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Characterization of three *Lactobacillus delbrueckii* subsp. *bulgaricus* phages and the physicochemical analysis of phage adsorption

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ABSTRACT

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Aims: Three indigenous *Lactobacillus delbrueckii* subsp. *bulgaricus* bacteriophages and their adsorption process were characterized.

Methods and Results: Phages belonged to Bradley's group B or the *Siphoviridae* family (morphotype B1). They showed low burst size and short latent periods. A remarkably high sensitivity to pH was also demonstrated. Indigenous phage genomes were linear and double-stranded DNA molecules of approx. 31–34 kbp, with distinctive restriction patterns. Only one phage genome appeared to contain cohesive ends. Calcium ions did not influence phage adsorption, but it was necessary to accelerate cell lysis and improve plaque formation. The adsorption kinetics were similar on viable and nonviable cells, and the adsorption rates were high between 0 and 50°C. SDS and proteinase K treatments did not influence the phage adsorption but mutanolysin and TCA reduced it appreciably. No significant inhibitory effect on phage adsorption was observed for the saccharides tested. This study also revealed the irreversibility of phage adsorption to their hosts.

Conclusions, Significance and Impact of the Study: The study increases the knowledge on phages of thermophilic lactic acid bacteria.

Keywords: adsorption, bacteriophages, *Lactobacillus delbrueckii*, phage morphology.

INTRODUCTION

Lactobacillus delbrueckii subsp. *bulgaricus* is one of the two bacteria required for the production of yoghurt, playing an essential role in the development of the organoleptic, hygienic and perhaps probiotic properties of this food (Hassan and Frank 2001). Bacteriophage infections are the most important cause of slow acid production by lactic acid bacteria (LAB) during industrial fermentations. This is a problem confronted by all dairy industries that manufacture cheeses and fermented milks (Neve 1996; Moineau 1999; Suárez *et al.* 2002). The economic losses and the public health consequences incurred when phage infection occurs may be very significant and have been documented (Josephsen and Neve 1998; Forde and Fitzgerald 1999).

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Despite the economic relevance of *Lact. delbrueckii* subsp. *bulgaricus*, phage adsorption process to cells of this species has not been well described, and no data on the nature of phage receptors was reported. The first step in the bacteriophage lytic cycle is a highly specific interaction between phage proteins located at the tip of the tail (Ravin *et al.* 2002) and specific binding sites (receptors) on the cell wall. A successful attachment may then be followed by the penetration of the phage nucleic acid into the cell, its intracellular replication and the release of phage progeny (Lindberg 1973). The specificity of phage adsorption to cell surface receptors has been well studied in *Escherichia coli* and other Gram-negative bacteria (Gehring *et al.* 1987). In Gram-positive bacteria, phage receptors almost always are

cell surface carbohydrates. Specific adsorption studies for phages of *Lactococcus* species were carried out (Oram and Reiter 1968; Oram 1971; Keogh and Pettingill 1983; Sijtsma *et al.* 1988; Valyasevi *et al.* 1994). However, knowledge on bacilli is very scarce and the nature of phage receptors was only identified in *Bacillus subtilis* (Young 1967) and some species of *Lactobacillus*, such as *Lact. plantarum* (Douglas and Wolin 1971), *Lact. casei* (Yokokura 1977; Ishibashi *et al.* 1982) and *Lact. helveticus* (Callegari *et al.* 1998; Quiberoni and Reinheimer 1998).

Moreover, only partial genetic analyses have been carried out for *Lact. delbrueckii* subsp. *bulgaricus*, because of a lack of an efficient gene transfer system in this species. Only two gene transfer systems for *Lact. delbrueckii* subsp. *bulgaricus* have recently been described, including a conjugation-based gene transfer system (Rantsiou *et al.* 1999; Thompson *et al.* 1999) and electroporation (Serror *et al.* 2002). Then, molecular biology studies on its bacteriophages and their interaction with sensitive strains became tools of great interest that will lead to further understanding of this organism. In addition, phage genomes can be exploited to develop genetic tools suitable to numerous biotechnological applications for industrial purposes.

The present study focused on the characterization of *Lact. delbrueckii* bacteriophages, including indigenous *Lact. delbrueckii* subsp. *bulgaricus* phages, isolated from yoghurts. The influence of physicochemical parameters on cell adsorption and the nature of receptors were also investigated.

MATERIALS AND METHODS

Bacterial strains, bacteriophages and culture conditions

The host strains and indigenous bacteriophages used in this study are listed in Table 1. Phages (isolated from 1997 to 2000 from yoghurt plants) were the sole *Lact. delbrueckii* subsp. *bulgaricus* ones isolated in Argentina so far. For specific studies, bacteriophages LL-H (Alatossava and Pythilá 1980) and lb539 (Auad *et al.* 1997), virulent of *Lact. delbrueckii* subsp. *lactis* LKT (CNRZ 700) and *Lact. delbrueckii* subsp. *lactis* CNRZ 326, respectively, were also used.

Table 1 Indigenous *Lactobacillus delbrueckii* subsp. *bulgaricus* phages and commercial host strains used in this study

Phage	Origin	Date of isolation (month/year)	Sensitive strain (*Lact. delbrueckii* subsp. *bulgaricus*)
BYM	Yoghurt – plant A	05/1997	YSD V
YAB	Yoghurt – plant B	08/1998	Ab_1
Ib_3	Yoghurt – plant B	12/2000	LB-Ib_3

Sensitive strains were isolated from commercial starters used in yoghurt manufactures. They were maintained as frozen stocks at −80°C in reconstituted (10%) commercial nonfat dried skimmed milk (RSM), or MRS broth (Biokar, Beauvais, France) supplemented with 15% of glycerol, and routinely reactivated overnight at 42°C in MRS broth. Phage stocks were prepared as described by Neviani *et al.* (1992) in MRS broth, adding 10 mmol l^{-1} $CaCl_2$ (MRS-Ca), and then stored at 4°C and frozen at −80°C in the presence of 15% of glycerol. Phage enumerations (PFU ml^{-1}) were performed by the double-layer plaque titration method (Svensson and Christiansson 1991), using MRS agar with 10 mmol l^{-1} $CaCl_2$ and 100 mmol l^{-1} glycine (Lillehaug 1997).

