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Detection and identification of *Lactobacillus helveticus* bacteriophages by PCR

Miriam Zago[1], Lia Rossetti[1], Jorge Reinheimer[2], Domenico Carminati[1] and Giorgio Giraffa[1]*

[1] CRA-FLC Centro di Ricerca per le Produzioni Foraggere e Lattiero Casearie, Lodi (Italy)
[2] Instituto de Lactología Industrial, Facultad de Ingeniería Química, Universidad Nacional del Litoral, Santa Fe (Argentina)

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A PCR protocol for detection of *Lactobacillus helveticus* bacteriophages was optimized. PCR was designed taking into account the sequence of the *lys* gene of temperate bacteriophage Φ-0303 and optimized to obtain a fragment of 222 bp using different *Lb. helveticus* phages from our collection. PCR was applied to total phage DNA extracted from 53 natural whey starters used for the production of Grana cheese and all gave the expected fragment. The presence of actively growing phages in the cultures was verified by traditional tests. Several PCR products of the *lys* gene were sequenced and aligned. The resulting sequences showed variable heterogeneity between the phages.

Keywords: *Lactobacillus helveticus*, lytic bacteriophages, endolysin gene, PCR, Grana cheese.

Bacteriophages are a common and constant threat to proper milk fermentation. In the modern dairy industry, the disruption of lactic acid fermentation by bacteriophages can lead to serious economic losses. Particularly in cheese manufacturing, phage attack has become a significant problem over recent years, especially for those technologies where the cheese curd and whey may contain high levels of active bacteria, thus providing ample opportunity for the growth and spreading of phage through the plant environment. The effects may range from outright failure of acid production through a pronounced decrease in activity to no visible effect at all (Cogan et al. 1991).

Phages of thermophilic dairy LAB have received less attention than those of lactococci. The biology, genetics, taxonomy, ecology, and origin of isolated phages of *Lactobacillus delbrueckii* subsp. *lactis*, *Lb. delbrueckii* subsp. *bulgaricus*, and *Streptococus thermophilus*, however, have been the subject of a number of investigations in the past few years (Desiere et al. 2002). In contrast, although *Lb. helveticus* is the dominant species present in starter cultures used in Italian, French, and Swiss cheese manufacturing, phages attacking this species still remain relatively neglected. An earlier study (Sozzi & Maret, 1975) describes the characteristics of *Lb. helveticus* phages isolated from Emmental starters. A comparative study on 35 *Lb. helveticus* bacteriophages was carried out on 23 phages isolated from cheese whey in French factories and 12 temperate phages (Séchaud et al. 1992). Quiberoni et al. (1999) studied the inactivation of *Lb. helveticus* phages by thermal and chemical treatments. More recently Zago et al. (2005) demonstrated the presence of *Lb. helveticus* phages in natural whey starters.

PCR-based approaches are increasingly used to detect bacteriophages because of their high sensitivity, specificity, and speed. The availability of PCR-based phage detection tools could lead to a rapid phage monitoring or be used to confirm the cause of acidification problems in cheese production. PCR methods are presently available for the detection of dairy bacteriophages and prophages infecting lactococci, *Strep. thermophilus*, and *Lb. delbrueckii* (Brussow et al. 1994; Labrie & Moineau, 2000; O'Sullivan et al. 2000; Craven et al. 2006; Zago et al. 2006, 2007; del Rio et al. 2007). However, no PCR methods have been developed to identify phages infecting *Lb. helveticus*. This work was therefore aimed at optimizing a PCR-based method to detect *Lb. helveticus* phages. The procedure was applied to evaluate phage presence in 53 whey starter cultures used for the production of Grana cheese.

Materials and Methods

Bacterial strains, bacteriophages, and culture conditions

Sixteen phages and their respective host strains used in this study are listed in Table 1. A further 40 *Lb. helveticus* strains from the CRA-FLC collection were used as

