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ORIGINAL ARTICLE

# Lysogeny in *Lactobacillus delbrueckii* strains and characterization of two new temperate prolate-headed bacteriophages

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## Abstract

**Aims:** Frequency of lysogeny in *Lactobacillus delbrueckii* strains (from commercial and natural starters) and preliminary characterization of temperate bacteriophages isolated from them.

**Methods and Results:** Induction of strains (a total of 16) was made using mitomycin C (MC) (0·5 $\mu$g ml$^{-1}$). For 37% of the MC-treated supernatants, it was possible to detect phage particles or presence of killing activity, but only two active bacteriophages were isolated. The two temperate phages isolated were prolate-headed phages which belonged to group *c* of *Lact. delbrueckii* bacteriophages classification. Different DNA restriction patterns were obtained for each phage, while the structural protein profiles and packaging sites were identical. Distinctive one-step growth curves were exhibited by each phage. An influence of calcium ions was observed for their lysis in broth but not on the adsorption levels.

**Conclusions:** Our study showed that lysogeny is also present in *Lact. delbrueckii* strains, including commercial strains.

**Significance and Impact of the Study:** Commercial strains could be lysogenic and this fact has a great practical importance since they could contribute to the dissemination of active-phage particles in industrial environments.

## Introduction

Phage infection is the most important cause of slow acid production by lactic acid bacteria (LAB) during industrial fermentations (Neve 1996; Moineau 1999; Suárez *et al.* 2002). The economic losses and the public health consequences incurred when a phage infection occurs may be very significant (Josephsen and Neve 1998; Forde and Fitzgerald 1999).

The lysogenic stage in LAB is considered the principal source of bacteriophages in dairy industrial environments. Temperate phages can disturb the normal process of fermentations when, by mutation, they become virulent phages capable of overcoming superinfection immunity. Another problem derived from the use of lysogenic strains is their potentially spontaneous induction, releasing phage particles that could infect sensitive strains of starter cultures (Davidson *et al.* 1990). The consequences of this fact are evident when commercial starters are used, because they are composed of a low number of strains.

In particular, *Lactobacillus delbrueckii* is one of the dairy LAB species (together with *Streptococcus thermophilus*) used in yogurt production and is a fundamental constituent of whey starter cultures used for hard cheeses (Curry and Crow 2003; Giraffa and Rossetti 2004). Because of the potential negative impact on dairy technology, phages of *Lact. delbrueckii* need further studies.

Virulent and temperate *Lact. delbrueckii* bacteriophages have been characterized and classified into four groups (*a* to *d*) on the basis of morphology, immunoblotting

tests and DNA–DNA hybridizations (Mata *et al.* 1986; Séchaud *et al.* 1988). Prolate-headed bacteriophages are included in group *c*. This group is constituted by a small number of phages which belong only to the species *Lact. delbrueckii* ssp. *lactis* and a few of them were characterized. Séchaud *et al.* (1988) described the morphology of two prolate-headed phages of *Lact. delbrueckii* ssp. *lactis*. More recently, the characterization of phage JLC 1032, based on its homology with isometric-headed phages, was reported by Forsman (1993).

In this study, we investigated the frequency of lysogeny in *Lact. delbrueckii* strains and characterized two newly isolated, temperate prolate-headed phages of *Lact. delbrueckii* ssp. *lactis*.

## Materials and methods

### Strains and culture conditions

Bacteria tested for induction are listed in Table 1. Stocks were maintained in MRS broth (Biokar, Beauvois, France) with the addition of 15% (v/v) of glycerol as a cryoprotective agent at −80°C, and in reconstituted (10% w/v) commercial dried skimmed milk (RSM), and routinely cultured overnight at 42°C in MRS broth.

### Phage induction

*Lactobacillus delbrueckii* presumptive lysogenic strains were selected on the basis of the presence of the integrase gene, according to Zago *et al.* (2007). Strains were induced by the addition of 0·5 $\mu$g ml$^{-1}$ mitomycin C (MC, Sigma Chemical Co., St Louis, MO, USA) to an early exponential–phase culture of the strain ($OD_{560\ nm}$ = 0·15) at 42°C. Incubation continued up to a good growth of control (medium without MC), which was evaluated by measuring absorbance at 560 nm. After centrifugation at 12 000 ***g*** (10 min at room temperature), the supernatants were filtered (Millipore, 0·45 $\mu$m) and tested for the presence of phages. Host range of temperate phages was also studied. The presumptive host strains used to propagate the temperate bacteriophages and evaluate their strain cross-sensitivity are listed in Table 1. The methodologies applied were: spot test (with and without thermal treatment for 15 min at 90°C), turbidity test (Svensson and Christiansson 1991), and microplate test (Zago *et al.* 2007). MRS broth or agar, which had been supplemented with 10 mmol l$^{-1}$ $CaCl_2$ (MRS-Ca when specified), was routinely used to propagate and count phages. In addition, the presence of killing activity was tested using the methodology described by Carminati *et al.* (1997).

