Journal of Dairy Research (2009) **76** 433–440. © *Proprietors of Journal of Dairy Research 2009*
doi:10.1017/S0022029909990112

Evidence for the presence of restriction/modification systems in *Lactobacillus delbrueckii*

Viviana Suárez[1], Miriam Zago[2], Giorgio Giraffa[2], Jorge Reinheimer[1] and Andrea Quiberoni[1]*

[1] Instituto de Lactología Industrial (UNL-CONICET), Facultad de Ingeniería Química, Santiago del Estero 2829, 3000 Santa Fe, Argentina
[2] Centro di Ricerca per le Produzioni Foraggere e Lattiero-Casearie, Settore di Ricerca 'Lattiero-Caseario', Via Lombardo 11, 26900 Lodi, Italy

Received 6 November 2008; accepted for publication 4 June 2009; first published online 29 July 2009

The bacteriophages Cb1/204 and Cb1/342 were obtained by induction from the commercial strain *Lactobacillus delbrueckii* subsp. *lactis* Cb1, and propagated on *Lactobacillus delbrueckii* subsp. *lactis* 204 (*Lb.l* 204) and *Lactobacillus delbrueckii* subsp. *bulgaricus* 342 (*Lb.b* 342), respectively. By cross sensitivity, it was possible to detect a delay in the lysis of *Lb.l* 204 with Cb1/342 phage, while the adsorption rate was high (99·5%). Modified and unmodified phages were isolated using phage Cb1/342 and strain *Lb.l* 204. The EOP (Efficiency of Plaquing) values for the four phages (Cb1/204, Cb1/342, Cb1/342modified and Cb1/342unmodified) suggested that an R/M system modified the original temperate phage, and the *Bgl*II-DNA restriction patterns of these phages might point out the presence of a Type II R/M system. Also, the existence of a Type I R/M system was demonstrated by PCR and nucleotide sequence, being the percentages of alignment homology with Type I R/M systems reported previously higher than 95%. In this study it was possible to demonstrate that the native phage resistant mechanisms and the occurrence of prophages in commercial host strains, contribute strongly to diversify the phage population in a factory environment.

Keywords: Restriction/modification system, lysogeny, bacteriophages, *Lactobacillus delbrueckii*.

Lactobacillus delbrueckii is a lactic acid bacterium (LAB) of great technological relevance. *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. delbrueckii* subsp. *lactis* are part of commercial yogurt cultures and they are also present in whey cultures used for the production of Argentinean (Reinheimer et al. 1995, 1996) and Italian (Neviani & Carini, 1994; Giraffa et al. 1997; Giraffa & Rosetti, 2004) hard cheeses.

Bacteriophage inhibition during dairy fermentations is an ever-present threat for industry. To minimize phage dissemination in dairy plants, various strategies are applied, such as direct vat-inoculation of starters, optimized sanitation, and rotation of phage-resistant starter cultures (Moineau et al. 2002). Unfortunately, with the constant use of LAB strains carrying phage defense mechanisms, new phages resistant to these systems have emerged, but only a few of them have been characterized (Labrie & Moineau, 2007).

*For correspondence; e-mail: aquibe@fiq.unl.edu.ar

Within host-related, phages defense mechanisms, restriction/modification (R/M) systems are one of the most commonly spread in LAB. Restriction/modification systems have been mainly studied in *Lactococcus* genus (Schouler et al. 1998), but they have also been demonstrated for *Lactobacillus helveticus* (de los Reyes-Gavilán et al. 1990), *Streptococcus thermophilus* (Allison & Klaenhammer, 1998), and *Lb. delbrueckii* (Auad et al. 1998; Bourniquel et al. 2002). In response to R/M systems, phages have evolved several anti-restriction measures to ensure their survival, such as modification of the phage genome, transient occlusion of restriction sites, subversion of host R/M activities and direct inhibition of restriction enzymes (Tock & Dryden, 2005).

The present study demonstrates the ability of R/M systems present in a commercial *Lb. delbrueckii* strain to modify a phage originally derived from another *Lb. delbrueckii* commercial strain, which was also lysogenic. A Type I R/M system was molecularly characterized and the *hsdR* and *hsdM* genes were sequenced after PCR amplification.

Table 1. Source and *Lactobacillus delbrueckii* strains used in this work

Strain	Genus/species	Source (culture/country)
Lb.l Cb1 (lysogenic strain)	*Lb. delbrueckii* subsp. *lactis*	Yogurt/Argentina
Lb.l 204 (host strain)	*Lb. delbrueckii* subsp. *lactis*	Provolone cheese whey starter/Italy
Lb.b 342 (host strain)	*Lb. delbrueckii* subsp. *bulgaricus*	Yogurt/Italy

Materials and Methods

Strains and culture conditions

The source and identification of the strains used in this work are listed in Table 1. Stocks were maintained at −80 °C, in MRS broth (Biokar, Beauvois, France) with the addition of 15% (v/v) glycerol as a cryoprotective agent, and in reconstituted (10% w/v) skimmed milk (RSM), and routinely cultured overnight at 42 °C in MRS broth.