*For correspondence; e-mail: ggiraffa@ilclodi.it

Table 1. Bacteriophages and bacterial strains used in this study

Species	Strains/host strains	Phages	Source
Strains and phages used for the PCR optimization			
Lb. helveticus	AA15	ϕ AA15	CRA-ILC collection
	AB3	ϕ AB3	CRA-ILC collection
	AB7	ϕ AB7	CRA-ILC collection
	AB21	ϕ AB21	CRA-ILC collection
	AE6	ϕ AE6	CRA-ILC collection
	AF3	ϕ AF3	CRA-ILC collection
	G8	ϕ G8	CRA-ILC collection
	H3	ϕ H3	CRA-ILC collection
	U9	ϕ U9	CRA-ILC collection
	U14	ϕ U14	CRA-ILC collection
	W4	ϕ W4	CRA-ILC collection
	PA44	ϕ PA44	CRA-ILC collection
	PS5	ϕ PS5	CRA-ILC collection
	CNRZ328	ϕ CNRZ328	CNRZ collection
	CNRZ493	ϕ CNRZ493	CNRZ collection
	CNRZ892	ϕ CNRZ892	CNRZ collection
Lb. delbrueckii	AI1	ϕ AI1	CRA-ILC collection
subsp. *lactis*	AI2	ϕ AI2	CRA-ILC collection
	AG8	ϕ AG8	CRA-ILC collection
	PS11	ϕ PS11	CRA-ILC collection
	PS14	ϕ PS14	CRA-ILC collection
Lb. fermentum	CNRZ1637	ϕ CNRZ1641	CNRZ collection
	CNRZ63	ϕ CNRZ064	CNRZ collection
Lb. plantarum	ATCC8014	ϕ B1	ATCC
	ATCC8014	ϕ B2	ATCC
	O36	ϕ K3	Argentinean kefir
	O36	ϕ K4	Argentinean kefir
Strep. thermophilus	St21	ϕ St21	Commercial starter
	St45	ϕ St45	Commercial starter
Phages isolated in this study			
	858	ϕ 858	Grana cheese whey starter
	988	ϕ 988	Grana cheese whey starter
	1312	ϕ 1312	Grana cheese whey starter
	1314	ϕ 1314	Grana cheese whey starter
	1608	ϕ 1608	Grana cheese whey starter
	1617	ϕ 1617	Grana cheese whey starter
	1623	ϕ 1623	Grana cheese whey starter

indicators to search for phages in Grana cheese whey starters (see below). Cells were maintained as frozen stocks at −80 °C in the presence of 150 ml glycerol/l as cryoprotective agent. Phage stocks were prepared by the addition of phages to an actively growing culture of the appropriate host in MRS (Merck, Germany) broth that had been supplemented with 10 mM-$CaCl_2$ (MRS-Ca^{++} when specified). Host cultures were incubated at 42 °C until lysis was complete. Unlysed cells were removed by centrifugation at 4000 ***g*** for 10 min at 4 °C. Cell-free phage lysates were prepared by filtration using a 0·45 μm syringe filter unit (Millipore S.p.A, Milano, Italy). Lysates were neutralized to pH 7·0 with 1 M-NaOH and stored at 4 °C for a maximum of two weeks. Phage titres were determined in MRS agar (14 g/l) and soft agar (2·5 g/l) by the agar spot test and the double layer plaque titration test (Svensson & Christiansson, 1991). Titres were expressed as plaque-forming units/ml (PFU/ml).

Extraction of phage DNA

Total phage DNA was extracted and purified from high titre, cell-free phage lysates by the method previously described (Zago et al. 2006). The same protocol was applied to extract total phage DNA from 53 whey starters. Before DNA extraction, cell-free whey starters (CFWS) were prepared by neutralizing whey starters to pH 7·0 with 1 M-NaOH. Neutralised samples were then pre-filtered through a Whatman Polycap 75 SPF with porosity of 1μm (Arbor Technologies Inc., MI, USA) and sterilized by filtration through a 0·22 μm Nalgene filter unit (Nalgene Company, Rochester, USA).

PCR amplification

PCR primers were designed on the basis of the *lys* gene sequence (accession no. AF495798) of *Lb. helveticus* temperate bacteriophage Φ-0303 (Deutsch et al. 2004). The primers had the following sequences (5′-3′): GGG-TAGCATCTTATAAAGTTAGCGG (endolys for, nucleotide position 482 to 506 of the *lys* gene) and CAC-TTGACTACGGGATGCTGAGA (endolys rev, nucleotide position 704 to 682).

Amplifications were performed in 25 µl volumes with 0·5 µM of each primer (Biotez, Berlin, Germany), 2·5 units/100 µl of AmpliTaq Gold DNA polymerase (Applera Italia, Monza, Italy), 1·5 mM-$MgCl_2$, 25 ng of total DNA, and 200 µM of each dNTP. DNA amplifications were performed in a Perkin Elmer thermal cycler (mod. 9700; Applied Biosystems) under the following conditions: initial denaturation at 95 °C for 10 min; 35 cycles of denaturation at 95 °C for 30 sec, annealing for 30 sec at 60 °C, and extension at 72 °C for 1 min; final extension at 72 °C for 7 min. PCR products were analysed by electrophoresis through 1·2% (w/v) agarose gels at 10 V/cm for 1 h in Tris-acetate EDTA (TAE) buffer (TAE: 40 mM-Tris acetate, 1 mM-EDTA, pH 8·0).