Phage stocks of temperate isolated phages were maintained at −80°C with addition of 15% (v/v) of glycerol.

**Table 1** Identification and origin of strains used in this study

| Strains* | Genus/species/subspecies | Origin (culture/country) |
|---|---|---|
| *L.l* 192† | *Lactobacillus delbrueckii* ssp. *lactis* | Grana Padano whey starter (Italy) |
| *L.l* 193† | | Grana Padano whey starer (Italy) |
| *L.l* 195† | | Grana Padano whey starter (Italy) |
| *L.l* 197† | | Grana Padano whey starter (Italy) |
| *L.l* 200† | | Grana Padano whey starter (Italy) |
| *L.l* 203† | | Provolone whey starter (Italy) |
| *L.l* 204† | | Provolone whey starter (Italy) |
| *L.l* 205 | | Provolone whey starter (Italy) |
| *L.l* 209 | | Provolone whey starter (Italy) |
| *L.l* 212 | | Provolone whey starter (Italy) |
| *L.l* 216 | | Grana Padano whey starter (Italy) |
| *L.l* 217† | | Grana Padano whey starter (Italy) |
| *L.l* 218 | | Grana Padano whey starter (Italy) |
| *L.l* 219 | | Grana Padano whey starter (Italy) |
| *L.l* 242 | | Grana Padano whey starter (Italy) |
| *L.l* 254† | | Provolone whey starter (Italy) |
| *L.l* Cb1† | | Yoghurt commercial starter (Argentina) |
| *L.l* Gb2† | | Yoghurt commercial starter (Argentina) |
| *L.l* M† | | Cremoso cheese (Argentina) |
| *L.l* CNRZ 326 | | Collection strain |
| *L.b.* 342 | *Lact. delbrueckii* ssp. *bulgaricus* | Yoghurt (Italy) |
| *L.b* c.332† | | Yoghurt commercial starter (Argentina) |
| *L.b* Hb2† | | Yoghurt commercial starter (Argentina) |
| *L.b* Eb4† | | Yoghurt commercial starter (Argentina) |
| *L.b* Bb1† | | Yoghurt commercial starter (Argentina) |

*All strains were used as presumptive host (except *L.l* CNRZ 326) and to perform the cross-sensitivity of bacteriophages.
†Strains induced.

### Electron microscopy

Micrographs of active temperate phages were obtained according to Bolondi *et al.* (1995). Phage suspensions were concentrated by centrifugation (1 h, 70 000 ***g***, 5°C) and then stained using uranyl acetate (2% w/v, pH 4·5) or phosphotungstic acid (2% w/v). Electron micrographs were taken with a JEOL 100-C electron microscope (Jeol USA, Inc. Peabody, MA, USA) operating at 80 kV. Phage morphologies and dimensions (capsid diameter, tail length, and width) were recorded.

### Phage DNA manipulation and analysis

Phages were propagated in a volume of 100 ml of MRS-Ca broth, treated for 30 min with DNase I (Sigma-Aldrich Corporation, St Louis, MO, USA) (1 $\mu$g ml$^{-1}$)

 

and RNase (USB Corporation, Cleveland, OH, USA) (1 $\mu$g ml$^{-1}$), centrifuged (10 min at 5000 ***g***) and filtered (Millipore membranes, 0·45 $\mu$m pore size). Phage particles were concentrated overnight at 4°C with PEG 8000 (10% w/v) and 0·5 mol l$^{-1}$ NaCl (Yamamoto *et al.* 1970), centrifuged (10 min at 10 000 ***g***) and resuspended in TE buffer (10 mmol l$^{-1}$ Tris-HCl and 1 mmol l$^{-1}$ EDTA, pH 8·0). Phage DNAs were obtained by three phenol–chloroform–isoamyl alcohol extractions and concentrated by ethanol precipitation. DNA pellets were resuspended in double-distilled and nuclease-free water. Phage DNAs were quantified by electrophoresis on agarose (0·8% w/v) gels (Quiberoni *et al.* 2004). Their visualization by ethidium bromide colouration was performed according to standard protocols (Sambrook *et al.* 1989). The DNA molecular weight marker 1kpb (Amersham Biosciences UK limited, UK) was used as a standard.