Bacteriophages

The bacteriophages Cb1/204 and Cb1/342 were obtained by induction with mitomycin C (0·5 mg/ml) (MC, Sigma Chemical Co., St. Louis, MO, USA) of the commercial strain *Lb. delbrueckii* subsp. *lactis* Cb1 (Suárez et al. 2008). After centrifugation at 12 000 ***g*** (10 min at room temperature), the supernatant was filtered (Millipore, São Paulo, Brazil) with a pore diameter 0·45 μm, and propagated on the strains *Lb. delbrueckii* subsp. *lactis* 204 (*Lb.l* 204) and *Lb. delbrueckii* subsp. *bulgaricus* 342 (*Lb.b* 342), respectively.

MRS broth or agar, which had been supplemented with 10 mM-$CaCl_2$ (MRS-Ca when specified), was routinely used to propagate and count phages (Svensson & Christiansson, 1991). Phage stocks were maintained at −80 °C with 15% (v/v) glycerol added.

Cross sensitivity and adsorption rates

The sensitivity of *Lb.l* 204 and *Lb.b* 342 against the bacteriophages Cb1/204 and Cb1/342 was investigated by the turbidity test (Svensson & Christiansson, 1991). Briefly, 5 ml MRS-Ca broth were inoculated with an overnight culture of each *Lb. delbrueckii* strain and each phage at a multiplicity of infection (m.o.i.) of 0·5. Tubes inoculated only with the strains were used as controls. A total of three subcultures were made to increase the concentration of phages. Tubes were incubated at 42 °C and the turbidity was compared with the control tubes.

Phage adsorption rates on bacterial cells were performed at 30 min in MRS-Ca broth as described by Séchaud et al. (1989). Results were expressed as percentages of the initial phage counts.

Detection of R/M systems in Lactobacillus delbrueckii *subsp.* lactis *204*

The presence of R/M systems was investigated as proposed by de los Reyes-Gavilán et al. (1990). The Cb1/342 bacteriophage was propagated on the strain *Lb.l* 204 and the presumptive modified phage was named Cb1/342m. This one was propagated on strain *Lb.b* 342, obtaining another phage named Cb1/342m-d. For all propagated phages, the EOP (Efficiency of Plaquing) values were calculated by using the double-layer plaque titration method (Svensson & Christiansson, 1991).

DNA manipulation and analysis

Phages were propagated in a volume of 100 ml MRS-Ca broth and phage particles were concentrated overnight (Yamamoto et al. 1970). Phage DNAs were obtained by three phenol-chloroform-isoamyl alcohol extractions and concentrated by ethanol precipitation (Quiberoni et al. 2004). Their quantification and visualization was performed according to Sambrook & Russell (2001). One-Kbp DNA ladder was used as molecular weight marker (Amersham Biosciences UK limited, UK).

For restriction analysis, phage DNA aliquots were cleaved with *Bgl*II, *Hin*dIII, *Eco*RI, *Eco*RV and *Sal*I (Amersham Biosciences UK Limited, UK) and restriction fragments were then treated for 10 min at 70 °C (Quiberoni et al. 2004). After heat treatment, gel electrophoresis in agarose (0·8% w/v) was performed (Sambrook & Russell, 2001) to resolve DNA fragments.

The total DNA of the strain *Lb. delbrueckii* subsp. *lactis* 204 was obtained by phenol-chloroform extractions as was previously described (Quiberoni et al. 2004). The alkaline lysis method was used for plasmid isolation from the strain *Lb. delbrueckii* subsp. *lactis* 204 (Quiberoni et al. 1998).

Primer design and PCR conditions

Pairs of primers were designed to characterize the R/M systems of *Lb.l* 204 (DNAMAN Sequence Analysis Package Program Version 5.2.9, Lynnon Corporation, Copyright 1994–2001). Primer sequences are detailed in Table 2. The primers to amplify the restriction and modification subunits (*hsdR* and *hsdM,* respectively) for Type I R/M system were designed on the basis of the complete genome sequence of *Lb. delbrueckii* subsp. *bulgaricus* ATCC 11842 (GenBank, accession number NC_008054). Type II R/M system, specifically Type II restriction enzyme *Ngo*F-VII, harbored in *Lb. delbrueckii* subsp. *bulgaricus* ATCC BAA-365 (GenBank, accession number NC_008529) was used to design another pair of primers. Finally, the methyltransferase enzyme gene sequence of *Lb. delbrueckii* subsp. *bulgaricus* ATCC 11842 (GenBank, accession

Table 2. Sequences of primers used in this study

Primer	Nucleotide sequence (5′→3′)	Fragment size (bp) expected
hsdS I for	TCT TGT CAG CGA ACA TCT TC	535
hsdS I rev	TCC TTG GGA GCA GTG TAA G	
hsdR I for	CTA TTC CCG ACG CTG ATT	494
hsdR I rev	GTA TCT GGT AAG GAC GGA GC	
hsdM I for	GCT GGG TCA AAA CTT GCA	575
hsdM I rev	CTT AGA CTT TGG CGG CAA	
R/M II for	CTG GAA AGA TGG CAA ACC	441
R/M II rev	GAA CCA GTT TCG TGG AGT AAC	
R/M III-1 for	AGC ACC TGT TTC ATC CTT G	582
R/M III-1 rev	TGT AGG AAT GAC AGC GGA	
R/M III-2 for	CCT TTC TTA CAG TCT CTG GGC T	400
R/M III-2 rev	CGA CTA TGG CTT CCG TTG T	
R/M III-3 for	AAT CAG TGT GAA AGC GTC C	449
R/M III-3 rev	AAG ACG GCA ATA CTG TTC G	
R/M III-4 for	TTC CAA CAT CAC TTA GCC AC	597
R/M III-4 rev	GGA CGC TTT CAC ACT GAT T	

number NC_008054) was used to design the four pairs of primers corresponding to Type III R/M system.