Specificity and detection limit of the PCR

PCR specificity was tested by amplifying phage DNAs extracted from *Lb. plantarum, Lb. fermentum, Lb. delbrueckii* subsp. *lactis*, and *Strep. thermophilus* phages (Table 1). In order to determine limits of detection of PCR, DNA of bacteriophage Φ-CNRZ 892 was extracted from an MRS broth lysate with a known titre (approx. 10^8 PFU/ml) and from decimal dilutions of the lysate and PCR was applied using 1 µl DNA. The lowest concentration visible on the gel was set as the detection limit of the PCR.

Phage detection in Grana cheese whey starters

PCR was applied to phage DNA extracted from 53 whey starter cultures according to previously described methods, primers, and amplification conditions. The presence of replicating phage particles was verified by the microtitre plate method on about half of the whey starter cultures which had resulted positive after PCR. To this end, a total of 40 *Lb. helveticus* strains were used as indicators in a microtitre plate assay (Zago et al. 2006). Briefly, 5 µl of an overnight culture from different host bacteria and 25 µl CFWS were used. CFWS were inoculated in microtitre plates, which had been filled with 170 µl MRS-Ca^{++} broth containing bromcresol purple as growth indicator. All plates were incubated at 42 °C under anaerobic conditions and, after 8 h, scored for lysis of indicator strains (no colour change of the indicator). Because low phage concentrations could give false negative results, different subcultures were performed and scored for lysis of the indicator strains. Control wells, i.e. wells without addition of CFWS samples, were always included.

DNA sequencing

DNA sequencing of 11 of the 222-bp PCR-amplified products of the *lys* gene was performed using an ABI PRISM 310 automated DNA sequencer (Applied Biosystems, Foster City, CA) as previously described (Zago et al. 2006). The sequences obtained were compared together and with the sequence of the reference phage Φ-0303 and grouped into clusters according to the sequence distance between all pairs. Clusters were aligned as pairs and then collectively as sequence groups to produce the overall alignment. After the multiple-sequence alignment was completed, the neighbour-joining method and the bootstrap analysis were used to construct a dendrogram showing the phylogenetic relationships. Sequence alignments were performed with the Sequence Navigator software using ClustalW algorithm (Applied Biosystems). Cluster analysis, neighbour-joining, and bootstrap analysis were performed with the MEGA software, version 3.1 (http://www.megasoftware.net).

Results and Discussion

Lactobacillus phage (both lytic and temperate) sequence data are available from five distinct species of lactobacilli (*Lb. delbrueckii, Lb. gasseri, Lb. plantarum, Lb. casei*, and *Lb. johnsonii*) but not from *Lb. helveticus* (Desiere et al. 2002). Therefore we optimized a PCR aimed at detecting *Lb. helveticus* phages on the basis of the available DNA sequence of the lysin-encoding *lys* gene of *Lb. helveticus* temperate phage Φ-0303 (Deutsch et al. 2004). The PCR was sensitive because amplicons of the expected size (222 bp) were always obtained using DNA from 16 phages of *Lb. helveticus* (Fig. 1), 13 of which came from our collection and were shown to be highly diverse according to restriction analysis and phage host range (Zago et al. 2005) and three were reference phages (Table 1). The PCR was also specific because no amplification products were observed from phages of *Lb. plantarum* and other LAB species, such as *Lb. delbrueckii*, *Lb. fermentum*, and *Strep. thermophilus* (Fig. 1; lanes 17–24 showing some of the non-*Lb. helveticus* phages). These are, together with *Lb. helveticus*, the four major species components of the Grana and Provolone cheese whey starters (Giraffa et al. 1998; Beresford et al. 2001; Gobbetti, 2004; Parente & Cogan, 2004). When DNA from serial dilutions in MRS broth of the purified phage Φ-CNRZ 892 were amplified, the detection limit of the method was 10^2–10^3 PFU/ml (data not shown). Since phage concentration below 10^5 PFU/ml in whey or milk is not considered a threat to fermentation (McIntyre et al. 1991; Suarez et al. 2002), the proposed PCR is well above the minimum sensitivity requirements.