Electron microscopy

Micrographs of Argentinian 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.

Strain cross-sensitivity

Strain cross-reactivity was investigated using the Spot and Turbidity Tests, as described Svensson and Christiansson (1991).

DNA manipulation and analysis

Phages were propagated in a volume of 50 ml of MRS-Ca broth, treated for 30 min at 37°C with DNase I (Sigma) (1 μg ml^{-1}) and RNase (Sigma) (1 μg ml^{-1}), centrifuged (10 min at 5000 *g*) and filtered (Millipore membranes, 0·45 μm pore size). Phage particles were concentrated overnight at 4°C with PEG 8000 (Sigma) (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 mol l^{-1} EDTA, pH 8). 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. Their visualization by ethidium bromide coloration was performed according to standard protocols (Sambrook *et al.* 1989).

For packaging sites studies, phage DNA aliquots, ligated (Kit Ready-to-go T4 DNA Ligase, Amersham Pharmacia,

Little Chalfont, UK) and not, were treated with endonuclease enzymes (*Bgl*II and *Hin*dIII), and restriction digests were then heated for 10 min at 70°C. 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 adding the *Bgl*II- and *Hin*dIII-digested DNA fragments, and the 1 kb DNA Ladder (Sigma) was used as marker.

One-step growth curves

Sensitive strains of *Lact. delbrueckii* strain cells, in exponential growth ($O.D._{560nm} = 0{\cdot}5$), were harvested and suspended in one-fifth of initial volume (2 ml) of MRS-Ca broth. Phages were added with a multiplicity of infection (m.o.i.) of 2, approximately. After adsorption at 42°C for 5 min, cells were harvested by centrifugation (10 000 *g* for 4 min), resuspended in 10 ml of MRS-Ca broth and decimal dilutions were incubated at 42°C. At intervals, 100 μl of the suspension were collected for bacteriophage counts (Chow *et al.* 1988). Latent periods, burst times and burst sizes were calculated from one-step growth curves.

pH sensitivity of phages

Each phage (approx. 10^6 PFU ml^{-1}) suspended in MRS-Ca broth, with a previous pH adjustment (pH 4–8), was placed into an Eppendorf tube and used to test the pH influence on the phage stability. After 30 min of incubation (25 and 42°C), tubes were removed and the surviving phages were immediately diluted and counted. The results were expressed as a percentage of the initial phage counts.

Influence of calcium ions

The influence of Ca^{2+} on cell lysis was investigated by incubation (42°C) of infected (multiplicity of infection, m.o.i.: 0·5) *Lact. delbrueckii* cultures in MRS broth, with and without $CaCl_2$ (10 mmol l^{-1}). Plaque formation was investigated using the double-layer plaque technique as previously described, in MRS agar, with and without $CaCl_2$.

The effect of calcium ions on phage adsorption on *Lact. delbrueckii* cells was investigated by determination of the adsorption kinetics in MRS and MRS-Ca broth as described by Séchaud *et al.* (1989) but modified as follows: exponentially growing ($O.D._{560nm}$: 0·5) host strain cultures in MRS broth were centrifuged and resuspended at a concentration of $3{\cdot}10^8$–$5{\cdot}10^8$ CFU ml^{-1} (determined by plate count) in MRS and MRS-Ca broths. Each phage, separately, was added at a multiplicity of infection of about 0·02, and the mixtures incubated at 42°C for adsorption, after distributing the infected culture in Eppendorf tubes. At intervals, tubes were removed and centrifuged (12 000 *g* for 4 min) to sediment the phage-adsorbed bacteria. Then, titres of unadsorbed free phages in the supernatant were assayed as indicated, and the results expressed as percentages of the initial phage counts.

Influence of temperature on adsorption

Phage adsorption rates on *Lact. delbrueckii* cells were determined at 0, 10, 20, 30, 37, 45, and 50°C, as follows. Exponentially growing ($O.D._{560nm}$: 0·5) host strain cultures in MRS broth were centrifuged and resuspended at a concentration of $3{\cdot}10^8$–$5{\cdot}10^8$ CFU ml^{-1} (determined by plate count) in MRS-Ca broth. Each phage, separately, was added at a m.o.i.: 0·02 and the mixtures incubated (30 min). After centrifugation (12 000 *g* for 4 min), the supernatants were assayed for unadsorbed phages (double-layer plaque titration) and the counts were compared with the titre of a control without cells. The results were expressed as percentages of adsorption.

Influence of physiological cell state on adsorption

The ability of viable and nonviable cells to allow phage adsorption was tested by determination of phage adsorption kinetics on *Lact. delbrueckii* cells. Nonviable cells were obtained by keeping a cell suspension in boiling water during 10 min (Quiberoni and Reinheimer 1998). Nonviability of treated cells was checked by plate counts, and a 100% of cell death was obtained after the treatment.

