737), MD2 (AF348736), MD4 (AF348735), Q5 (AF348734), ϕ7201 (AF145054), ϕO1205 (U88974), ϕSfi11 (AF158600), ϕSfi19 (AF115102), ϕSfi21 (AF115103), 858 (AY699705), and 2972 (EF529515). The alignment grouped the VR2 sequences of the 12 Argentinian phages analysed into six typing profiles (>96% similarity; Fig. 2). One of the clusters (G.1) including a large group of phages was formed by two subgroups sharing 98%

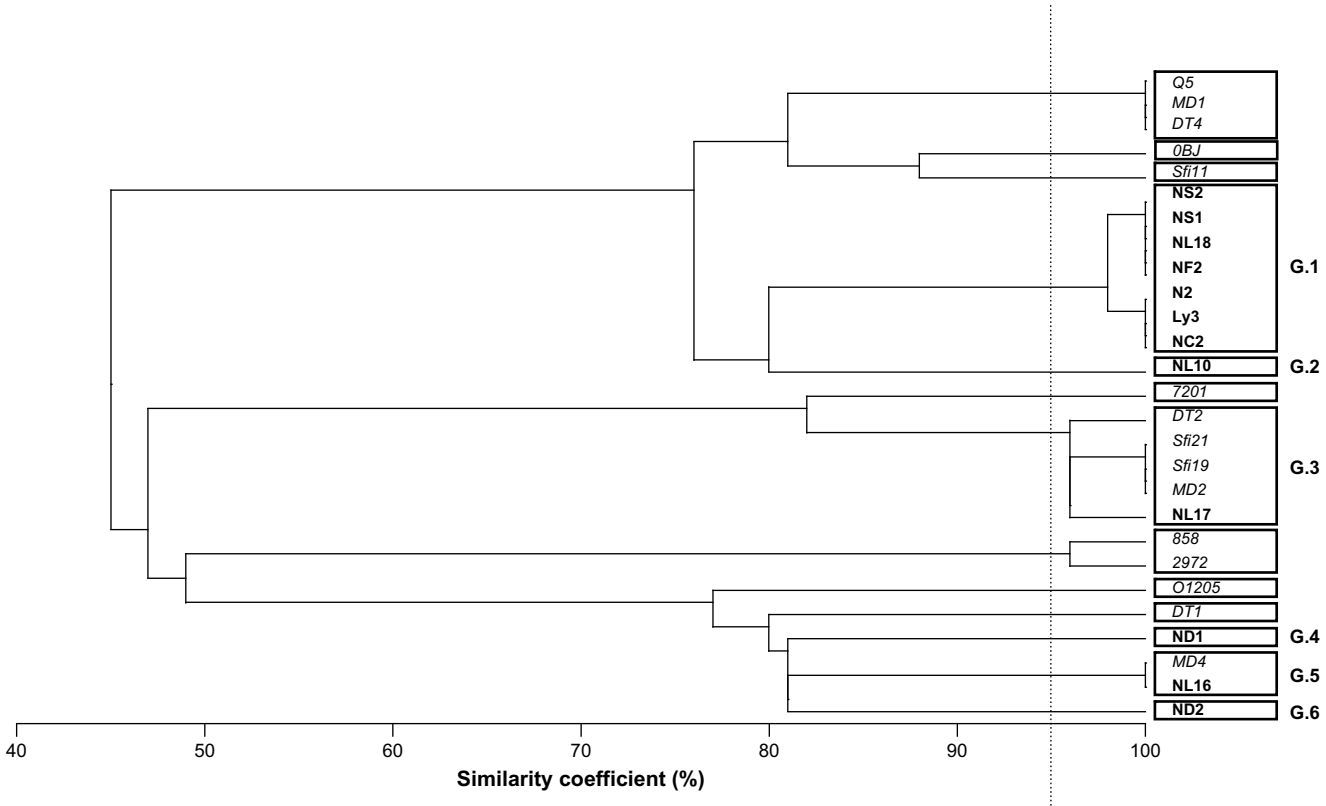

Fig. 2. Dendrogram obtained by the neighbour-joining method for the antireceptor VR2 sequences from 12 out of 19 *Streptococcus thermophilus* phages used in this work, phage 0BJ (Binetti et al., 2005) and those whose sequences are available in GenBank database: DT1 (AF085222), DT2 (AF348739), DT4 (AF348738), MD1 (AF348737), MD2 (AF348736), MD4 (AF348735), Q5 (AF348734), ϕ7201 (AF145054), ϕO1205 (U88974), ϕSfi11 (AF158600), ϕSfi19 (AF115102), ϕSfi21 (AF115103), 858 (AY699705), and 2972 (EF529515). Boxes and Groups (G. 1 to G. 6) indicate highly similar corresponding sequences (>96% similarity) in the phages. *S. thermophilus* bacteriophages used in this study are indicated in boldface. *S. thermophilus* bacteriophages previously studied are indicated in italics.

similarity. One of them included phages ϕNC2, ϕNS1, ϕNS2, ϕNL18 and ϕNF2, which have highly related host strains, indicating that VR2 regions could be implied in host specificity for these phages. However, no correlation was showed by phages that belong to the other subgroup of G.1, since ϕN2 and ϕLy3 have very different host ranges when compared with members that shared high VR2 sequence similarity (>96%).

These results seem to indicate that additional phage factors could be involved in host specificity of these bacteriophages, as previously reported by other authors (Duplessis and Moineau, 2001; Duplessis et al., 2006). Alternatively, host factors (such as resistant mechanisms) may also influence phage infection (Sturino and Klaenhammer, 2006). ϕNL17 shared a high (97%) sequence similarity with phages DT2, ϕSfi21, ϕSfi19 and MD2 (G.3), while ϕNL16 was clustered with phage MD4 (G.5), showing a complete homology between their VR2 sequences.

