

## ORIGINAL ARTICLE

**Adsorption of temperate phages of *Lactobacillus delbrueckii* strains and phage resistance linked to their cell diversity**

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dairy processes, lactic acid bacteria, *Lactobacillus delbrueckii*, phage resistance, temperate bacteriophages.

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**Abstract**

**Aims:** The aim of this work was to study the adsorption step of two new temperate bacteriophages (Cb1/204 and Cb1/342) of *Lactobacillus delbrueckii* and to isolate phage-resistant derivatives with interesting technological properties.

**Methods and Results:** The effect of divalent cations, pH, temperature and cell viability on adsorption step was analysed. The Ca<sup>2+</sup> presence was necessary for the phage Cb1/342 but not for the phage Cb1/204. Both phages showed to be stable at pH values between 3 and 8. Their adsorption rates decreased considerably at pH 8 but remained high at acid pH values. The optimum temperatures for the adsorption step were between 30 and 40°C. For the phage Cb1/342, nonviable cells adsorbed a lower quantity of phage particles in comparison with the viable ones, a fact that could be linked to disorganization of phage receptor sites and/or to the physiological cellular state. The isolation of phage-resistant derivatives with good technological properties from the sensitive strains and their relationship with the cell heterogeneity of the strains were also made.

**Conclusions:** Characterization of the adsorption step for the first temperate *Lact. delbrueckii* phages isolated in Argentina was made, and phage-resistant derivatives of their host strains were obtained.

**Significance and Impact of the Study:** Some phage-resistant derivatives isolated exhibited good technological properties with the prospective to be used at industrial level.

**Introduction**

Lactic acid bacteria (LAB) are widely used in fermented dairy products to give them certain qualities and protect them against the action of food pathogenic and spoilage micro-organisms. *Lactobacillus delbrueckii*, in particular, is one of the most important industrial species as it is used, together with *Streptococcus thermophilus*, for yogurt production, and it is a fundamental component of the starters used for hard cheese manufacturing (Reinheimer *et al.* 1995, 1996; Curry and Crow 2003; Giraffa and Rossetti 2004).

Phage infections are the most important cause for slow acid production by LAB during industrial fermentations (Moineau 1999; Suárez *et al.* 2002; Moineau and Lévesque 2005; Emond and Moineau 2007). 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). Fermentative dairy industries are, in that sense, those that show the best documented failed fermentations caused by phage infections (Neve 1996; Brussow 2001; Suárez *et al.* 2002; Moineau and Lévesque 2005).

The lysogenic state in LAB is considered a fundamental cause of bacteriophage entry into industrial environments (Zago *et al.* 2005, 2008). Thus, temperate phages can disturb the normal fermentation process when, by mutation, they become virulent phages against the host strain, overcoming the lysogenic immunity. Moreover, these released phages could find sensitive strains among the other starter strains and so attack them (Davidson *et al.* 1990).

On the other hand, the cell heterogeneity in pure LAB cultures, particularly in *Lact. delbrueckii*, has been known for a long time. However, few studies have reported morphological diversity when the strains are cultured in complex media under optimal incubation conditions. Vescovo *et al.* (1990) have reported morphological and phenotypic variants for *Lact. delbrueckii* ssp. *bulgaricus* LB6 with different phage resistance. Suárez *et al.* (2008a) have reported the isolation of phage-resistant derivatives from morphological variants isolated from *Lact. delbrueckii* ssp. *lactis* Ab1, the phage resistance mechanisms involved and the derivatives obtained with good technological properties.

In this study, two temperate phages with their sensitive strains were used to characterize the adsorption step, taking into account different variables. In addition, the study aimed to isolate phage-resistant derivatives with interesting technological properties from the morphological variants obtained from the sensitive strains.