Preparation of cell walls

Cell walls were prepared by the procedure reported by Quiberoni *et al.* (2000), modified as follows: cells were grown in MRS broth ($O.D._{560nm}$: 0·5–0·8) and centrifuged at 2000 *g* for 5 min. The supernatant was removed, and the cells were washed twice with 10 mmol l^{-1} phosphate buffer (pH 6·8) and centrifuged (2000 *g* for 10 min). Then, the cells were suspended in a phosphate buffer and glass beads (0·10–0·15 mm diameter) were added in a ratio of 1 : 1 (vol/vol). The mixture was then vortexed for 45 min, during which the suspension was cooled in an ice bath every 30 s (Ranhand 1974). Optic microscopy and bacterial counts (MRS agar) were used to investigate the effectiveness of cell disruption. Glass beads were removed by four successive sedimentation steps (2 h at 4°C), and the supernatants were collected. Whole cells and cell debris were removed by centrifugation at 3000 *g* for 10 min. Cell walls, sedimented by centrifugation at 12 000 *g* for 15 min and resuspended in 50 mmol l^{-1} Tris–HCl (pH 7·5), were treated with DNase (0·10 mg ml^{-1}) and RNase (0·15 mg ml^{-1}) for 30 min at 37°C. Cell walls were collected by centrifugation at 12 000 *g* for 15 min and washed by 10 successive resuspensions in

phosphate buffer. The purified cell walls were finally sedimented by centrifugation as described above, and stored at −20°C.

Phage adsorption on cell walls

Cell walls able to bind approx. 90% of the phages were mixed with phages (10^6 PFU ml^{-1}) in MRS-Ca broth and incubated at 42°C for 30 min. The mixtures were then centrifuged (12 000 *g* for 10 min) and free phage counts in the supernatants were performed as indicated above. The results were expressed as percentages of the initial phage counts (Valyasevi *et al.* 1990).

Chemical and enzymatic treatments of cell walls

Cell wall suspensions in 50 mmol l^{-1} Tris–HCl (pH 7·5), at a concentration that had been found to bind approx. 90% of a specific amount of phage (10^6 PFU ml^{-1}) were treated with SDS (1%), mutanolysin (50 U ml^{-1}) and proteinase K (0·10 mg ml^{-1}) at 37°C for 30 min (Valyasevi *et al.* 1990), and trichloroacetic acid (TCA) (5%) at 100°C for 15 min (Callegari *et al.* 1998). Treated cell walls were then washed by 10 successive resuspensions in phosphate buffer and centrifuged at 12 000 *g* for 10 min (Callegari *et al.* 1998). Cell walls were assayed for phage-adsorption as described above, in comparison with a control of nontreated cell walls.

Phage inhibition by saccharides

The ability of various saccharides to inactivate phages was determined as it was described by Valyasevi *et al.* (1990). Either glucose, galactose, mannose, rhamnose, ribose, glucosamine or *N*-acetylglucosamine to a final concentration of 500 mmol l^{-1} was mixed with each phage, separately (10^6 PFU ml^{-1}) in 50 mmol l^{-1} Tris–HCl (pH 7·5) added of 10 mmol l^{-1} of $CaCl_2$, for 30 min at 42°C. The mixtures were diluted adequately and assayed for plaques by the agar double-layer method. The results were compared with titres of control samples without saccharides, and then expressed as a percentage of phage inactivation (Quiberoni and Reinheimer 1998).

Reversibility of phage binding on cell walls

After the phage-adsorption test performed on the cell wall control samples (cell walls without treatment), the mixtures of adsorbed-phage cell walls and unadsorbed phages were centrifuged (12 000 *g* for 10 min). Pellets, which contained cell wall-adsorbed phages, were resuspended in 1 ml of 50 mmol l^{-1} Tris–HCl (pH 7·5), supplemented with 10 mmol l^{-1} $CaCl_2$, and incubated at room temperature. At intervals, the mixtures were centrifuged and free phage enumerations were carried out in the supernatant by the double-layer plaque titration method. The results were expressed as free phage particles at different intervals (Valyasevi *et al.* 1990).

Statistical analysis

Experiments were replicated three times. All data were analysed using the one-way ANOVA procedure of SPSS. The differences among means were detected by the Duncan's multiple range test (Lizasoain and Joaristi 1995).

RESULTS

Phage morphology

All indigenous *Lact. delbrueckii* subsp. *bulgaricus* phages showed isometric heads (phage BYM, 50 ± 2·1 nm; phage YAB, 54·5 ± 1·7 nm; phage Ib_3, 57·3 ± 2·3 nm, in diameter; $n = 10$) and long flexible tails (phage BYM, 181 ± 1·7 nm; phage YAB, 251·2 ± 2 nm; phage Ib_3, 272·8 ± 2·4 nm, in length, and phage BYM, 5·1 ± 0·3 nm; phage YAB, 7·6 ± 0·4 nm; phage Ib_3, 9·1 ± 0·6 nm, in width; $n = 10$) with noncontractile sheaths (Fig. 1). Phages belonged to Bradley's group B (Bradley 1967) or the *Siphoviridae* family (morphotype B1) of the International Committee on Taxonomy of Viruses (Matthews 1982). A characteristic collar structure, not always present in phages of other species (Jarvis 1989), was exhibited by phage BYM.

Strain cross-sensitivity

For the strain group used in this study (Table 1), each phage infected only their host strain.

DNA characterization

DNAs from the three indigenous *Lact. delbrueckii* subsp. *bulgaricus* phages were studied to determine their restriction patterns with enzymes *Bgl*II or *Hin*dIII. Distinctive patterns were obtained (Fig. 2) for each phage.

