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Short communication

Phage-resistance linked to cell heterogeneity in the commercial strain *Lactobacillus delbrueckii* subsp. *lactis* Ab1

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ABSTRACT

The aim of this work was to study the relationship between the cell morphological heterogeneity and the phage-resistance in the commercial strain *Lactobacillus delbrueckii* subsp. *lactis* Ab1. Two morphological variants (named C and T) were isolated from this strain. Phage-resistant derivatives were isolated from them and the percentage of occurrence of confirmed phage-resistant cells was 0.001% of the total cellular population. Within these phage-resistant cell derivatives there were T (3 out of 4 total isolates) and C (1 out of 4 total isolates) variants. The study of some technological properties (e.g. proteolytic and acidifying activities) demonstrated that most of phage-resistant derivatives were not as good as the parental strain. However, for one derivative (a T variant), the technological properties were better than those of the parental strain. On the other hand, it was possible to determinate that the system of phage-resistance in the T variants was interference in adsorption step, with adsorption rates <15%. For the C variant derivative it was possible to demonstrate the presence of a restriction/modification system and, moreover, to determinate that this system could be Type I R/M.

1. Introduction

Lactobacillus delbrueckii is a lactic acid bacteria (LAB) species of great technological importance. This species is a fundamental constituent in the Italian (Neviani and Carini, 1994; Giraffa et al., 1997; Giraffa and Rosetti, 2004) and Argentinian (Reinheimer et al., 1995b, 1996) whey starters used for hard cheese manufacture. The obtention of spontaneous phage-resistant derivatives in LAB is one of the various strategies applied in the dairy industry to minimize phage infections (Limsowtin and Terzaghi, 1976; Neviani et al., 1992; Carminati et al., 1993; Weimer et al., 1993; Quiberoni et al., 1998a,b; Viscardi et al., 2003; Guglielmotti et al., 2006; Binetti et al., 2007). To be applied to industrial processes, phage-resistant derivatives must conserve the technological properties (e.g. proteolytic and acidifying activities) of the parental strain. Diverse studies were made on this topic (Carminati et al., 1993; Reinheimer et al., 1995a; Quiberoni et al., 1998a; Viscardi et al., 2003; Guglielmotti et al., 2006; Binetti et al., 2007).

Phenotypic variations within single-strain LAB populations have been known for a long time. These variations, which can be dependent of the growth conditions as temperature, pH, oxygen concentration and different components in culture medium, are generally reversible (Deibel et al., 1956; Holden and Holman, 1957; Kojima et al., 1970; Rhee and Pack, 1980). Morphological variations have also been reported when strains are cultured in complex media under optimal incubation conditions. Vescovo et al. (1990) related morphological and phenotypic variants of *L. delbrueckii* subsp. *bulgaricus* LB6 with the phage-resistance phenotype.

The natural defense mechanisms in LAB against phage infections are often plasmid-coded and may be classified into four broad categories: adsorption interference, injection blocking, restriction/modification (R/M) systems and abortive-infection (Forde and Fitzgerald, 1999; Coffey and Ross, 2002). Adsorption interference is the mechanism prevailing in spontaneous phage-resistant mutants of thermophilic LAB (Quiberoni et al., 1998a; Guglielmotti et al., 2006; Binetti et al., 2007), whereas R/M systems are widely present in *Lactococcus* genus (Forde and Fitzgerald, 1999, Moineau, 1999). Restriction/modification systems are classified into Type I, Type II, and Type III on the basis of their composition and cofactor requirements, nature of their target sequence, and the position of the site of DNA cleavage with respect to the target sequence (Bickle and Krüger, 1993; Murray, 2000).

The aim of this study was to investigate the relationship between morphological diversity and phage-resistance within cell population of the commercial strain *L. delbrueckii* subsp. *lactis* Ab1.

2. Materials and methods

2.1. Bacterial strain, bacteriophage and culture conditions

L. delbrueckii subsp. *lactis* Ab1 is a commercial strain isolated from a yogurt starter. Its specific virulent phage YAB was isolated from a

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failed industrial production of yogurt. The strain, its morphological variants and the phage-resistant derivatives were maintained as frozen stocks at −80 °C in reconstituted (10%, w/v) skimmed milk (RSM) and MRS broth supplemented with 15% (v/v) of glycerol, and routinely reactivated overnight at 42 °C. Phage stocks and phage enumerations were made as reported by Guglielmotti et al. (2006).

2.2. Isolation of morphological variants

The strain *L. delbrueckii* subsp. *lactis* Ab1 was streaked on four different agar media: MRS (BIOKAR, Beauvois, France), Triptone Soy Agar (TSA, Britania, Argentina), LAPTg (Raibaud et al., 1961) and 1006M (Vescovo et al., 1990). The plates were incubated at 42 °C for 48 h. Morphological variants were scored, isolated and cultured in MRS broth.