For packaging site studies, phage DNA aliquots, whether ligated or not (T4 DNA ligase, USB Corporation), were cleaved with endonuclease enzymes (*Hind*III, *Bgl*II and *Eco*RV), 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 *et al.* 1989) to resolve DNA fragments. Sizes of the phage genomes were estimated by summing the *Bgl*II- and *Eco*RV-digested DNA fragments, using 1kpb as DNA molecular weight marker.

### Bacteriophage structural proteins

Phage particles (approx. 10$^{10}$ PFU ml$^{-1}$) were suspended in EDTA 20 mmol l$^{-1}$ (pH = 8·0), disrupted by boiling (3 min) and separated by electrophoresis on polyacrylamide gels with sodium dodecyl sulfate (SDS-PAGE) according to Candioti *et al.* (2002). Acrylamide was used to prepare stacking (4%, w/v) and separation (13%, w/v) gels. The procedure was carried out at alkaline pH using a Tris-glycine buffer. An electrophoretic chamber Mini Protean II (Bio-Rad) was used. After electrophoresis, the protein bands were stained for 1 h at room temperature, in 0·2% (w/v) Coomassie Brilliant Blue R-250 dissolved in methanol : acetic acid :water, 40 : 16 : 44 (v/v/v). The gels were de-stained in ethanol : acetic acid : water, 25 : 10 : 65 (v/v/v) until a clear background was obtained. Broad-range protein molecular weight marker (Promega) was used to estimate the molecular weight of major phage proteins.

### One-step growth curves

*Lactobacillus delbrueckii* sensitivity strains, in exponential growth ($OD_{560\ nm}$ = 0·5), were harvested and suspended in one-fifth of the initial volume of MRS-Ca broth. Phages were added with a multiplicity of infection (m.o.i.) of 2, approximately. After adsorption (30 min at 42°C), cells were harvested by centrifugation (10 000 ***g*** for 5 min), resuspended in 10 ml of MRS-Ca broth and decimal dilutions of this suspension were carried out. Suspension and dilutions were then incubated at 42°C. At regular intervals, 100 $\mu$l of each dilution were collected for bacteriophage counts (Chow *et al.* 1988). Latent periods, burst times and burst sizes were calculated from one-step growth curves. Values are the mean of three determinations.

### Influence of calcium ions

The influence of calcium ions on cell lysis was studied by incubation (42°C) of infected (m.o.i. = 0·5) sensitive strains in MRS broth, with and without 10 mmol l$^{-1}$ $Ca^{2+}$.

The effect of calcium ions on phage adsorption to bacterial cells was performed by determination of the adsorption rate (30 min) in MRS and MRS-Ca broth as described by Séchaud *et al.* (1989) but modified as follows: exponentially growing ($OD_{560\ nm}$ = 0·5) host strain cultures in MRS broth were centrifuged and resuspended at a concentration of $5 \times 10^8$ CFU ml$^{-1}$, approximately, in MRS and MRS-Ca broth. Each phage was added (m.o.i. = 0·02) and the mixtures were incubated at 42°C for adsorption, after distributing the infected cultures in Eppendorf tubes. At 30 min, tubes were centrifuged (10 000 ***g*** for 5 min) and supernatants were titred to enumerate unadsorbed free phage particles. The results were expressed as percentages of the initial phage counts.

## Results

### Phage induction

All strains used for induction presented a DNA amplification fragment of 315 bp (data not shown), which corresponded to that expected for the *Lact. delbrueckii* ssp. *lactis* integrase gene (Zago *et al.* 2007). All strains tested for induction showed a steady decrease of the $OD_{560\ nm}$ values of cultures treated with MC, compared with a control without MC. However, it was only possible to find a sensitive strain for only six MC-treated supernatants (37% out of the total strains) (Table 2). After treatment at 90°C, all MC culture supernatants gave a negative signal with the agar spot test demonstrating the presence of active viral particles or heat-labile bactericidal molecules. Proliferating phages able to infect and lyse indicator host strains were found only for three MC-treated supernatants