The molecular weight of phage genomes were: phage BYM, 31·3 ± 1·9 kbp; phage YAB, 34 ± 1·9 kbp, and phage Ib_3, 33·2 ± 1·3 kbp ($n = 3$). When packaging sites were investigated, the loss of two bands (1 and 2·9 kbp) and the intensity increase of one band (4 kbp), indicated cohesive ends for phage Ib_3. The BYM and YAB phage genomes did not appear to contain cohesive ends because heating of ligated and not ligated restriction digests did not alter the restriction patterns of *Bgl*II or *Hin*dIII (Fig. 2).

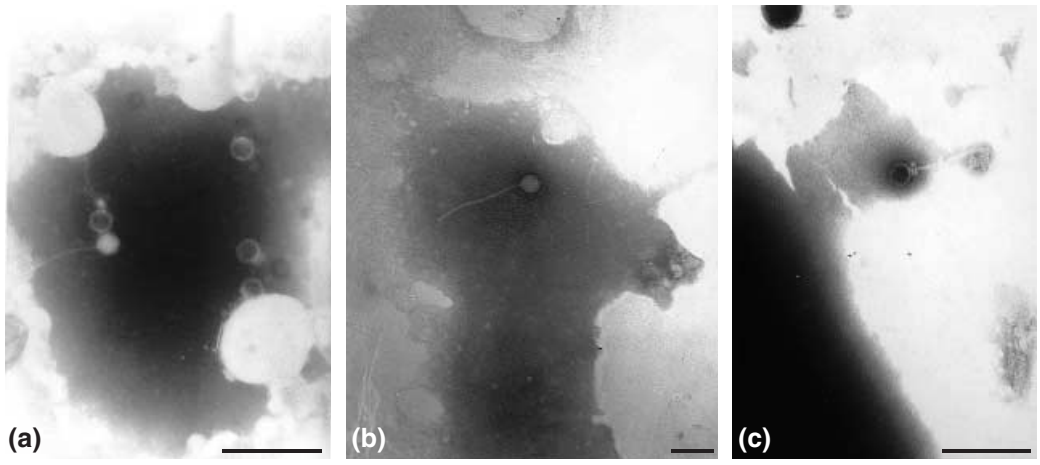

Fig. 1 Electron micrographs of indigenous *Lactobacillus delbrueckii* subsp. *bulgaricus* phages YAB (a), Ib_3 (b) and BYM (c). Bar represents 50 nm

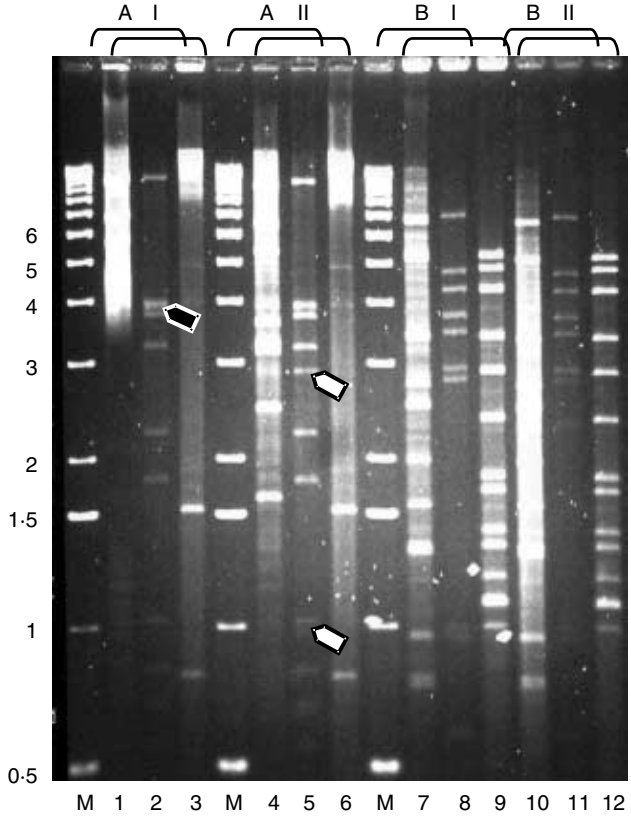

Fig. 2 Agarose gel electrophoresis of *Bgl*II- (A) and *Hin*dIII- (B) generated DNA fragments of phages BYM (lanes 1, 4, 7 and 10), Ib_3 (lanes 2, 5, 8 and 11) and YAB (lanes 3, 6, 9 and 12). Lanes I and II, ligated or not ligated DNAs of phages, respectively. Lane M: 1 kbp DNA Ladder. The position of DNA fragments lost upon heating (open arrowheads) and the position of the DNA fragment intensified on heating (filled arrowheads) are indicated

One-step growth curves

From one-step growth curves, multiplication phage parameters were calculated. The burst sizes (mean values) were 23, 27 and 48 PFU per infection centre (phages BYM, Ib_3 and YAB, respectively); burst times were 60, 80 and 80 min (phages YAB, BYM and Ib_3, respectively), and latent periods were lower than 40 min.

pH sensitivity of phages

At room temperature (25°C, 30 min) an excellent viability and stability was observed for all phages in the pH range investigated (pH values from 4 to 8) (data not shown). At 42°C, both parameters were dependent on phage (Fig. 3). For