## Material and methods

### Bacterial strains, phages and culture conditions

Two temperate phages isolated at the INLAIN (Instituto de Lactología Industrial, Facultad de Ingeniería Química, Santa Fe, Argentina) from the commercial strain *Lact. delbrueckii* ssp. *lactis* Cb1 were used. These phages were named Cb1/204 and Cb1/342 and their sensitive strains were *Lact. delbrueckii* ssp. *lactis* 204 (*L.l* 204) and *Lact. delbrueckii* ssp. *bulgaricus* 342 (*L.b* 342), respectively. These phages were partially characterized by our group in a previous work (Suárez *et al.* 2008c). Sensitive strains were maintained as frozen stocks at  $-80^{\circ}\text{C}$  in reconstituted (10% w/v) commercial nonfat dried skimmed milk (RSM), or Mannose-Rogosa-Sharpe (MRS) broth (Biokar, Beauvais, France) supplemented with 15% (v/v) of glycerol, and routinely reactivated overnight at  $42^{\circ}\text{C}$  in MRS broth. Phage stocks were prepared as described by Neviani *et al.* (1992) in MRS broth, adding  $10\text{ mmol l}^{-1}$  of  $\text{CaCl}_2$  (MRS-Ca), and then stored at  $4^{\circ}\text{C}$  and frozen at  $-80^{\circ}\text{C}$  in the presence of 15% of glycerol. Phage enumerations ( $\text{PFU ml}^{-1}$ ) were performed by the double-layer plaque titration method (Svensson and Christiansson 1991), using MRS-Ca agar added with  $100\text{ mmol l}^{-1}$  of glycine (Lillehaug 1997).

### Adsorption studies

#### *Influence of calcium ion*

The effect of calcium ions on the phage adsorption on *Lact. delbrueckii* cells was studied by the determination of

adsorption kinetics in MRS and MRS-Ca broths (Biokar) according to Suárez *et al.* (2008b). Exponentially growing cultures ( $\text{OD}_{600\text{nm}} = 0.5$ ) of the strains were centrifuged ( $12\ 000\text{ g}$  for 4 min) and resuspended at a concentration from  $3 \times 10^8$  to  $5 \times 10^8\text{ CFU ml}^{-1}$  (determined by plate counts) in MRS and MRS-Ca broths. Each phage was added at a multiplicity of infection (m.o.i.) of about 0.02, and the mixtures were incubated for adsorption at  $37^{\circ}\text{C}$ . At time intervals, an aliquot was centrifuged ( $12\ 000\text{ g}$  for 4 min) to sediment the phage-adsorbed cells. Then, the number of free phages in the supernatant was determined, and the results were expressed as percentages of the initial phage counts.

#### *Influence of pH*

Cells prepared according to the same protocol used in the previous experience were resuspended in MRS-Ca broth, adjusted to the desired pH values (3, 4, 5, 6, 7 and 8). Phages were added (m.o.i.: 0.02) and the mixtures incubated for 30 min at  $37^{\circ}\text{C}$ . After centrifugation, the supernatants were assayed for unadsorbed phages and their counts compared with the initial titre. Results were expressed as percentages of adsorption and plotted against pH values. Phage suspensions in pH-adjusted MRS-Ca broth were included as controls to determine the pH effect on phage infectivity.

#### *Influence of temperature*

Phage adsorption rates on *Lact. delbrueckii* cells were determined at 0, 10, 20, 30 and  $40^{\circ}\text{C}$  as follows: exponentially growing cells ( $\text{OD}_{600\text{nm}}: 0.5$ ) were centrifuged and resuspended in MRS-Ca broth. Phages were added (m.o.i.: 0.02) and the mixtures incubated for 30 min. After centrifugation, supernatants were assayed for unadsorbed phages and counts were compared with the control titre without cells. Results were expressed as percentage of adsorption.

#### *Influence of cell viability*

The adsorption kinetics of viable and nonviable *Lact. delbrueckii* cells were also determined. Cells were prepared according to the same protocol used in the previous experiences. Nonviable cells (checked by plate counts) were obtained by keeping a cell suspension in boiling water for 10 min (Quiberoni and Reinheimer 1998).

### Isolation of phage-resistant derivatives from morphological variants

*Lactobacillus delbrueckii*-sensitive strains were streaked on MRS (Biokar) agar (Suárez *et al.* 2008a). The plates were incubated at  $37^{\circ}\text{C}$  for 48 h. Morphological variants were scored, isolated and cultured in MRS broth.