**Table 2** Detection of phage particles from *Lactobacillus delbrueckii* strains induced by MC, using different methodologies

| | Methodology | | | |
|---|---|---|---|---|
| | Spot-test† | | | |
| Induced strains* | Induced without thermal treatment | Induced with thermal treatment¶ | Microplate test‡ | Turbidity test§ |
| *L.l* 192 | + (*L.l* 219)** | – | + (*L.l* 209, 2°)†† | + (*L.l* 209, 2°)†† |
| *L.l* 193 | + (*L.l* 218)** | – | – | – |
| *L.l* 195 | + (*L.l* 219)** | – | + (*L.l* 209, 2°)†† | + (*L.l* 209, 2°)†† |
| *L.l* 197 | – | – | – | – |
| *L.l* 200 | – | – | – | – |
| *L.l* 203 | – | – | – | – |
| *L.l* 204 | – | – | – | – |
| *L.l* 217 | – | – | – | – |
| *L.l* 254 | – | – | – | – |
| *L.l* Cb1 | – | – | + (*L.l* 204, 2°)†† | + (*L.l* 204, 2°)†† |
| | | | + (*L.b* 342, 3°)†† | + (*L.b* 342, 3°)†† |
| *L.l* c.332 | + (*L.l* 218)** | – | – | – |
| *L.l* M | – | – | – | – |
| *L.l* Gb2 | – | – | – | – |
| *L.b* Hb2 | – | – | – | – |
| *L.b* Eb4 | + (*L.l* 218)** | – | – | – |
| *L.b* Bb1 | – | – | – | – |

*L.l*, *Lact. delbrueckii* ssp. *lactis*; *L.b*, *Lact. delbrueckii* ssp. *bulgaricus*.
*0·5 $\mu$g ml$^{-1}$ MC.
†MRS–Ca agar, 24 h – 42°C.
‡MRS–Ca–bromocresol purple broth.
§MRS–Ca broth.
¶15 min – 90°C.
()**Sensitive strains.
()††Sensitive strains, subculture number when lysis was observed.

**Table 3** Killing activity of MC culture supernatants on non-proliferating cells of their respective host strains*

| Host strain | Untreated cells counts (log orders) (control) | Viable cells after treatment with MC culture supernatants ($x \pm \delta$)† (log orders) | | | | | |
|---|---|---|---|---|---|---|---|
| | | I193 | IEb4 | Ic.332 | I192 | I195 | ICb1 |
| *L.l* 218 | 7·62 ± 0·06 | 6·60 ± 0·07‡ | 6·92 ± 0·03‡ | 6·43 ± 0·04‡ | nd | nd | nd |
| *L.l* 219 | 7·16 ± 0·12 | nd | nd | Nd | 7·38 ± 0·07§ | 7·35 ± 0·15§ | nd |
| *L.l* 204 | 7·69 ± 0·06 | nd | nd | Nd | nd | nd | 7·60 ± 0·05§ |
| *L.b* 342 | 7·58 ± 0·08 | nd | nd | Nd | nd | nd | 7·61 ± 0·04§ |

nd, no determined; *L.l*, *Lactobacillus delbrueckii* ssp. *lactis*; *L.b*, *Lactobacillus delbrueckii* ssp. *bulgaricus*
*Killing activity was evaluated by a decrease of viable cells after treatment with MC culture supernatants for 60 min at 42°C compared with the decrease of untreated cells (control) incubated at the same condition and with the same concentration of MC.
†One-way ANOVA. Results are media ± SD ($x \pm \delta$) of three determinations.
‡Different from the respective control ($P < 0·01$).
§Not different from the respective control ($P > 0·01$).

(*Ll* 192, *Ll* 195, *Ll* Cb1; Table 2). From the commercial strain *Lact. delbrueckii* ssp. *lactis* Cb1, it was possible to isolate active viral particles, which were able to propagate on two different host strains. The bacteriophages which were able to propagate on *Lact. delbrueckii* ssp. *lactis* 204 and *Lact. delbrueckii* ssp. *bulgaricus* 342 were named Cb1/204 and Cb1/342, respectively.

The killing activity was positive for three MC-treated strains (Table 3). These presumptive lysogenic strains (*L.l* 193, *L.b* Eb4 and *L.b* c.332) showed a positive reaction in a spot test (without thermal treatment), but it was not possible to isolate phages from them (Table 2). No killing activity was detected with the other three MC culture supernatants (*Ll* 192, *Ll* 195, *Lb* Cb1).