The PCR reactions were performed in 25 µl containing 200 µM-deoxynucleoside triphosphate, 0·5 µM of each primer, 1·25 U *Taq* DNA polymerase (Invitrogen), *Taq* buffer (20 mM-Tris-HCl, pH 8·4, 1·5 mM-magnesium chloride, 50 mM-potassium chloride) and 1 µl of total bacterial DNA. The PCR conditions were as follows: 5 min at 94 °C followed by 30 cycles (30 s at 94 °C, 30 s at 53 °C, 1 min at 72 °C) and a final step of 7 min at 72 °C. The PCR products were separated on a 0·8% (w/v) agarose gel, stained with ethidium bromide, and visualized under UV light.

DNA sequencing

DNA sequencing of the PCR-amplified, infragenic fragment of the *hsdR* and *hsdM* genes was performed. Before PCR extension, amplified products were excised with the NucleoSpin Extract (Macherey Nagel, Germany). After a second amplification, the amplified products were purified with the Exo-SAP-IT (GE Healthcare, Italy) according to the manufacturer's recommendations. The PCR extension reactions were performed in 20 µl volumes with 3·2 pM of each primer (BioTez, Berlin, Germany), 4 µl of sequencing mix (Applera, Italia), and 7 µl of purified, amplified product.

PCR extension consisted of 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. Rapid thermal ramps (1 deg C/s) between steps were performed. Prior to sequencing, extended products were purified with Big-Dye X-Terminator (Applera, Italia) according to the supplier's indications. Nucleic acid sequences were obtained using an ABI PRISM 310 automated DNA sequencer (Applied Biosystems, Foster City, CA). Sequence alignments and analyses were performed with the Sequence Navigator software (Applied Biosystems). Consensus sequences obtained were also compared with *hsdR* and *hsdM* gene sequences of *Lb. delbrueckii* subsp. *lactis* strain NCC82 (GenBank, accession number AJ938156) and *Lb. delbrueckii* subsp. *lactis* strain NCC88 (GenBank, accession number AJ938155) (DNAMAN Sequence Analysis Package Program).

Table 3. Adsorption rates (%) and cross sensitivity in broth (MRS-Ca) for phages Cb1/204 and Cb1/342, on *Lactobacillus delbrueckii* host strains

Values are the means ± SD for three replicate trials

Host Strain	Phage	
	Cb1/204	Cb1/342
Lb.l 204	99·7 ± 1·5† (+, 1°)‡	99·5 ± 1·8† (+, 2°)‡
Lb.b 342	94·0 ± 1·2† (+, 1°)‡	94·0 ± 0·7† (+, 1°)‡

† Adsorption rate (30 min – 42 °C)
‡ +: Lysis in broth, subculture number when lysis was observed

Results

Cross sensitivity and adsorption rates

Both phages, Cb1/204 and Cb1/342, were able to produce lysis of both *Lb. delbrueckii* strains, *Lb.l* 204 and *Lb.b* 342. However, the lysis of *Lb.l* 204 strain with phage Cb1/342 was slightly delayed (i.e. in the second subculture) with respect to that observed on its host strain *Lb.b* 342, which was observed in the first subculture (Table 3).

The adsorption rates (%) of both bacteriophages to *Lb.l* 204 and *Lb.b* 342 were similar, with values ranging between 94·0 ± 0·7% (both phages on *Lb.b* 342) and 99·7 ± 1·5% (both phages on *Lb.l* 204) (Table 3). Specifically for strain *Lb.l* 204 with phage Cb1/342, the adsorption rate was higher than 99%, but the lysis in MRS broth was not observed until the second subculture. This fact may indicate the presence of a R/M system in the *Lb.l* 204 strain, active against phage Cb1/342.

Detection of R/M systems in Lactobacillus delbrueckii *subsp.* lactis *204*

The EOP values for phages Cb1/342, Cb1/342m and Cb1/342m-d on the restrictive strain *Lb.l* 204 are shown in Table 4. A restrictive development of bacteriophage Cb1/342 after propagation on the original host was observed when it was subsequently propagated on the *Lb.l* 204 strain. After a second passage on the restrictive strain, the progeny of the bacteriophage (named Cb1/342m) grew on this strain as well as on the host strain (*Lb.b* 342). When this new phage was propagated back through its original host, a drop of the EOP value on *Lb.l* 204 was observed

Table 4. EOP (Efficiency of Plaquing) of phage Cb1/342 and the corresponding propagated isolates on the *Lb.b* 342 host strain and the *Lb.l* 204 restrictive strain

Values are the means ± SD for three replicate trials

Phage	Strain†	EOP‡ on *Lb.l* 204
Cb1/342	*Lb.b* 342	$2{\cdot}0\ 10^{-3} \pm 3{\cdot}0\ 10^{-4}$
	Lb.b 342; *Lb.l* 204	$8{\cdot}9\ 10^{-1} \pm 4{\cdot}0\ 10^{-2}$
	Lb.b 342; *Lb.l* 204; *Lb.b* 342	$5{\cdot}0\ 10^{-3} \pm 7{\cdot}0\ 10^{-4}$

† Strain succession of phage propagation
‡ EOP (phage count on the restrictive strain/phage count on the host strain)