The secondary culture method (SC) (Carminati *et al.* 1993) was used to isolate phage-resistant derivatives, which is modified as follows: an overnight culture of each strain in MRS-Ca broth was infected, at different infection ratios (m.o.i. of 1, 0.1 and 0.01), with suspensions of the corresponding lytic phage. After incubation at 37°C for 6–8 h, cultures exhibited complete lysis. SCs were obtained after further incubation for up to 48 h at 37°C and streaked on MRS agar plates. After incubation for 48 h at 37°C, colonies of each variant were isolated and cultured in MRS broth. These isolates were purified by three consecutive streakings on MRS agar. Phage resistance was confirmed by confronting each isolate cultivated in MRS-Ca broth with the corresponding lytic phage. Three subcultures were prepared, and isolates that were able to grow normally under these conditions were considered as true phage-resistant derivatives (Reinheimer *et al.* 1993) and stored at –80°C. Phage resistance stability was assayed by seven sequential subcultures (2%) of the derivatives in MRS-Ca broth with independent infection with phages at each subculture (Carminati *et al.* 1993). The loss of phage resistance was determined by the culture lysis in comparison with a control (mutant subculture without phage addition). The subculture at which lysis occurred was recorded.

### Technological characterization of phage-resistant derivatives

The proteolytic and acidifying activities were determined according to Guglielmotti *et al.* (2006).

### Statistical analysis

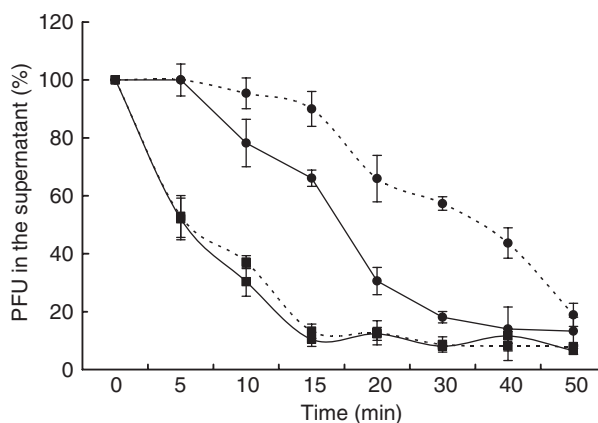
To compare the results of technological properties obtained for the parental strains and their phage-resistant derivatives, a statistical one-way ANOVA analysis was made. The significance level (*P*) taken to consider significant differences was 0.05.

## Results

### Adsorption studies

#### *Influence of calcium ion.*

The adsorption kinetics in the presence and absence of calcium for the systems Cb1/204–*L.l.* 204 and Cb1/342–*L.b.* 342 are shown in Fig. 1. For Cb1/204–*L.l.* 204, it was observed that both adsorption kinetics, either in the presence or absence of the cation, showed the same trend. By contrast, a different behaviour was observed for Cb1/342–*L.b.* 342. In this case, the absence of calcium ion delayed the phage adsorption on the sensitive



**Figure 1** Calcium ion influence on the adsorption kinetics (at 37°C) for phages Cb1/204 (■) and Cb1/342 (●), on their sensitive strains *Lactobacillus delbrueckii* ssp. *lactis* 204 and *Lact. delbrueckii* ssp. *bulgaricus*, respectively, with (—) and without (---) Ca<sup>2+</sup> (10 mm l<sup>-1</sup>). Values are the mean and standard deviation of three determinations.

strain, although the adsorption rates at 50 min were similar.

#### *Influence of pH*

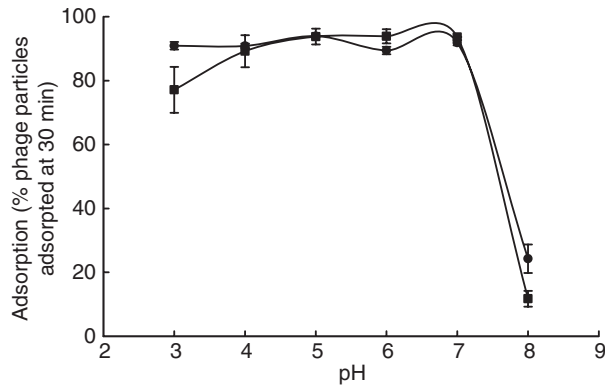
It was found that the pH did not affect the survival of both phages (data not shown). Decreased adsorption rates were observed for the phage Cb1/204 on its host strain *L.l.* 204 when pH values were extreme (3 and 8). Furthermore, the maximum adsorption occurred at pH values between 5 and 7. Regarding the other system, the adsorption rate of the phage Cb1/342 on its host strain *L.b.* 342 was affected only at pH 8 (Fig. 2).