 

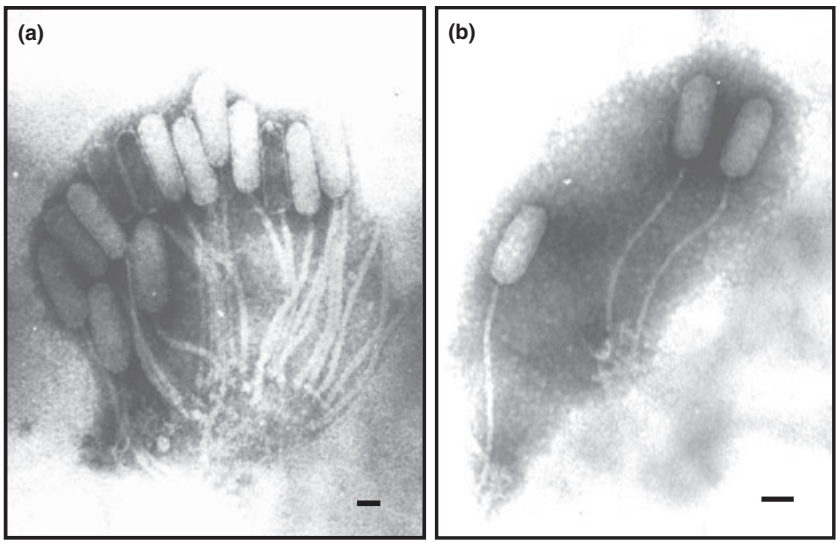


**Figure 1** Electron micrographs of temperate prolate-headed *Lactobacillus delbrueckii* ssp. *lactis* phages Cb1/204 (a) and Cb1/342 (b). Bars represent 50 nm.

### Electron microscopy

Temperate bacteriophages, Cb1/204 and Cb1/342, showed prolate heads and long non-contractile tails with transversal striations (Fig. 1). Dimensions of both phages were similar, showing a capsid of 125 ± 2 nm long and 58 ± 2 nm wide, and a tail of 334 ± 3 nm long and 12 ± 0·5 nm wide. These phages were classified as members of the *Siphoviridae* family (morphotype B1) (Matthews 1982), which correspond to group B of Bradley (Bradley 1967). According to their morphology, these phages belong to group *c* (prolate-headed) (Séchaud *et al.* 1988).

### Host range (strain cross-sensitivity)

Temperate bacteriophages, Cb1/204 and Cb1/342, were tested against several strains of *Lact. delbrueckii* (20 *Lact. delbrueckii* ssp. *lactis* strains and 5 *Lact. delbrueckii* ssp. *bulgaricus* strains). Both phages were able to produce lysis on both indicator strains (*L.l* 204 and *L.b* 342), but the lysis of phage Cb1/342 on *L.l* 204 occurred later (Table 4). Besides, both bacteriophages were capable of producing lysis on collection strain CNRZ 326, which is a sensitive strain of temperate bacteriophages lb539 and mv4 (both members of group *a* of *Lact. delbrueckii* bacteriophage classification) (Séchaud *et al.* 1988; Auad *et al.* 1997). The other strains used were not sensitive to these newly isolated bacteriophages.

### Genetic characterization

The Cb1/204 and Cb1/342 bacteriophage genomes consisted of a linear, double-stranded DNA. Genome sizes were estimated as approximately 53·8 ± 1·79 kbp (Cb1/204) and 58·2 ± 4·41 kbp (Cb1/342) by adding the sizes of DNAs fragments generated by the restriction enzymes *Eco*RV and *Bgl*II. Different restriction patterns were exhibited when DNA of Cb1/204 and Cb1/342 were treated with *Hind*III and *Bgl*II (Fig. 2).

The Cb1/204 and Cb1/342 genomes did not appear to contain cohesive ends because heating for 10 min at 70°C

**Table 4** Strain cross-sensitivity in broth and adsorption rate (%) of host strains with phages Cb1/204 and Cb1/342

| | Adsorption rate (30 min – 42°C)* | | | | Lysis in broth (MRS)† | | | |
|---|---|---|---|---|---|---|---|---|
| | Cb1/204 | | Cb1/342 | | Cb1/204 | | Cb1/342 | |
| Strain | $Ca^{2+}$ (10 mmol $l^{-1}$) | No $Ca^{2+}$ | $Ca^{2+}$ (10 mmol $l^{-1}$) | No $Ca^{2+}$ | $Ca^{2+}$ (10 mmol $l^{-1}$) | No $Ca^{2+}$ | $Ca^{2+}$ (10 mmol $l^{-1}$) | No $Ca^{2+}$ |
| *L.l* 204 | 99·7 ± 0·2 | 99·5 ± 0·1 | 99·5 ± 3·5 | 99·8 ± 0·1 | 1 | – | 2 | – |
| *L.b* 342 | 94·0 ± 1·9 | 92·5 ± 2·2 | 94·0 ± 1·9 | 95·5 ± 0·2 | 1 | 4 | 1 | 3 |

*Results are the media ± SD ($x \pm \delta$) of three determinations.
†Subculture number when lysis was observed – no lysis.
*L.l*, *Lactobacillus delbrueckii* ssp. *lactis*; *L.b*, *Lactobacillus delbrueckii* ssp. *bulgaricus*.