The optimized PCR was tested to ascertain if *Lb. helveticus* bacteriophages could be detected directly from Grana cheese whey starters. Fifty-three cultures were sampled from different Grana cheese plants and DNA was

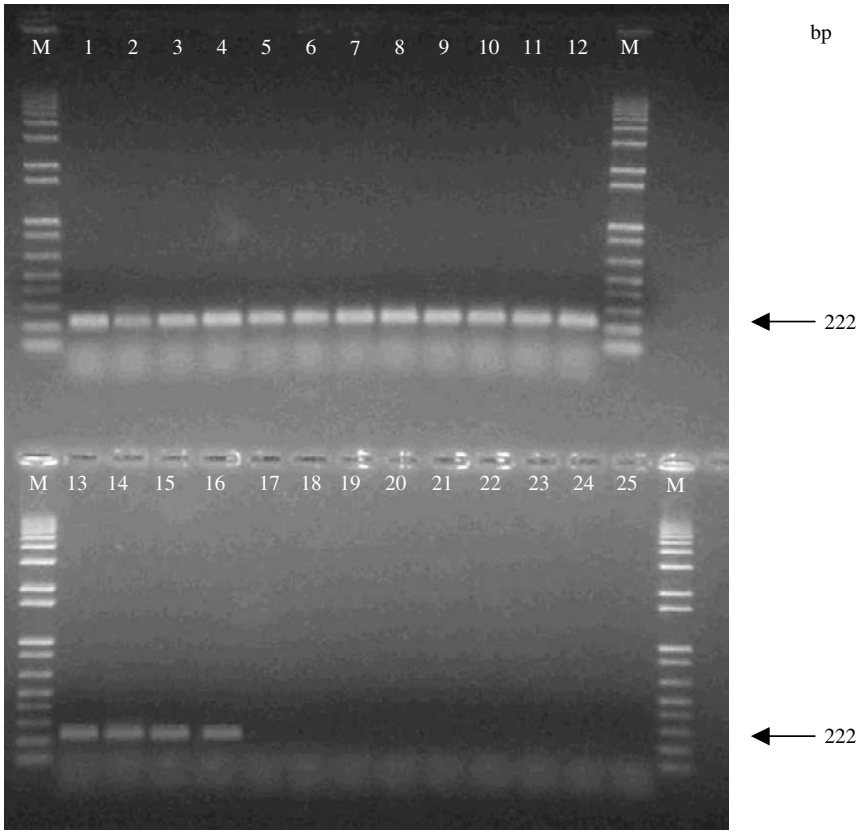

Fig. 1. Agarose gel electrophoresis of the products from PCR amplification of total phage DNA extracted from different phages of the CRA-FLC collection. Primers were directed against a 222-bp intragenic fragment of the *lys* gene of *Lactobacillus helveticus* temperate bacteriophage Φ-0303 (accession no. AF495798). Lanes 1–16: *Lb. helveticus* phages; lanes 17–18: *Lb. delbrueckii* subsp. *lactis* phages; lanes 19–20: *Lb. fermentum* phages; lanes 21–22: *Lb. plantarum* phages; lanes 23–24: *Streptococcus thermophilus* phages; lane 25: negative control (no DNA added). M: molecular size DNA marker (1 Kb plus DNA ladder; Invitrogen Italia, Milan, Italy). Arrows highlight the PCR product.

extracted as described in the methods. After amplification, all the samples were confirmed positive for the presence of the 222-bp *lys* gene fragment (data not shown), indicating a presumptive presence of *Lb. helveticus* phages. About half of the 53 PCR positive whey cultures were searched for the presence of active phage particles. Proliferating phages were found in five cultures and a total of seven new lytic phages (named Φ858, Φ988, Φ1312, Φ1314, Φ1608, Φ1617, and Φ1623; Table 1) were isolated from them. The finding of *Lb. helveticus* phage DNA in all the starters confirms previous studies on *Lb. delbrueckii* subsp. *lactis* phages (Zago et al. 2006) and suggests a frequent presence of phages in these cultures even without apparent performance failures. In natural whey cultures used for Grana Padano and Provolone cheeses, which did not show evidence of failures in acidifying activities, a coexistence of phages and sensitive strains of *Lb. helveticus* and *Lb. delbrueckii* subsp. *lactis* has been demonstrated (Zago et al. 2005).