#### *Influence of temperature*

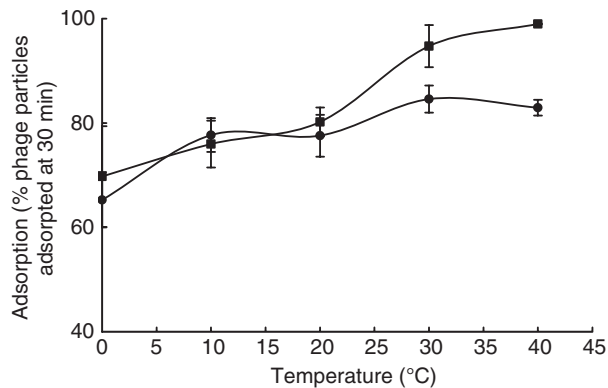
Adsorption rates for phages Cb1/204 and Cb1/342 at different temperatures on their sensitive strains *L.l.* 204 and *L.b.* 342, respectively, are shown in Fig. 3. For both cases, it was observed that the adsorption processes were influenced by the incubation temperature and that high rates of phage adsorption were attained even at low temperatures. In addition, it was found that the optimum temperatures for phage adsorption were 40°C for phage Cb1/204, with an adsorption rate of 99%, and 30–40°C for phage Cb1/342, with an adsorption rate of 85%.

#### *Influence of cell viability*

Figure 4 shows the phage adsorption kinetics on viable and nonviable cells. A higher percentage of phage Cb1/204 particles was adsorbed/was seen to be adsorbed on nonviable than on viable cells at early times, but after 15 min, the adsorption rates on viable and nonviable *L.l.* 204 cells were the same. Contrary to the system



**Figure 2** pH value influence on the adsorption step (30 min at 37°C) for phages Cb1/204 (■) and Cb1342 (●) on their sensitive strains *Lactobacillus delbrueckii* ssp. *lactis* 204 and *Lact. delbrueckii* ssp. *bulgaricus*, respectively. Values are the mean and standard deviation of three determinations.

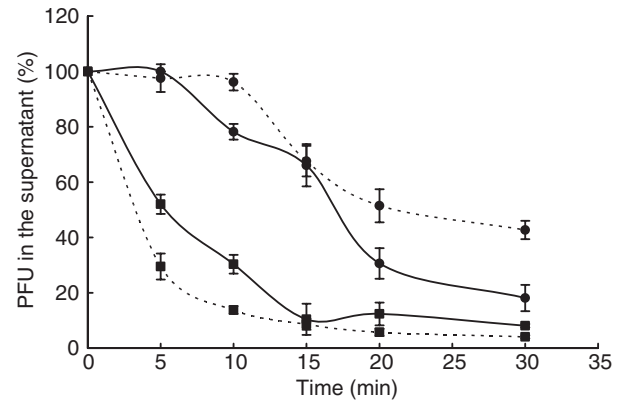


**Figure 3** Temperature influence on the adsorption step (30 min at 0, 10, 20, 30 and 40°C) for phages Cb1/204 (■) and Cb1342 (●) on their sensitive strains *Lactobacillus delbrueckii* ssp. *lactis* 204 and *Lact. delbrueckii* ssp. *bulgaricus*, respectively. Values are the mean and standard deviation of three determinations.

Cb1/204–*L.l* 204, a higher percentage of phage Cb1/342 particles was adsorbed on viable cells compared with non-viable cells, this being significant at 30 min. At this time, only 57% of phage particles was adsorbed on nonviable cells, while the adsorption percentage on viable cells was 82%, approximately.