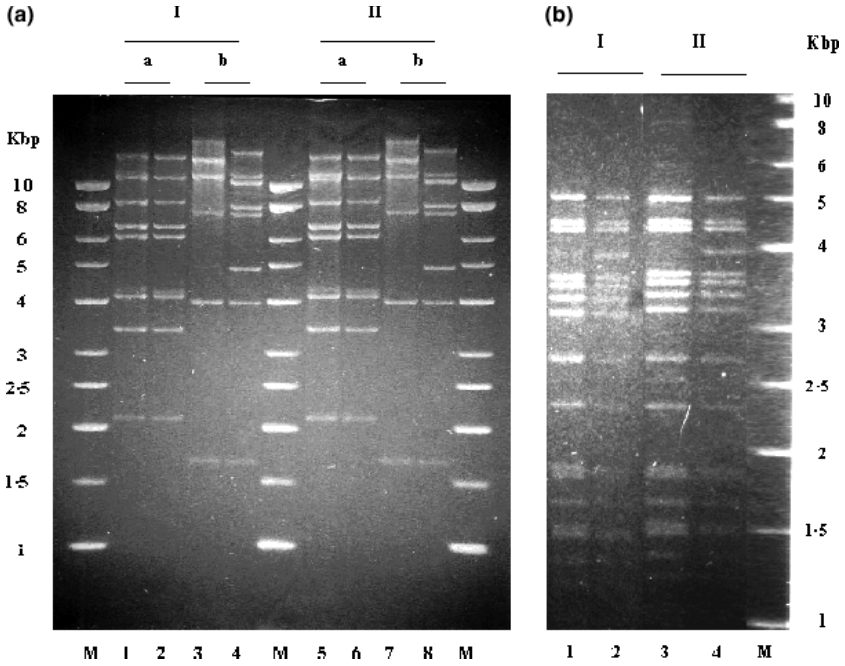


**Figure 2** (a) Agarose gel electrophoresis of *Eco*RI (i) and *Bgl*II (ii) generated DNA fragments of phages Cb1/204 (lanes 1, 3, 5 and 7) and Cb1/342 (lanes 2, 4, 6 and 8). Lanes I and II, not ligated and ligated, respectively. (b) Agarose gel electrophoresis of *Hind*III generated DNA fragments of phages Cb1/204 (lanes 1 and 3) and Cb1/342 (lanes 2 and 4). Lanes I and II, not ligated and ligated, respectively. Lane M: 1Kbp DNA marker.

before electrophoresis did not alter the restriction patterns of *Hind*III, *Eco*RV, *Bgl*II (Fig. 2), *Bam*HI and *Eco*RI (data not shown). However, submolar fragments were not observed as a consequence of DNA digestions with the same restriction endonucleases.

### Bacteriophage structural proteins

Patterns of structural proteins obtained from both temperate bacteriophages were identical. Three strong (23, 31 and 60 kDa) and one weak (49 kDa) bands were observed for each phage (data not shown).

### One-step growth curves

Multiplication parameters of the lytic cycle of both temperate bacteriophages were determined by one-step growth curves (Fig. 3). Latent periods were 60 min for Cb1/204 and 120 min for Cb1/342, while burst periods were 110 and 140 min, respectively. Burst size values of Cb1/204 and Cb1/342 were estimated as 61·7 ± 2·5 and 29·0 ± 2·0 PFU per infected cell, respectively.

### Influence of calcium ions

Calcium ions were indispensable to complete the lytic cycle in broth of Cb1/204 and Cb1/342 on strain *L.l* 204. On the contrary, it was possible to observe a delayed lysis of *L.b* 342 strain with both bacteriophages, in broth without $Ca^{2+}$, at the fourth and third subculture (Table 4).

Adsorption rates (%) were similar with and without $Ca^{2+}$ (Table 4). The values were high and ranged between 92·5% and 99·8%. For *L.b* 342 with both phages, with and without $Ca^{2+}$, adsorption rates were lower than those obtained for *L.l* 204. Particularly, for Cb1/342, the adsorption rate on *L.l* 204 was high (99·5%), but lysis was not observed until a second subculture in comparison with a lysis control on its host strain *L.b* 342.