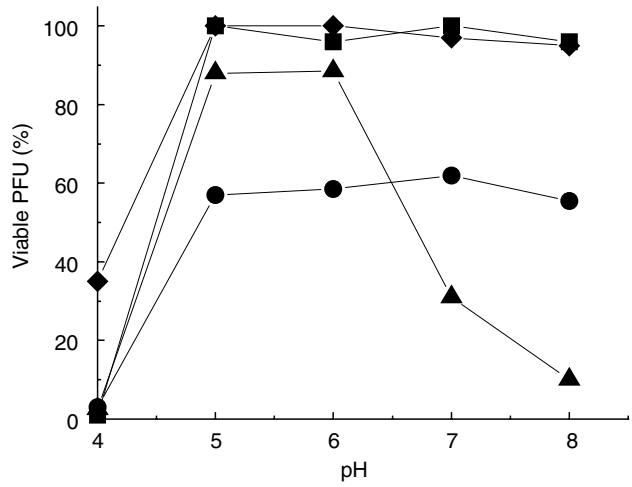

Fig. 3 Effect of pH on viability (after 30 min at 42°C, in MRS-Ca broth) of phages YAB (■), Ib_3 (●), BYM (♦) and LL-H (▲). Values are the mean of three determinations

all phages, the lowest viability values (<40%) were observed at pH 4. An excellent viability and stability was exhibited at pH values ≥5 by phages YAB and BYM (96–100% of viable viral particles). Phage Ib_3 was equally stable against pH but only 60% of viral particles remained viable. Regarding the collection phage LL-H, it showed an acceptable survival at pH 5 and 6 that fell dramatically at pH 7 and 8.

Influence of calcium ions on cell lysis and phage adsorption

For phages BYM and LL-H, cell lysis in MRS broth was achieved even without added Ca^{2+}, but the process was faster in its presence. In contrast, calcium ions were indispensable to obtain the complete lysis of *Lact. delbrueckii* subsp. *bulgaricus* Ab_1 and LB-Ib_3 by phages YAB and Ib_3, respectively. All indigenous phages produced visible lysis plaques in MRS agar only if Ca^{2+} was added. Phages BYM and Ib_3 produced very clear plaques (0·5–1 or 2–2·5 mm for phages BYM and Ib_3, respectively), while heterogeneous and diffuse plaques were exhibited by phage YAB. In contrast, visible and clear plaques – although smaller – were yielded by the collection phage LL-H, even in the absence of calcium ions.

In general, no influence of Ca^{2+} was observed on phage adsorption kinetics (Fig. 4). Regarding indigenous phages, after 5 min of incubation, more than 98% of the initial viral particles were adsorbed, while the maximum adsorption rates were achieved at 15–45 min (99–99·9% of phages adsorbed). A distinctive behaviour was exhibited by the collection phage LL-H, as a lower adsorption rate (94%) was achieved after 45 min of incubation, even in the presence of Ca^{2+}.

Influence of temperature on phage adsorption

The effect of temperature was only significant for phage BYM, as adsorption rates reached values of 77·2 and 98·2% at 0 and 20°C, respectively, while the maximum (99%) was achieved at 30°C and maintained up to 50°C. The rate adsorption of phages YAB, Ib_3 and LL-H were not significantly influenced by temperature.

Influence of physiological cell state on phage adsorption

Figure 5 shows that phage adsorption values were not significantly different ($P > 0·05$) on viable and nonviable cells. More than 98% of the initial viral particles (indigenous phages) were adsorbed after only 5 min of incubation, on viable and nonviable cells, while the maximum adsorption rates were achieved at 45 min (99–99·9% of bacteriophages adsorbed). The collection phage LL-H exhibited a similar behaviour regarding the independence of physiological cell state, but the low adsorption rates reached (89–92%) were again a distinctive characteristic.

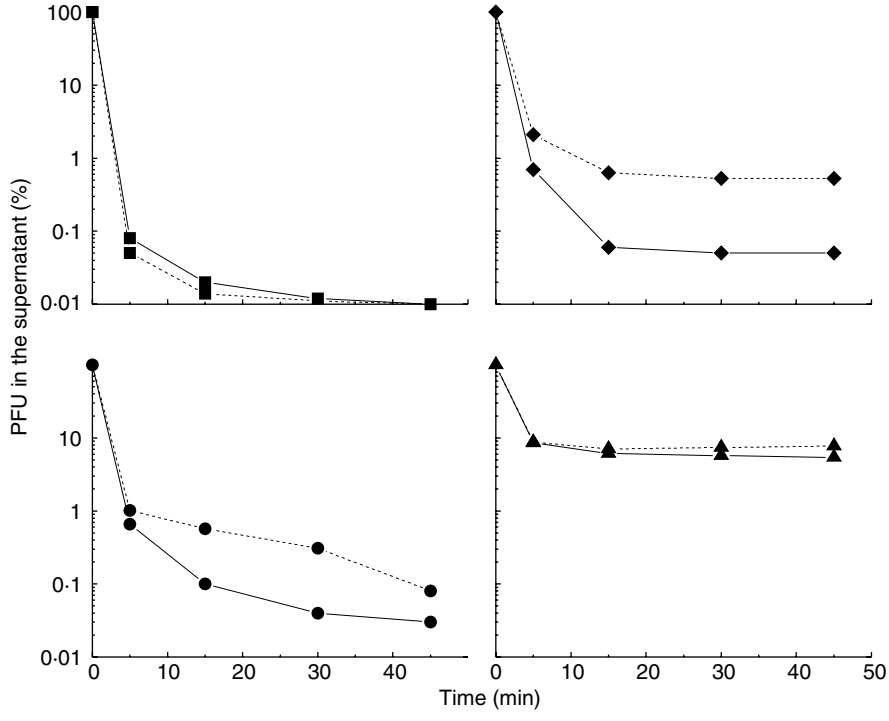

Fig. 4 Adsorption kinetics of phages YAB (■), Ib_3 (●), BYM (♦) and LL-H (▲) on *Lactobacillus delbrueckii*-sensitive strains at 42°C in MRS broth with (—) and without (- - -) Ca^{2+} (10 mmol l^{-1}). Values are the mean of three determinations