#### Isolation of morphological variants

For the strain *L.l* 204 (Fig. 5a), it was possible to obtain two colony morphologies: variant A (44% of the total colonies) appeared as a large colony with a white centre and irregular edges, while variant B showed a smaller size, with regular edges and whitish. For the strain *L.b* 342, it was possible to isolate two morphological variants: variant



**Figure 4** Cellular viability on the adsorption kinetics (at 37°C) for phages Cb1/204 (■) and Cb1/342 (●) on their sensitive strains *Lactobacillus delbrueckii* ssp. *lactis* 204 and *Lact. delbrueckii* ssp. *bulgaricus*, respectively, viable (—) and nonviable (---) cells. Values are the mean and standard deviation of three determinations.

A (32% of the total colonies) was smaller and white, while the variant B was large and whitish (Fig. 5b). Variants maintained their morphology after subcultures.

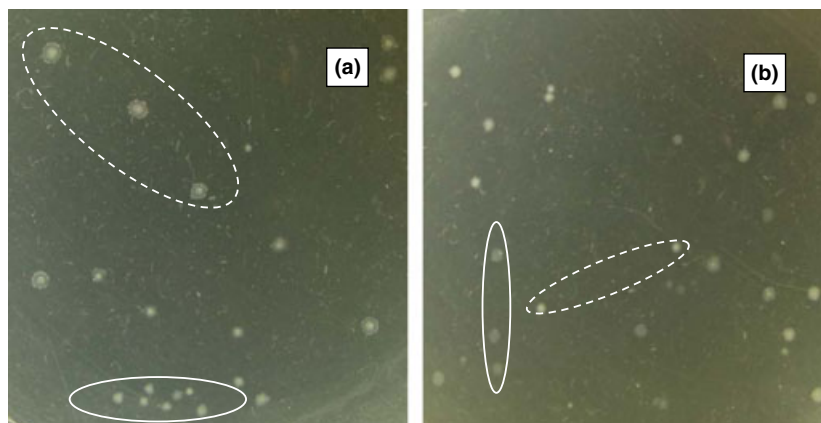
#### Isolation of phage-resistant derivatives

The percentages of phage-resistant derivatives isolated from each morphological variant for the two phage–host strain systems are shown in Table 1. For both strains, at low m.o.i values, the most phage-resistant derivatives belonged to the variants B (67% for *L.l* 204 and 57% for *L.b* 342). When the value of m.o.i. increased, the percentage of phage-resistant derivatives varied according to the strain.

All the phage-resistant derivatives obtained (ten from both strains, five for each morphological variant) showed stability for this phenotype (by seven sequential subcultures with independent infection with phages at each subculture).

#### Technological characterization of phage-resistant derivatives

Acidifying and proteolytic activities obtained for both sensitive strains and their phage-resistant derivatives are shown in Table 2. For the strain *L.l* 204, derivatives with higher acidifying activity were isolated from both variant types but mainly for the variant B. Furthermore, phage-resistant derivatives with a good acidifying activity were clearly isolated from variant B of the strain *L.b* 342. According to the statistical analysis, the differences were significant between derivatives from A and B variants ( $P < 0.05$ ) of both strains. As regards the proteolytic activity, significant differences between A and B variants were found only for the strain *L.b* 342.



**Figure 5** Morphological variants obtained from MRS agar for *L.l* 204 (a) and *L.b* 342 (b) strains. Variant A is shown as a dotted line (- - -) and variant B as a full line (—).

**Table 1** Percentages of phage-resistant derivatives obtained from variants A and B of *L.l* 204- and *L.b* 342-sensitive strains, when infected with their specific phages

Multiplicity of infection (m.o.i.)	Strain			
	<i>L.l</i> 204		<i>L.b</i> 342	
	Derivatives (%) from variant A	Derivatives (%) from variant B	Derivatives (%) from variant A	Derivatives (%) from variant B
1	—	—	12.5	87.5
10 <sup>-1</sup>	57.0	43.0	42.0	58.0
10 <sup>-2</sup>	62.0	38.0	43.0	57.0
10 <sup>-3</sup>	33.0	67.0	—	—

## Discussion

The first step in the phage biology is the adsorption on the host cell surface. Phage adsorption process was studied for several species of LAB (Sijtsma *et al.* 1988; Valsayevi *et al.* 1990; Monteville *et al.* 1994; Caso *et al.* 1995; Foschino *et al.* 1995; Quiberoni and Reinheimer 1998; Binetti *et al.* 2002; Quiberoni *et al.* 2004; Capra *et al.* 2006; Müller-Merbach *et al.* 2007; Suárez *et al.* 2008b; Briggiler Marcó *et al.* 2010). All these studies have been performed for lytic phages, the temperate ones being the least studied.