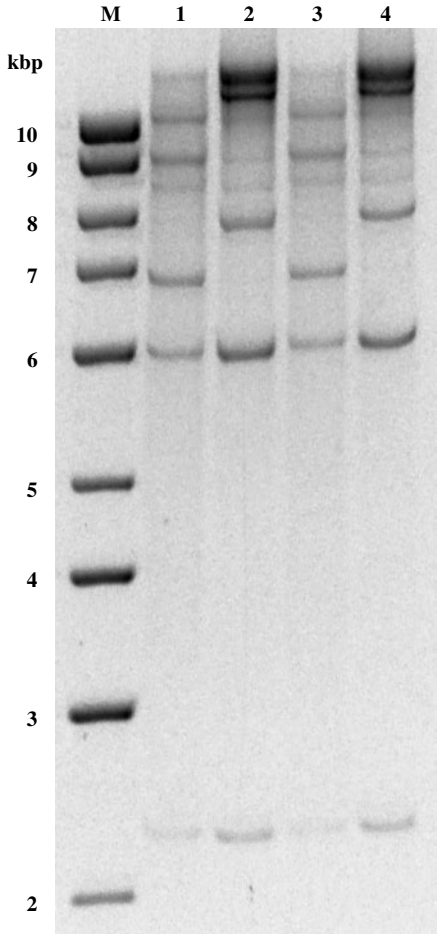


Fig. 1. Agarose gel electrophoresis of DNA fragments obtained from cleavage with the *Bgl*II restriction enzyme of Cb1/342 (line 1), Cb1/204 (line 2), Cb1/342m-d (line 3) and Cb1/342m (line 4) phages. Lines M: 1 kbp molecular weight marker.

again. These data could indicate the presence of R/M systems in the strain *Lb.l* 204, active on phage Cb1/342.

Phage DNA analysis

The DNA restriction profile obtained with *Bgl*II on the bacteriophage Cb1/342m (presumptively modified by

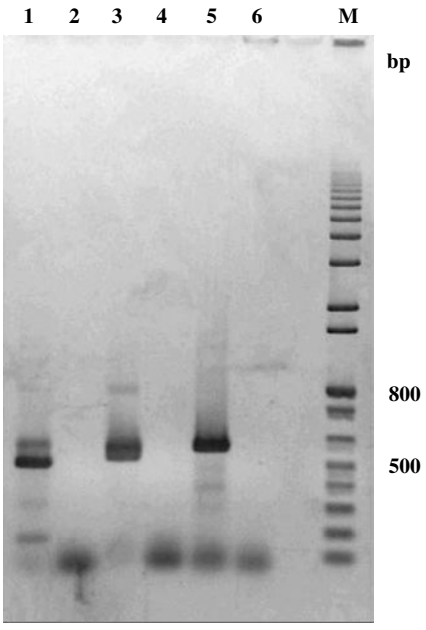


Fig. 2. Agarose gel electrophoresis of amplified DNA fragments from *hsdR* (line 1), *hsdS* (line 3) and *hsdsM* (line 5) genes. Lines 2, 4 and 6: negative controls. Line M: 1 kbp molecular weight marker.

Lb.l 204) was identical to that found for the phage Cb1/204, originally isolated as temperate phage from the commercial strain *Lb. delbrueckii* subsp. *lactis* Cb1 (Fig. 1). More specifically, phage Cb1/204 was obtained by propagation of an induced prophage from *Lb.l* Cb1 on *Lb. delbrueckii* subsp. *lactis* 204. The DNA restriction profile of phage Cb1/342m-d (propagated again on the original strain *Lb.b* 342) reverted to that exhibited by the originally isolated phage (Cb1/342). These results might indicate the existence of an operative Type II R/M system in *Lb.l* 204 strain.

The profiles obtained with the rest of the restriction enzymes did not show any differences among the four bacteriophages (data not shown).

Characterization of R/M systems

A Type I R/M system was amplified from total DNA of strain *Lb.l* 204 (Fig. 2). Fragments with the sizes expected for the *hsdS* (535 bp), *hsdR* (494 bp) and *hsdM* (575 bp) subunits were obtained. No amplification products were observed with primers designed for Type II and III R/M systems (data not shown).