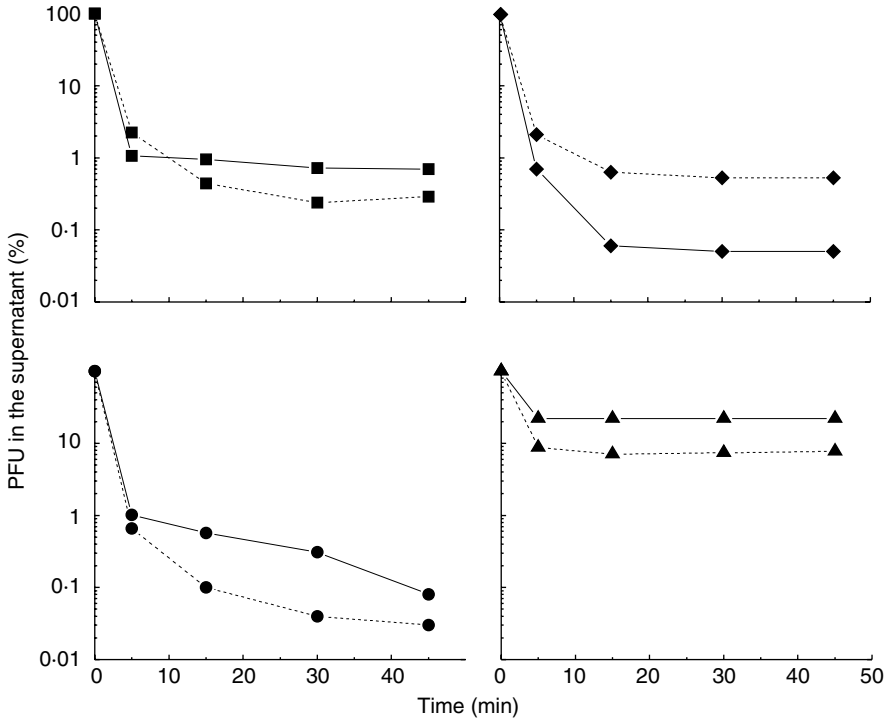

Fig. 5 Adsorption kinetics of phages YAB (■), Ib_3 (●), BYM (♦) and LL-H (▲) on viable (—) and nonviable (- - -) cells of *Lactobacillus delbrueckii*-sensitive strains at 42°C in MRS-Ca broth. Values are the mean of three determinations

Phage adsorption on treated cell walls

The cell treatments with SDS and proteinase K did not reduce significantly ($P > 0{\cdot}05$) the adsorption rates of phages (Table 2). However, phage binding was drastically reduced (adsorption rates ranged from 0 to 38%) for all phages when the cell walls were treated with mutanolysin. Furthermore, TCA cell wall treatments influenced the binding of phages appreciably ($P < 0{\cdot}05$), as adsorption rates lower than 21% were obtained.

Phage inhibition by saccharides

No significant inhibitory effect on adsorption was observed for the saccharides tested (Table 3). Only minimal effects were evidenced when glucose was assayed on phage YAB (25·5% of inhibition), mannose on phage lb539 (21·9% of inhibition) or rhamnose and *N*-acetylglucosamine on phage BYM (17·5 and 13·7% of inactivation, respectively). Noticeably, almost all saccharides tested produced a slight effect on phage Ib_3, which was gently inhibited (10·1–18%).

Table 2 Adsorption (MRS-Ca broth, 30 min at 42°C) of phages on chemically and enzymatically treated cell walls of *Lactobacillus delbrueckii* strains

	Phage adsorption (%)* (mean ± S.D.)			
	Phages			
Treatment	YAB	Ib_3	BYM	lb539
None (control)	97·8 ± 0·6	99·5 ± 0·3	98·6 ± 0·5	90·0 ± 1·1
Proteinase K 0·10 mg ml^{-1} (30 min, 37°C)	95·4 ± 0·4	99·7 ± 0·2	89·0 ± 1·5	78·5 ± 1·3
SDS 1% (30 min, 37°C)	96·0 ± 1·4	99·5 ± 0·3	93·0 ± 1·7	85·5 ± 1·1
Mutanolysin 50 U ml^{-1} (30 min, 37°C)	27·5 ± 1·3	38·0 ± 1·5	0·4 ± 0·2	25·5 ± 0·7
TCA 5% (15 min, 100°C)	0	21·5 ± 1·3	17·0 ± 1·9	0

*Values are the mean of three determinations.

Table 3 Inhibition of phage adsorption by different saccharides

	Phage inhibition (%)* (mean ± S.D.)			
	Phages			
Saccharides	YAB	Ib_3	BYM	lb539
None (control)	0	0	0	0
Galactose	0	17·0 ± 0·4	0	0
Glucose	25·5 ± 1·3	10·1 ± 0·7	0	0
Mannose	0	15·2 ± 0·3	0	21·9 ± 0·3
Glucosamine	0	16·5 ± 0·5	0	5·2 ± 0·7
N-acetylglucosamine	0	10·2 ± 0·6	17·5 ± 1·2	6·5 ± 0·4
Rhamnose	0	12·4 ± 1·1	13·7 ± 0·9	0
Ribose	0	0	0	0

*Values are the mean of three determinations.

Reversibility of phage binding to the cell walls

The binding of phages BYM, YAB, Ib_3 and lb539 on the respective *Lact. delbrueckii* sensitive strains was irreversible, as it can be seen in Figure 6. After 30 min of adsorption, more than 89% of phage particles were bound to the cell walls. After adsorbed phage cell wall complexes were collected by centrifugation and resuspended in a buffer, no significant amount ($P > 0{\cdot}05$) of viral particles was released into the supernatants.