The remaining phages included in this study (ϕNL10, ϕND1 and ϕND2) constituted individual clusters (G.2, G.4 and G.6, respectively). According to this analysis, phages with very different (from 41 to 81% similarity) VR2 sequences, showed clearly different host ranges. One of the exceptions was represented by ϕND1, that showed a VR2 sequence different (<96%, G.4) from the remaining phages, and a host range that overlapped with those of ϕNC2, ϕNS1 and ϕNS2 (G.1). Similarly, phages ϕNL16 (G.5) and ϕND2 (G.6) exhibited different VR2 sequences, although they have one overlapping host strain. Considering all these cases, in practice, there will be at least one strain resistant to a group of phages that share similar VR2 sequence.

When a phage showing any of the VR2-type sequences is detected in a dairy plant, all strains included in the associated host ranges can be easily discarded. It is important to highlight that one phage-resistant strain, which would be infected by only one or two non-related phages, could be recommended for all analysed cases. Strains that are infected by several phages (ST NL1 is attacked by 11 phages; ST NL12 by 10 phages) or those which are infected by a high number of related phages (strains ST NL13 and ST NL18) should be suggested cautiously as starter cultures.

4. Conclusions

Data from this study show a significant phage biodiversity found in a single cheese plant that implements a starter rotation program without knowledge of the phage ecology contaminating the factory. Comprehension of this genetic diversity and phage monitoring are essential for the correct implementation of phage control measures. In such a sense, relevant information about host ranges of bacteriophages may be inferred from the classification based on the VR2 sequence, allowing the selection of those *S. thermophilus* strains that would be sensitive to the lowest number of phages present in that particular dairy environment.

Acknowledgements

This research was supported by projects PIP-CONICET N° 5321 (Consejo Nacional de Investigaciones Científicas y Tecnológicas), C.A.I. + D. N° 37–200 (Universidad Nacional del Litoral) and PICT ANPCyT N° 31991 (Agencia Nacional de Promoción Científica y Tecnológica).

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