DNA sequencing

The two sequences obtained from the amplified fragments of the putative *Lb.l* 204 *hsdR* (494 bp) and *hsdM* (575 bp) genes showed similarities higher than 90% with other *hsdR* and *hsdM* *Lb. delbrueckii* genes reported in GenBank (Fig. 3 & 4). The alignments of the sequenced

```
ATCC 11842   CTATTCCCGACGCTGATTTGCTGATATTAGACAATACCAACATTGCTGGTGGGAACTCAGTTTATGAAGTTGTACACCAGG      81
NCC82        CTATTCCCGACGCTGATTTGCTGATATTAGACAATACCAACATTGCTGGTGGGAACTCAGTTTATGAAGTTGTACACCAGG      81
NCC88        CTATTCCCGACGCTGATTTGCTGATATTAGACAATACCAACATTGCTGGTGGGAACTCAGTTTATGAAGTTGTACACCAGG      81
L.l.204      ..............GATTTGCTGATTTTAGACAATACCAACATTGCGGGTGGAAACTCAGTTTATGAAGTTGTACACCAGG      67
Consensus    ctattcccgacgctgatttgctgatattagacaataccaacattgctggtgggaactcagtttatgaagttgtacaccagg

ATCC 11842   GTTCAGCTACAGAAAAAGACCGCGCTTAATCGAGACCGTCGTTTTGACGTTAGTTTGTTGATCAACGGCTTGCCGGTGATT      161
NCC82        GTTCAGCTACAGAAAAAGACCGCGCTTAATCGAGACCGTCGTTTTGACGTTAGTTTGTTGATCAACGGCTTGCCGGTGATT      161
NCC88        GTTCAGCTACAGAAAAAGACCGCGCTTAATCGAGACCGTCGTTTTGACGTTAGTTTGTTGATCAACGGCTTGCCGGTGATT      161
L.l.204      GTTCAGCTACAGAAGAAGACCGCGCTTAATCAAGACCGTCGTTTCGACGTTAGTTTGTTGATCAACGGCTTGCCGGTAATT      147
Consensus    gttcagctacagaaaaagaccgcgcttaatcgagaccgtcgttttgacgttagtttgttgatcaacggcttgccggtgatt

ATCC 11842   CACATTGAGCTTAAAGCGCCAAATGTTCCTTATAGGAAGGCCTTTAACCAAATTCAAAAGTATATCGATGAAGGGCAATT      241
NCC82        CACATTGAGCTTAAAGCGCCAAATGTTCCTTATAGGAAGGCCTTTAACCAAATTCAAAAGTATATCGATGAAGGGCAATT      241
NCC88        CACATTGAGCTTAAAGCGCCAAATGTTCCTTATAGGAAGGCCTTTAACCAAATTCAAAAGTATATCGATGAAGGGCAATT      241
L.l.204      CACATTGAGCTTAAAGCTCCAAATGTTCCTTATAAGAAGGCCTTTAACCAAATTCAAAAGTATATCGACGAAGGACAATT      227
Consensus    cacattgagcttaaagcgccaaatgttccttataggaaggcctttaaccaaattcaaaagtatatcgatgaagggcaatt

ATCC 11842   ACTGACATTTACAGCTTCGTAGAAATGTTTGTGGTAACTAATGGTACTCAAACAAGATATATATCTGCTGGGCAGAATT      321
NCC82        ACTGACATTTACAGCTTCGTAGAAATGTTTGTGGTAACTAATGGTACTCAAACAAGATATATATCTGCTGGGCAGAATT      321
NCC88        ACTGACATTTACAGCTTCGTAGAAATGTTTGTGGTAACTAATGGTACTCAAACAAGATATATATCTGCTGGGCAGAATT      321
L.l.204      ACTGACATTTACAGCTTCGTAGAAATGTTTGTGGTAACTAATGGTACTCAAACAAGATATATATCTGCTGGGCAGAATT      307
Consensus    actgacatttacagcttcgtagaaatgtttgtggtaactaatggtactcaaacaagatatatatctgctgggcagaatt

ATCC 11842   TGAATGCCAAGTTTTTAACGGCCTGGGTTGATAAGAATAATAAGCGGGTAGACAATTATCTGAGTTTTGCTGAAGAGGTC      401
NCC82        TGAATGCCAAGTTTTTAACGGCCTGGGTTGATAAGAATAATAAGCGGGTAGACAATTATCTGAGTTTTGCTGAAGAGGTC      401
NCC88        TGAATGCCAAGTTTTTAACGGCCTGGGTTGATAAGAATAATAAGCGGGTAGACAATTATCTGAGTTTTGCTGAAGAGGTC      401
L.l.204      TGAATGCCAAGTTTTTAACGGCCTGGGTTGATAAGAATAATAAGCGGGTAGACAATTATCTGAGTTTTGCAGAAGAGGTT      387
Consensus    tgaatgccaagttttaacggcctgggttgataagaataataagcgggtagacaattatctgagttttgctgaagaggtc

ATCC 11842   TTGTCAATTCCTGCTGCTCACCATATGATTGCCGACTATGTGGTCTTAGACAGCGAAAACAAGAGCGTTATCCTGCTCCGTCCTTACCAGATAC      495
NCC82        TTGTCAATTCCTGCTGCTCACCATATGATTGCCGACTATGTGGTCTTAGACAGCGAAAACAAGAGCGTTATCCTGCTCCGTCCTTACCAGATAC      495
NCC88        TTGTCAATTCCTGCTGCTCACCATATGATTGCCGACTATGTGGTCTTAGACAGCGAAAACAAGAGCGTTATCCTGCTCCGTCCTTACCAGATAC      495
L.l.204      TTGTCAATACCTGCTGCTCACCATATGATTGCCGACTATGTGGTTTTAGACAGCGAAAGCAAGAGCGTTATCCTGCTC..................      465
Consensus    ttgtcaattcctgctgctcaccatatgattgccgactatgtggtcttagacagcgaaaacaagagcgttatcctgctccgtccttaccagatac
```