DISCUSSION

In this work, three indigenous *Lact. delbrueckii* subsp. *bulgaricus* phages were characterized. Phages YAB, Ib_3 and BYM showed small isometric heads and long tails with noncontractile sheaths. A collar appearing just below the head was a distinctive structure in phage BYM. So, they were classified as belonging to the *Siphoviridae* family, morphotype B1 (Bradley 1967). This same basic morphology was also reported for other *Lact. delbrueckii* bacteriophages (Alatossava and Pythilá 1980; Cluzel *et al.* 1987; Chow *et al.* 1988; Auad *et al.* 1997) and it closely resembles those of other lactobacilli phages (Accolas and Spillman 1979).

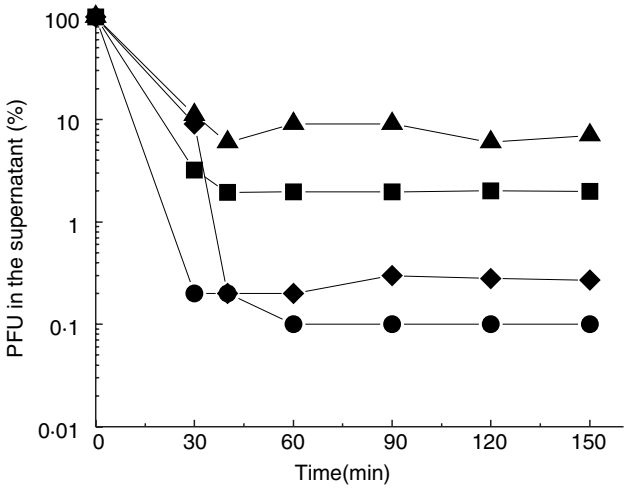

Fig. 6 Irreversibility of binding for phages YAB (■), Ib_3 (●), BYM (◆) and LL-H (▲) to *Lactobacillus delbrueckii*-sensitive strains. Values are the mean of three determinations

Genomes of indigenous *Lact. delbrueckii* subsp. *bulgaricus* bacteriophages YAB, Ib_3 and BYM consist of linear and double-stranded DNAs from 31 to 34 kbp, and the presence of *cos* sites was revealed in the Ib_3 phage genome. Restriction patterns, obtained with endonucleases, turned out to be very different amongst them. Studies to compare these phage genomes with those belonging to phages isolated from other countries, are in progress. The genome sizes reported in this study are comparable with those determined for other *Lact. delbrueckii* phages (Chow *et al.* 1988; Auad *et al.* 1997). Specifically, the genome size of the *Lact. delbrueckii* subsp. *lactis* phage LL-H has been calculated in 34 659 bp (Alatossava *et al.* 1998), constituting the first *Lactobacillus* phage, of which the complete nucleotide sequence has been determined.

The infection cycle of the indigenous bacteriophages was studied by its one-step growth curves, and the events in bacteriophages development were determined: latent time ranging 20–40 min, rise periods of 60–80 min and burst sizes calculated in 23 to 48 phage particles per cell. The burst size and latent time of a number of lactobacilli phages have been reported (De Klerk and Coetzee 1963; Watanabe *et al.* 1970; Sarimo *et al.* 1976; Chow *et al.* 1988; Jarvis 1989) and the burst size of indigenous phages YAB, Ib_3 and BYM are certainly within this range.

Phages studied in this work only tolerate the pH values closest to neutrality (pH 5–7), and the viability declined notably at pH values lower than 5. This behaviour could explain the low phage numbers found in yoghurt samples (data not shown). In contrast, phage lb539, inducible from *Lact. delbrueckii* subsp. *bulgaricus* CRL 539 (Auad *et al.* 1997), showed an excellent survival within a pH range from 4 to 10. Its infectivity only declined at pH 3 and it was only inhibited at extreme pH values (pH 2 and 13).

The lytic cycle of a phage begins with its adsorption on the cell wall receptors of a sensitive bacteria host, a highly specific event. The phage adsorption process in mesophilic LAB was well studied in a few cases (Budde-Niekiel and Teuber 1987; Sijtsma *et al.* 1988; Valyasevi *et al.* 1990; Monteville *et al.* 1994; Foschino *et al.* 1995) but thermophilic LAB has been less investigated and data on the nature of their phage receptors are still very scarce (Watanabe *et al.* 1993; Callegari *et al.* 1998; Quiberoni and Reinheimer 1998; Lucchini *et al.* 2000; Quiberoni *et al.* 2000; Binetti *et al.*

2002). So far, phage adsorption on *Lact. delbrueckii* strains has not been well described.

There are some recognized factors affecting the phage adsorption process, such as the presence of Ca^{2+} ions, the physiological state of bacterial cells, pH and temperature. The role of inorganic cations is noteworthy. According to Séchaud *et al.* (1988), the Ca^{2+} (or Mg^{2+}) requirement not only stabilizes the coiled DNA inside the phage capsid and greatly improves the adsorption rate, but also controls the penetration efficiency of phage DNA into the bacterial cells. The results of our study demonstrated that while the adsorption was not affected by Ca^{2+} for all phages, the completion of vegetative cycle became slower or null in broth and no visible or very small plaques were produced in the absence of calcium ions. For other *Lactobacillus* phages, the requirement of Ca^{2+} for either adsorption or subsequent lysis was variable (Séchaud *et al.* 1989; Quiberoni *et al.* 2000).

The phages adsorbed on *Lact. delbrueckii* cells even at 0°C but adsorption rate reached its maximum at 37°C. It was also interesting to note that the adsorption maximum for all phages was achieved at the temperature range (37–42°C) in which *Lact. delbrueckii* strains are used to manufacture fermented dairy products. Watanabe *et al.* (1993) have also demonstrated that adsorption at 0°C was almost identical to that at 37°C for phage PL-1 (*Lact. casei*). However, the number of ghost particles did not increase during incubation at 0°C, suggesting that actively functioning bacteria is required for the injection of DNA from this phage.