## Discussion

The study of lysogeny in LAB is very necessary for a number of reasons. At a practical level, lysogenic strains are a reservoir of lytic phages in dairy plants (Klaenhammer and Fitzgerald 1994). Lysogeny is widespread in lactococcal and lactobacilli, and several studies have demonstrated high frequencies of lysogenic strains (Séchaud *et al.* 1988, 1992; Davidson *et al.* 1990; Carminati *et al.* 1997; Josephsen and Neve 1998). Apparently, lysogeny is less common in *Strep. thermophilus* since Carminati and Giraffa (1992) found only one strain inducible by MC out of 45 strains tested. In *Lactobacillus helveticus*, the inducible strains ranged from 10% to 80% according to Carminati *et al.* (1997). In the present study, lysogeny has also been confirmed for *Lact. delbrueckii*. Host strains were found for 19% of the MC-treated supernatants (3 out of 16). An important fact was that one of the inducible strains was a commercial one. Even if in lactobacilli a high percentage of strains are inducible, complete phage particles able to propagate on host strains are less

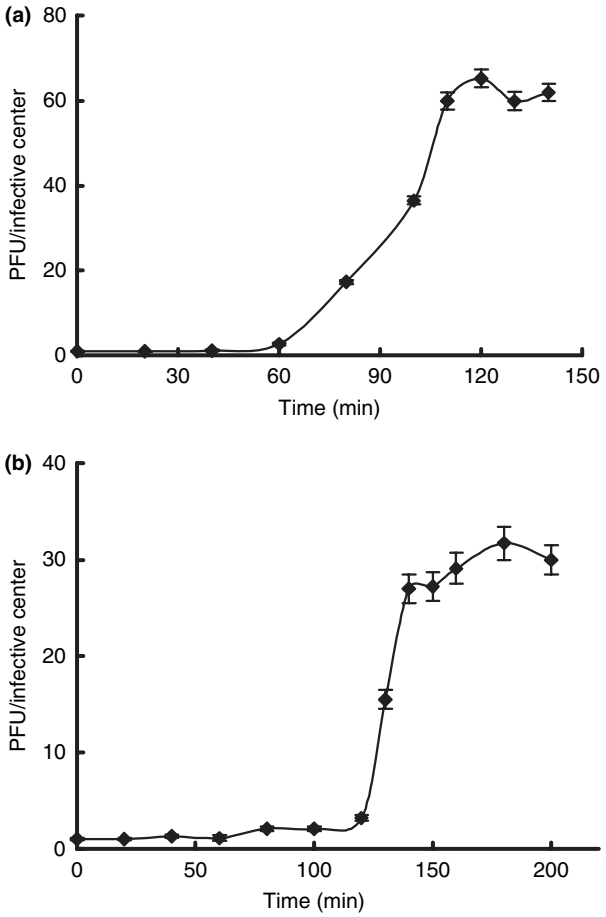


**Figure 3** One-step growth curve of phages Cb1/204 (a) and Cb1/342 (b) on *L.l* 204 and *L.b* 342, respectively. Values are the mean of three determinations.

frequently present (Carminati *et al.* 1997). The presence of defective phages or 'killer' particles unable to propagate on suitable indicators was previously reported (Davidson *et al.* 1990; Carminati *et al.* 1997). Our study showed the presence of a killing activity in three out of six MC culture supernatants. In the absence of direct electron microscopy confirmation, the presence of defective phages, endolysin or bacteriocins could be supposed.

Phages included in group *c* (Séchaud *et al.* 1988) have a prolate head and belong to *Lact. delbrueckii* ssp. *lactis*. Forsman (1993) described phage JCL 1032, which has a prolate head (group *c*) but has intermediate molecular characteristics between groups *a* and *b*. Both temperate phages (Cb1/204 and Cb1/342) isolated in our study are included, morphologically, in group *c*. Cb1/204 and Cb1/342 phages presented a longer head width and tail length than *Lact. delbrueckii* ssp. *lactis* JCL 1032 (Forsman 1993) and 0235 (Séchaud *et al.* 1988) and their dimensions were more similar to those reported for the prolate-headed temperate phage of *Lactobacillus acidophilus* y8 (Kilic *et al.* 1996).

The genome size of phages Cb1/204 and Cb1/342 (53·8 and 58·2 kb, respectively) was higher than that previously reported for temperate and virulent phages specific of *Lact. delbrueckii* (Mata *et al.* 1986; Forsman and Alatossava 1991; Auad *et al.* 1997; Quiberoni *et al.* 2004), and other thermophilic lactobacilli (Capra *et al.* 2006). The genome size of prolate-headed *Lact. delbrueckii* ssp. *lactis* phage JCL 1032 (45·8 kpb) was higher than that of phages with isometric head (31–37 kpb). However, the genome sizes of our phages were more similar to the prolate-headed temperate *Lact. acidophilus* phage y8 (54·3 kpb) (Kilic *et al.* 1996).