Homology matrix of 4 sequences

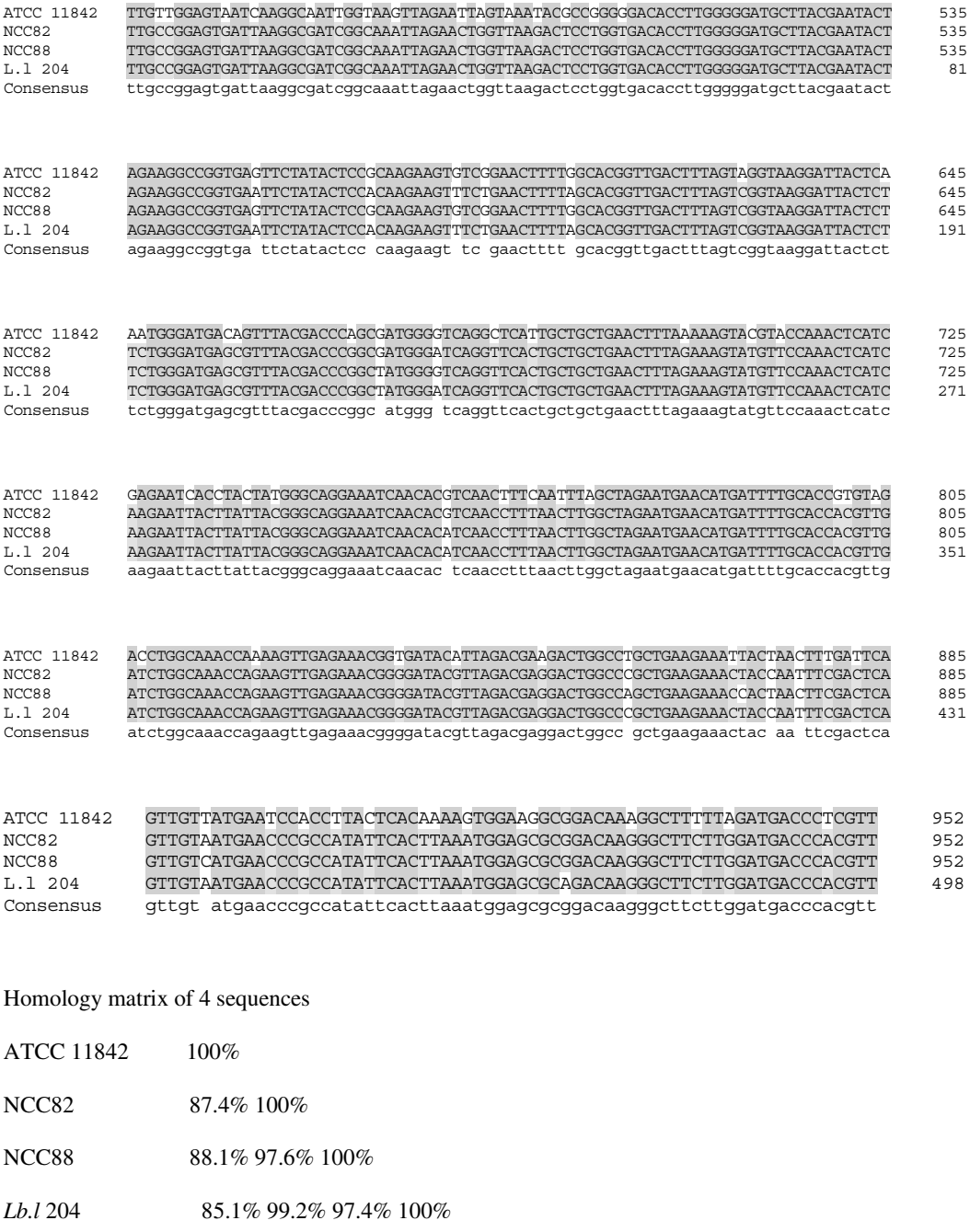
ATCC 11842	100%			
NCC82	100.0%	100%		
NCC88	100.0%	100.0%	100%	
Lb.l 204	96.6%	96.6%	96.6%	100%

Fig. 3. Sequence alignments of the *hsdR* gene for *Lactobacillus delbrueckii* susbsp. *bulgaricus* ATCC 11842, *Lb. delbrueckii* subsp. *lactis* NCC82 and *Lb. delbrueckii* subsp. *lactis* NCC88, with the corresponding amplicon sequence obtained from *Lb. delbrueckii* subsp. *lactis* 204.

hsdR gene fragment obtained from *Lb.l* 204 with published sequences of the same gene present in other *Lb. delbrueckii* strains gave a consensus of 96·6% (*Lb. delbrueckii* subsp. *bulgaricus* ATCC 11842, *Lb. delbrueckii* subsp. *lactis* NCC82 and *Lb. delbrueckii* subsp. *lactis* NCC88). On the other hand, the similarity percentages of the sequenced *hsdM* gene fragment obtained from *Lb.l* 204 with the other published *Lb. delbrueckii hsdM* genes were highest with *Lb. delbrueckii* subsp. *lactis* NCC82 (99·2%) and *Lb. delbrueckii* subsp. *lactis* NCC88 (97·4%) and the lowest with *Lb. delbrueckii* subsp. *bulgaricus* ATCC 11842 (85·1%).

Discussion

Lysogeny is widespread in lactococci and lactobacilli (Séchaud et al. 1988; Davidson et al. 1990; Carminati & Giraffa, 1992; Séchaud et al. 1992; Klaenhammer &



Homology matrix of 4 sequences

ATCC 11842	100%
NCC82	87.4% 100%
NCC88	88.1% 97.6% 100%
Lb.l 204	85.1% 99.2% 97.4% 100%

Fig. 4. Sequence alignments of the *hsdM* gene for *Lactobacillus delbrueckii* susbsp. *bulgaricus* ATCC 11842, *Lb. delbrueckii* subsp. *lactis* NCC82 and *Lb. delbrueckii* subsp. *lactis* NCC88, with the corresponding amplicon sequence obtained from *Lb. delbrueckii* subsp. *lactis* 204.