In this study, it was demonstrated that Ca<sup>2+</sup> was necessary either for adsorption or for completion of the lytic cycle of the phage Cb1/342. The final adsorption values (50 min) were similar, but the previous values were substantially lower when the calcium ion was not used. This fact would mean that, for this system, the stage of adsorption would be improved in the presence of the cation, whereas its absence would lead to a delayed lysis of the sensitive strain in liquid medium. On the contrary, the phage Cb1/204 adsorption on its sensitive strain

**Table 2** pH values and proteolytic activity (OPA test) determined in LDR (24 h at 37°C) for the sensitive strains and their phage-resistant derivatives

Strain/derivatives	pH*	Proteolytic activity* (A <sub>340nm</sub> )
<i>L.l</i> 204	4.07 ± 0.03	0.050 ± 0.010
RA204/1	4.42 ± 0.02	0.300 ± 0.100
RA204/2	4.19 ± 0.02	0.220 ± 0.050
RA204/3	4.31 ± 0.05	0.310 ± 0.060
RA204/4	4.82 ± 0.04	0.037 ± 0.008
RA204/5	4.55 ± 0.02	0.270 ± 0.010
RB204/1	4.16 ± 0.06	0.210 ± 0.030
RB204/2	3.79 ± 0.01	0.260 ± 0.040
RB204/3	4.21 ± 0.02	0.183 ± 0.003
RB204/4	4.05 ± 0.03	0.250 ± 0.060
RB204/5	3.85 ± 0.02	0.250 ± 0.020
<i>L.b</i> 342	3.87 ± 0.01	0.434 ± 0.001
RA342/1	4.74 ± 0.03	0.348 ± 0.002
RA342/2	4.03 ± 0.04	0.170 ± 0.010
RA342/3	4.62 ± 0.03	0.060 ± 0.003
RA342/4	4.92 ± 0.05	0.106 ± 0.006
RA342/5	4.87 ± 0.02	0.041 ± 0.004
RB342/1	3.71 ± 0.04	0.420 ± 0.010
RB342/2	3.66 ± 0.06	0.460 ± 0.001
RB342/3	3.81 ± 0.03	0.503 ± 0.006
RB342/4	3.74 ± 0.04	0.342 ± 0.003
RB342/5	3.65 ± 0.05	0.461 ± 0.004

\*Values are the mean and standard deviation of three determinations.

*Lact. delbrueckii* ssp. *lactis* 204 was independent of the calcium presence. Unfortunately, the use of phage inhibitory media that contain chelating agents (phosphates and citrates) might not be useful to control those bacteriophages, because they do not require cations to complete their lytic cycle (Suárez *et al.* 2007). Séchaud *et al.* (1988) reported that Ca<sup>2+</sup> is useful for the phage DNA penetration into the cells. However, this does not seem true for all phage-sensitive strain systems. For other lactobacilli

phages, the requirement of calcium either for adsorption or for lysis was variable (Quiberoni and Reinheimer 1998; Binetti *et al.* 2002; Quiberoni *et al.* 2004; Capra *et al.* 2006; Suárez *et al.* 2008b; Briggiler Marcó *et al.* 2010).