The *Bgl*II and *Hind*III restriction patterns showed that phages Cb1/204 and Cb1/342 presented minor differences between them and are closely related. Similar results were found for temperate phages mv1 and mv4, isolated from two *Lact. delbrueckii* ssp. *bulgaricus* strains and obtained from the same factory (Mata *et al.* 1986). Restriction endonuclease patterns were almost identical for mv1 and mv4; however, the two phages differed in their DNA size (Alatossava *et al.* 1995). The packaging sites for our temperate bacteriophages seem to be *pac* ends, as for temperate bacteriophages mv4 (Vasala *et al.* 1993) and lb 539 (Auad *et al.* 1997), both included in morphological group *a* of *Lact. delbrueckii* bacteriophages. For the other well-characterized prolate-headed JLC 1032 (Forsman 1993), its extremities were cohesive (*cos*).

In general, two major structural proteins are present in *Lact. delbrueckii* bacteriophages. The major structural proteins of lytic *Lact. delbrueckii* ssp. *lactis* LL-H phage are the major capsid protein of 34 kDa and the major tail protein of 19 kDa (Trautwetter *et al.* 1986). The same results were found by Forsman and Alatossava (1991) for other lytic phages (LL-K and LL-S) and by Auad *et al.* (1999) for the temperate bacteriophage lb 539. For temperate bacteriophages mv1 and mv4, the molecular weight of major tail protein is 18 kDa (Mata *et al.* 1986). In the same work, the phages included in group *b* (all belonging to *Lact. delbrueckii* ssp. *bulgaricus* species) showed two bands of 31 and 23 kDa, corresponding to the major capsid and tail proteins, respectively. Phage JCL 1032 (group *c*) showed four major structural proteins of 26, 32, 42 and 50 kDa (Forsman 1993). In our study, the isolated bacteriophages showed three major structural proteins exhibiting molecular weights of 23, 31 and 60 kDa. These protein sizes were more similar to those reported for *Lact. delbrueckii* ssp. *bulgaricus* phages included in group *b*, even when this phage is morphologically classified as a member of group *c*. Similarly, phage JLC 1032 belongs to

group *c* by head morphology, but has some DNA homology with phages grouped as *a* and *b* (Forsman 1993).

Burst size, latent period and burst period of a number of lactobacilli phages have been reported (Watanabe *et al.* 1970; Sarimo *et al.* 1976; Chow *et al.* 1988; Jarvis 1989; Quiberoni *et al.* 2004; Capra *et al.* 2006), and values obtained in this study fall within this range.

The role of inorganic cations ($Ca^{2+}$ and $Mg^{2+}$) in cell lysis is noteworthy. It was observed that chelating agents are able to inhibit bacteriophage proliferation by binding divalent cations (Shafia and Thompson 1964; Das and Marshal 1967; Sadashiva Karnik and Gopinathan 1980; Hicks and Surjawan 2002; Suárez *et al.* 2007), interfering with phage adsorption (Lorenz *et al.* 1997; Luscher-Mattli 2000; Capra *et al.* 2006) or inactivating phage particles (Van Vunakis and Herriot 1961; Yamamoto *et al.* 1968; Bassel *et al.* 1971; Issinger and Falk 1976). Our results demonstrated that while phage adsorption was not affected, the lytic cycle of our phages was not complete in the absence of $Ca^{2+}$. According to Séchaud *et al.* (1988), the $Ca^{2+}$ (or $Mg^{2+}$) ions not only stabilize the coiled DNA inside the phage capsid and greatly improve the adsorption rate, but also control the penetration efficiency of phage DNA into the bacterial cells. For our phages, the $Ca^{2+}$ presence was important after adsorption, possibly within injection step. At this level, the divalent cations could act as counterions during the translocation of the phage DNA across the cellular membrane (Josephsen and Neve 1998), or be involved in the DNA stabilization following the injection step. For other *Lactobacillus* phages, the requirement of $Ca^{2+}$ for either adsorption or lysis was variable (Séchaud *et al.* 1989; Quiberoni and Reinheimer 1998; Quiberoni *et al.* 2004; Capra *et al.* 2006).

Our study showed that lysogeny is also present in *Lact. delbrueckii* strains, including commercial strains. This fact has a great practical importance as they could contribute to the dissemination of active phage particles in industrial environments. Besides, it was possible to isolate and preliminarily characterize two new phages belonging to group *c* (composed of a small number of phages).

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