Fitzgerald, 1994; Carminati et al. 1997; Josephsen & Neve, 1998) and, specifically, a high frequency of lysogenic strains has been demonstrated for *Lb. delbrueckii* (Suárez et al. 2008).

Temperate phages can disturb the normal process of fermentations when they infect sensitive strains in starter cultures (Davidson et al. 1990). The source for the bacteriophages used in this study is the commercial strain *Lb. delbrueckii* subsp. *lactis* Cb1 (Suárez et al. 2008). The problem of phage release from lysogenic strains in the dairy industry environment and its consequences are clearly demonstrated in this work.

One effective way to control phages in dairy processes is through the use, in rotation, of strains harboring phage defense mechanisms. Based on their general modes of action, these mechanisms are classified as follows: inhibition

of phage adsorption, DNA ejection blocking, restriction/modification systems and abortive infection mechanisms (Abi) (Labrie & Moineau, 2007). More recently, CRISPRs (Clustered regularly interspaced short palindromic repeats) have been demonstrated as the latest defense mechanism revealed in prokaryotes (Barrangou et al. 2007; Deveau et al. 2008).

Restriction/modification systems are found in many bacteria where they act to protect the cell from foreign DNA such as bacteriophage DNA. In this work, the lysis of Cb1/342 phage on *Lb.l* 204 strain was slower than on its host strain *Lb.b* 342. This fact, in addition to a high adsorption level in both cases, suggested that *Lb.l* 204 strain had intracellular, chromosome-coded (since no plasmids were detected in the strain) phage resistance mechanisms. The activity of R/M mechanisms in *Lb.l* 204 was proven by obtaining the modified and unmodified Cb1/342 bacteriophages.

Restriction/modification systems of LAB collectively fall into Types I, II and III systems (Coffey & Ross, 2002). In Type I R/M systems, the active enzyme comprises three subunits encoded by three closely linked genes, *hsdR* (restriction), *hsdM* (methylation), and *hsdS* (specificity) (Giraffa et al. 1997; Bourniquel, 2000). In *Lb. delbrueckii*, Type I R/M is widespread but a few complete genes were sequenced (Bourniquel, 2000; Van de Guchte et al. 2006). The amplicons obtained by PCR on *Lb.l* 204 DNA using the primers designed for the three R/M systems (I, II and II) demonstrated the presence of a Type I system. The results obtained after sequence alignments suggested that all systems belong to the same family, because the *hsdR* and *hsdM* subunits are much conserved into the Type I R/M families (Murray, 2000). Conversely, the identical *Bgl*II-restriction profiles shown for Cb1/204 – Cb1/342m and Cb1/342 – Cb1/342m-d allowed us to hypothesize that a Type II R/M system is also present in *Lb.l* 204. These data seem to indicate that both Type I and Type II R/M systems may be present in *Lb.l* 204, with the latter being operative within the cell. Further research is needed to better clarify the type(s) of restriction/modification systems actively involved in *Lb.l 204* phage resistance.

In this work we proved that R/M systems of *Lb.l* 204 generate a modified phage progeny from the original phage Cb1/342, which continue the infectious process. This fact becomes even more critical when considering that the original bacteriophage is a prophage isolated from another *Lb. delbrueckii* commercial strain. These facts contribute strongly to diversify the phage population in a factory environment.

This work was supported by the Universidad Nacional del Litoral (Santa Fe, Argentina) (Programación CAI+D 2006, Secretaría de Ciencia y Técnica, Proyecto N° 37-200), the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina) (Project PIP 2004 N° 5321) and the Agencia Nacional de Promoción Científica y Técnológica (ANCyT, Argentina) (Project PICT 2004 N° 20358).

References

Allison G & Klaenhammer T 1998 Phage resistance mechanisms in lactic acid bacteria. *International Dairy Journal* **8** 207–226

Auad L, Ascárate Peril A, Ruiz Holgado A & Raya R 1998 Evidence of a restriction/modification system in *Lactobacillus delbrueckii* subsp. *lactis* CNRZ 326. *Current Microbiology* **36** 271–273

Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA & Horvath P 2007 CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes. *Science* **315** 1709–1712

Bourniquel A 2000 Molecuar insights into the metabolism and physiology of the lactic acid bacterium *Lactobacillus delbrueckii* subsp. *lactis*. Thesis Department of Molecular Microbiology Biozentrum der Universitaet Basel Basel, Switzerland

Bourniquel A, Casey M, Mollet B & Pridmore D 2002 DNA sequence and functional analysis of *Lactobacillus delbrueckii* subsp. *lactis* plasmids pN42 and pJBL2. *Plasmid* **47** 153–157

Candioti MC, Hynes ER, Perotti MC & Zalazar CA 2002 Proteolytic activity of commercial rennets and pure enzymes on whey proteins. *Milchwissenschaft* **57** 546–550

Carminati D & Giraffa G 1992 Evidence and characterization of temperate bacteriophage in *Streptococcus salivarius* subsp. *thermophilus* St18. *Journal of Dairy Research* **59** 71–79

Carminati D, Mazzucotelli G, Giraffa G & Neviani E 1997 Incidence of inducible bacteriophage in *Lactobacillus helveticus* strains isolated from natural whey starter cultures. *Journal of Dairy Science* **80** 1505–1511

Coffey A & Ross P 2002 Bacteriophage-resistance systems in dairy starters strains: molecular analysis to application. *Antoine van Leeuwenhoek* **82** 303–321