Both phages showed to be stable at pH values between 3 and 8. This fact could be explained by the origin of these phages. Temperate phages might have a high acidic resistance because acidity is a factor that induces these phages to release from the lysogenic strain, as a defence mechanism. The adsorption rates for both phages decreased considerably at pH 8, as opposed to the results reported by Suárez *et al.* (2008b) who demonstrated an efficient adsorption of *Lactococcus* phages on their host strains. Binetti *et al.* (2002) reported the adsorption rates for six *Strep. thermophilus* phages, and the results showed a high adsorption at pH 8. Similar results were found by Briggiler Marcó *et al.* (2010) for four *Lactobacillus plantarum* bacteriophages. At acid pH values (up to pH 3), temperate phages evidenced high adsorption rates. In general, bacteriophages infecting other LAB showed a lower adsorption at this pH value (Binetti *et al.* 2002; Capra *et al.* 2006; Briggiler Marcó *et al.* 2010).

Regarding the effect of incubation temperatures on the adsorption step, it was possible to observe that this process was dependent on this parameter and, even at 0°C, the adsorption rates were high (60–70%). Previous reports showed that *Lactococcus lactis* (Suárez *et al.* 2008b) and *Lact. delbrueckii* (Quiberoni *et al.* 2004) phages did not modify significantly the adsorption rates on their host strains with incubation temperature. On the contrary, *Strep. thermophilus* (Binetti *et al.* 2002), *Lactobacillus paracasei* (Capra *et al.* 2006) and *Lact. plantarum* (Briggiler Marcó *et al.* 2010) phages showed a remarkable dependence on temperature, especially at low ones.

In this work, the ability of thermally treated cells to adsorb phages was also evaluated. For the system Cb1/204–*Lb* 204, the adsorption rate on heat-treated cells was higher than that obtained on untreated cells, but this difference was not significant. On the contrary, the adsorption rate was affected by the temperature in the system Cb1/342–*Lb* 342. Nonviable cells adsorbed a lower quantity of phage particles in comparison with the viable ones. A behaviour similar to that of the phage Cb1/204 was exhibited by specific phages of *Lact. delbrueckii* (Quiberoni *et al.* 2004), *Strep. thermophilus* (Quiberoni and Reinheimer 1998; Binetti *et al.* 2002), *Lactobacillus casei* (Capra *et al.* 2006) and *L. lactis* (Suárez *et al.* 2008b). The lower phage adsorption observed on thermally treated cells could be linked to the disorganization of phage receptor sites and/or to the physiological cellular state (lack of bacterial energy). Further studies would be needed to discern the mechanisms involved in the decrease in the adsorption on nonviable cells.

Cell heterogeneity in cultures growing under unchanging and nutritionally nonlimiting environmental conditions has been poorly studied in the *Lactobacillus* genus. A large cell variability leading the clonal multiplication of subpopulations with differences in the phenotypic expression of lysozyme resistance was described in *Lactobacillus helveticus* (Veaux *et al.* 1991). Suárez *et al.* (2008a) reported the isolation of two clearly different colony morphologies for the commercial strain *Lact. delbrueckii* ssp. *lactis* Ab1. These results were similar to those found in *Lact. delbrueckii* ssp. *bulgaricus* LB6 (Vescovo *et al.* 1990) and *Lactobacillus acidophilus* RL8K (Klaenhammer and Kleeman 1981). In this work, two morphological variants were found from both sensitive strains. As regards the phage-resistant phenotype, the proportion of derivatives belonging to the different morphological variants was dependent on the m.o.i. used.

The isolation of phage-resistant derivatives with good technological properties is a simple resource to select 'new' strains from those frequently used in the industry. Many studies have been reported about this topic, mainly based on the effective isolation of phage-resistant derivatives for lytic phages (Neviani *et al.* 1992; Carminati *et al.* 1993; Viscardi *et al.* 2003a,b; Binetti *et al.* 2006, 2007; Guglielmotti *et al.* 2006). However, no information is available on this topic for temperate phages. The results obtained in this work showed that it was possible to isolate phage-resistant derivatives with technological properties similar or better than the strains from which they were obtained. The best technological derivatives, for both sensitive strains, belonged to the morphological variant B.

It is concluded that this work makes a significant contribution to the knowledge of the adsorption stage for temperate phages of *Lact. delbrueckii*, which would permit the 'regulation' of this stage, for example, using phage inhibitors media. It was possible to corroborate that the phage resistance derivatives for the both host strains that presented the best technological properties always belonged to one morphological variant.

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