In this work, the ability of thermally killed cells to allow adsorption of phages YAB, Ib_3, BYM and LL-H was observed, demonstrating the thermostable nature of the phage receptors and the independence of this process from the physiological state of the cells. Other authors have also demonstrated the ability of thermally killed cells to adsorb phages of *Lact. helveticus* (Quiberoni and Reinheimer 1998), *Lact. casei* (Watanabe *et al.* 1993) and *Streptococcus thermophilus* (Quiberoni *et al.* 2000; Binetti *et al.* 2002).

Purified cell walls of *Lact. delbrueckii*-sensitive strains were treated chemically and enzymatically, and scored for phage adsorbing abilities in order to identify the cell wall structures involved in phage adsorption. As it is known, SDS (1%) treatments remove either proteinaceous membrane components or proteins covalently bound to the cytoplasmic membrane (Valyasevi *et al.* 1990; Callegari *et al.* 1998). No significant effect on adsorption was observed for neither phage used in this study on the respective SDS treated cell walls. Similarly, the proteinase K treatment, which hydrolyses peptidic bindings, did not affect the adsorbing abilities of phages. Therefore, protein components could be excluded as phage receptors in our strains. For other thermophilic LAB, such as *Lact. helveticus*, the S-layer protein of several strains was found to be essential for the adsorption of the phage CNRZ 832-B1 (Ventura *et al.* 1999). Mutanolysin treatments appreciably reduced the adsorption of phages used in our work. This enzyme hydrolyses glycosidic bindings, mainly peptidoglycan structure components. However, mutanolysin has a restricted hydrolytic specificity, and we could not rule out the possibility that glycoproteins, membrane-bound lipoteichoic acids or accessory wall polysaccharides were hydrolysed (Valyasevi *et al.* 1991). The phage binding decrease that was observed due to this treatment suggests that the adsorption of *Lact. delbrueckii* phages on the cell surfaces may involve some of these envelope components. This hypothesis was further confirmed as the TCA treatment also reduced the phage adsorption on the cell walls. The use of TCA to extract peptidoglycan-associated cell wall polymers from Gram-positive bacteria had been well established (Gopal and Reilly 1995). The marked decreases observed in the adsorption of phages on cell walls treated with TCA 5% suggest that the accessory polysaccharide–peptidoglycan complex was involved in the phage receptor sites.

No significant inhibition of phages was observed when they were incubated with monossacharides known to be part of the bacterial cell wall. This fact suggested that the carbohydrates tested are not essential components of phage receptor structures or that their conformation is not recognized by phages. For *Lact. casei* phages, it was reported (Watanabe *et al.* 1991, 1993) that the receptor was the rhamnose of the cell wall polysaccharide.

Our results also revealed the irreversibility of the adsorption of *Lact. delbrueckii* phages to their hosts. This step was an efficient process for all phages, as over 89% of phage particles were adsorbed after 30 min of incubation at 42°C. This fact suggested an irreversible mechanism for the earlier steps of the infective cycle. The irreversibility of binding was also demonstrated for phage kh on *L. lactis* subsp. *cremoris* KH (Valyasevi *et al.* 1994) and five *Strep. thermophilus* bacteriophages (Binetti *et al.* 2002). In contrast, the initial adsorption step on the cell wall was a reversible process for seven phages on *L. lactis* subsp. *lactis* C2 (Monteville *et al.* 1994).

The isolation frequency for *Lact. delbrueckii* subsp. *bulgaricus* phages from Argentinian yoghurt plants was very low. While 59 phages of *Strep. thermophilus* were isolated in our laboratory from 96 yoghurt samples, only the three *Lact. delbrueckii* subsp. *bulgaricus* indigenous phages characterized in this work were obtained from these samples (Suárez *et al.* 2002). As both bacteria integrate yoghurt starters, this fact is, at least, curious. Two factors might be responsible of significant difference for the isolation frequency: (i) the proportion of *Lact. delbrueckii* subsp. *bulgaricus* cells in Argentinian yoghurt starters is less and less in order to obtain mild products, and (ii) their very low resistance to pH values below 5 at 42°C. According to our hypothesis, when a

phage infection occurs, only a moderate number of phage particles could be found, as the lactobacilli cell multiplication is reduced and then, a fast viability loss occurs in the yoghurt because the pH is lower than 5 (pH values from 4 to 4·5 are normal in Argentinian yoghurts). In fact, phages YAB and Ib_3 were counted at low numbers (1×10^2 and $2{\cdot}9 \times 10^3$ PFU ml^{-1}, respectively). Curiously, the phages of *Lact. delbrueckii* subsp. *bulgaricus* studied in the present work, showed a thermal and chemical resistance higher than that exhibited by phages of other LAB, including *Strep. thermophilus* (Quiberoni *et al.* 1999, 2003; Binetti and Reinheimer 2000). Thus, *Lact. delbrueckii* subsp. *bulgaricus* phages would exhibit distinctive features from an evolutionary point of view. These phages might survive a long time in dairy plants because of their high resistance to heat and biocides but at low numbers because of their low resistance under the acidic conditions used in yoghurt fermentation.

Furthermore, a detailed understanding of the biology of indigenous *Lact. delbrueckii* phages represents an alternative to study the genetics of this species as phage genomes can be exploited to develop genetic tools for biotechnological applications. Furthermore, knowledge on environmental factors that influence their binding to sensitive cells is very important to develop strategies to obtain more phage resistant strains for industrial uses.

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