Davidson B, Powell IB & Hillier AJ 1990 Temperate bacteriophages and lysogeny in lactic acid bacteria. *FEMS Microbioogyl Reviews* **87** 79–90

de los Reyes-Gavilán C, Linsowtin G, Séchaud L, Veaux M & Accolas J-P 1990 Evidence for a plasmid – linked to restriction – modification system in *Lactobacillus delbrueckii*. *Applied and Environmental Microbiology* **56** 3412–3419

Deveau H, Barrangou R, Garneau JE, Labonté J, Fremaux C, Boyaval P, Romero DA, Horvath P & Moineau S 2008 Phage Response to CRISPR-Encoded Resistance in *Streptococcus thermophilus*. *Journal of Bacteriology* **190** 1390–1400

Giraffa G, De Vecchi P & Reinheimer JA 1997 Population dynamics of thermophilic lactobacilli in mixed starter whey cultures. *Food Research International* **302** 137–140

Giraffa G & Rosetti L 2004 Monitoring of the bacterial composition of dairy starter cultures by RAPD-PCR. *FEMS Microbiology Letters* **237** 133–138

Josephsen J & Neve H 1998 Bacteriophages and lactic acid bacteria. In *Lactic Acid Bacteria Microbiology and Functional Aspects*, pp. 385–436 (Eds S Salminen & A von Wright). New York: Marcel Decker Inc

Klaenhammer TR & Fitzgerald GF 1994 Bacteriophages and bacteriophage resistance. In *Genetics and Biotechnology of Lactic Acid Bacteria*, pp. 106–158 (Eds MJ Gasson & WN de Vos). London: Chapman & Hall

Labrie S & Moineau S 2007 Abortive influence mechanisms and prophage sequences significantly influence genetic makeaup of emerging lytic lactococal phages. *Journal of Bacteriology* **189** 1482–1487

Moineau S, Tremblay D & Labrie S 2002 Phages of lactic acid bacteria: from genomics to industrial applications. *American Society for Microbiology News* **68** 388–393

Murray N 2000 Type I restriction systems: sophisticated molecular machines a legacy of Bertani and Weigle. *Microbiology and Molecular Biology Reviews* **64** 2 412–434

Neviani E & Carini S 1994 Microbiology of Parmesan cheese. *Microbiologie Aliments Nutrition* **12** 1–8

Quiberoni A, Guglielmotti D, Binetti A & Reinheimer J 2004 Characterization of three *Lactobacillus delbrueckii* subsp *bulgaricus* phages and the physicochemical analysis of phage adsorption. *Journal of Applied Microbiology* **96** 340–351

Quiberoni A, Reinheimer J & Tailliez P 1998 Characterization of *Lactobacillus helveticus* phage resistant mutants by RAPD fingernger-prints and phenotypic parameters. *Food Research International* **31** 537–542

Reinheimer JA, Suárez VB, Bailo NB & Zalazar CA 1995 Microbiological and technological characteristics of natural whey cultures for Argentinian hard-cheese production. *Journal of Food Protection* **58** 7 796–799

Reinheimer J, Quiberoni A, Tailliez P, Binetti A & Suárez V 1996 The lactic acid microflora of natural whey starters used in Argentina. *International Dairy Journal* **6** 869–879

Sambrook J & Russell DW 2001 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Schouler C, Clier F, Lerayer AL, Ehrlich DS & Chopin M-L 1998 A type IC restriction-modification system in *Lactococcus lactis*. *Journal of Bacteriology* **180** 2 407–411

Séchaud L, Callegari M-L, Rousseau M, Muller M-C & Accolas J-P 1989 Relationship between temperate bacteriophage 0241 and virulent phage 832-B1 of *Lactobacillus helveticus*. *Netherlands Milk and Dairy Journal* **43** 261–277

Séchaud L, Cluzel P-J, Rousseau M, Baumgartner A & Accolas J-P 1988 Bacteriophages of lactobacilli. *Biochimie* **70** 401–410

Séchaud L, Rousseau M, Fayard B, Callegari ML, Quénée P & Accolas J-P 1992 Comparative study of 35 bacteriophages of *Lactobacillus helveticus*: morphology and host range. *Applied and Environmental Microbiology* **58** 1011–1018

Suárez V, Zago M, Quiberoni A, Carminati D, Giraffa G & Reinheimer J 2008 Lysogeny in Lactobacillus delbrueckii strains and characterization of two new temperate prolate-headed bacteriophages. *Journal of Applied Microbiology* **105** 1402–1411

Svensson V & Christiansson A 1991 Methods for phage monitoring. *FIL-IDF Bulletin* **263** 29–39

Tock M & Dryden D 2005 The biology of restriction and anti-restriction. *Current Opinion in Microbiology* **8** 466–472

Van de Guchte M, Penaud S, Grimaldi C, Barbe V, Bryson K, Nicolas P, Robert C, Oztas S, Mangenot S, Couloux A, Loux V, Dervyn R, Bossy R, Bolotin A, Batto JM, Walunas T, Gibrat JF, Bessières P, Weissenbach J, Ehrlich SD & Maguin E 2006 The complete genome sequence of *Lactobacillus bulgaricus* reveals extensive and ongoing reductive evolution. *Proceedings of the National Academy of Sciences of the United States of America* **103** 9274–9279

Yamamoto KR, Alberts BM, Benzinger R, Lawhorne L & Treiber G 1970 Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. *Virology* **40** 734–744