The PCR assay reported here has the advantage of detecting phage DNA directly from artisanal whey starter samples of undefined composition. Therefore the proposed method, together with already published PCR protocols for detection of phage active against *Lb. delbrueckii* (and its subspecies 'lactis' and 'bulgaricus') and *Strep. thermophilus* (Zago et al. 2006; del Rio et al. 2007), allows us to have a full diagnostic PCR package to detect dairy bacteriophages attacking the dominant thermophilic LAB species present in milk and cheese starters. Like all the other PCR-based phage detection assays, however, PCR can not establish whether the phages are able to infect and lyse sensitive bacterial cells. This aspect has particular relevance concerning *Lb. helveticus* since defective phages and phage particles with killer activity against this species have been described in natural whey starters for Grana and Provolone cheeses (Carminati et al. 1997). In this regard, the low incidence of cultures containing actively growing phage particles can be explained by either the lack of effective host to be used as indicators or the presence of defective *Lb. helveticus* phage particles.

Most of the partial *lys* gene fragments, including two of the seven newly isolated phages (i.e. Φ-1608 and Φ-1617), were sequenced. After alignment and BLAST analysis they appeared to be 99% similar to the portion of the *lys* gene

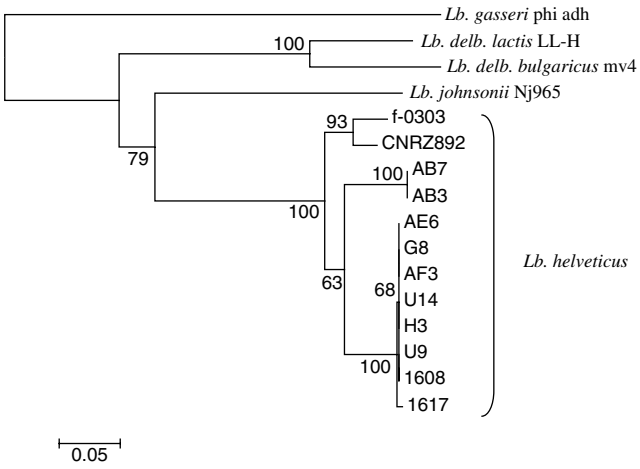

Fig. 2. Phylogenetic tree of the partial *lys* gene amplified from different *Lactobacillus helveticus* phages as inferred by the neighbour joining method and the ClustalW algorithm. Sequence alignment was performed with the Sequence Navigator software (Applied Biosystems, Foster City, CA). Consensus sequences were compared with the *lys* gene of *Lb. helveticus* temperate bacteriophage Φ-0303 (accession no. AF495798). Distance matrix and phylogenetic tree were calculated with the MEGA software, version 3.1 (http://www.megasoftware.net). Bootstrap probability values (percentages of 1000 tree replications) are indicated at branch-points. Bar, 5% sequence divergence. The phylogenetic distance between sequences is the sum of the horizontal segments.

of Φ-0303 extending from nucleotide position 482 to 704 (data not shown). The comparison with muramidase genes of other taxonomically related species available from the Genbank enabled allocation of *Lb. gasseri* (accession no. AJ131519), *Lb. delbrueckii* subsp. *lactis* (accession no. EF455602), *Lb. delbrueckii* subsp. *bulgaricus* (accession no. Z26590), *Lb. johnsonii* (accession no. AY459535), and all the *Lb. helveticus* sequences into four separated branches within the phylogenetic tree (Fig. 2). The relatively low sequence similarity with other *Lactobacillus* muramidases is explained by the choice, especially in reverse primer designing, of the C-terminal region of the protein which has poor homology score with *Lactobacillus* endolysins (Deutsch et al. 2004). This low sequence similarity suggests that the Φ-0303-like *lys* gene is highly conserved within *Lb. helveticus*. Sequence variability of the *lys* gene enabled the *Lb. helveticus* phages to be subdivided into three subclusters. One included the phage Φ-0303, chosen as reference for PCR design, and the phage Φ-CNRZ 892; a second subcluster included the two phages Φ-AB3 and Φ-AB7; finally, a third subcluster grouped all the other phages, including the newly isolated phages Φ-1608 and Φ-1617 (Fig. 2).

Taken together, data showed that whey starter cultures are complex ecosystems where lytic bacteriophages may play an active role in microbial composition and population dynamics. In particular, data of both the present study and a previous investigation (Zago et al. 2006) demonstrate that *Lb. helveticus* and *Lb. delbrueckii* subp. *lactis* lytic phages can be frequently present in whey starter cultures, although their number is not known and lytic effectiveness is demonstrated for only a fraction of PCR positive cultures tested. Because the *lys* gene codes for a broad-spectrum endolysin of *Lb. helveticus* and was sequenced from a temperate bacteriophage (Deutsch et al. 2004), the proposed PCR could be applied to also detect lysogenic *Lb. helveticus* strains. Preliminary data in our laboratory seem to substantiate this possibility.

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