2.3. Isolation of phage-resistant derivatives

To isolate phage-resistant derivatives from morphological variants, the cellular remnant obtained after infection with phage YAB was streaked on MRS agar. The colonies obtained were tested against the same phage (Guglielmotti et al., 2006). Only the isolates that were able to grow in the presence of the phage after three subcultures were considered as true phage-resistant derivatives (Reinheimer et al., 1993). For each morphological variant, the percentage of phage-resistant derivatives was calculated.

2.4. Characterization of the phage-resistance phenotype

Efficiency of plaquing (EOP) values for phage YAB on the morphological variants and their phage-resistant derivatives, phage-resistance stability and adsorption rates for parent, variant and derivatives strains were determined according to Guglielmotti et al. (2006).

The presence of R/M systems in strains that presented a high adsorption rate but did not show lysis in the turbidity test was tested according to De los Reyes-Gavilán et al. (1990).

2.5. Characterization of morphological variants and phage-resistant derivatives

Cells were examined microscopically under phase contrast illumination (1000×, Microscope Jenamed 2 CARL ZEISS), while colony morphologies were scored on MRS agar. Sugar fermentation patterns were determined by API 50 CHL test strips, according to the manufacturer's instructions (API System, Montalieu-Vercieu, France). The proteolytic and acidifying activities were determined according to Guglielmotti et al. (2006).

2.6. Genetic analysis

Total bacterial DNAs were extracted as detailed by Giraffa et al. (2000). PCR-ARDRA and RAPD-PCR patterns were obtained for the sensitive strain, their morphological variants and their phage-resistant derivatives according to Guglielmotti et al. (2006).

Some pairs of primers were designed to characterize the R/M system of the phage-resistant derivative *L. delbrueckii* subsp. *lactis* Ab1 C(a) (DNAMAN Sequence Analysis Package Program Version 5. 2. 9, Lynnon Corporation, Copyright 1994–2001). Primer sequences and amplicon sizes are detailed in Table 1. The primers used to amplify the subunits *hsdS* and *hsdR* of Type I system were designed according to the completely sequenced genome of *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 (GeneBank, accession number NC_008054).

PCR reactions were performed in 25 µl containing 200 µM deoxynucleoside triphosphate, 0.5 µM of each primer, 1.25 U of *Taq* DNA polymerase (Invitrogen), *Taq* buffer (20 mM Tris–HCl, pH 8.4, 1.5 mM magnesium chloride, 50 mM potassium chloride) and 1 µl of bacterial DNA. PCR conditions were as follows: 5 min at 94 °C followed by 30 cycles (30 s at 94 °C, 30 s at 53 °C, 1 min at 72 °C) and a final step of 7 min at 72 °C. PCR products were separated on a 0.8% (w/v) agarose gel in TAE buffer, stained with ethidium bromide, and visualized under UV light. The positive control for Type I R/M system was strain *L. delbrueckii* subsp. *lactis* 204 (Suárez et al., data not published).

Table 1
Primer sequences used in this study

Primer	Sequence (5′→3′)	Amplicon size (bp)
hsdS I for	TCT TGT CAG CGA ACA TCT TC	535
hsdS I rev	TCC TTG GGA GCA GTG TAA G	
hsdR I for	CTA TTC CCG ACG CTG ATT	494
hsdR I rev	GTA TCT GGT AAG GAC GGA GC	

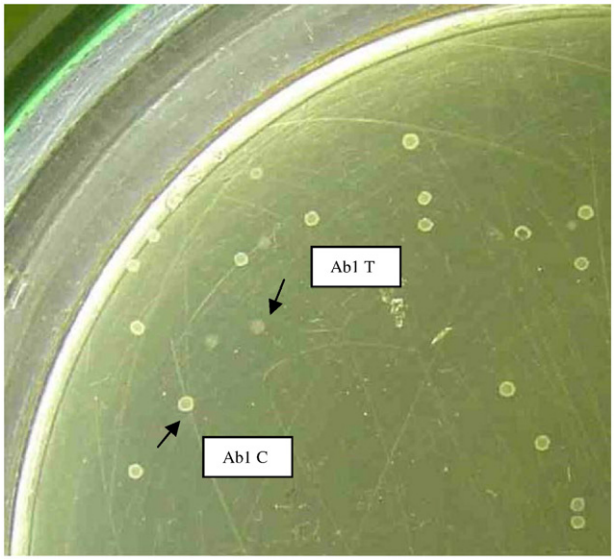

Fig. 1. *Lactobacillus delbrueckii* subsp. *lactis* Ab1 morphological variants visualized on MRS Agar.

3. Results and discussion

3.1. Colony heterogeneity

Cell heterogeneity in cultures growing under unchanging and nutritionally non-limiting environmental conditions has been poorly studied in the *Lactobacillus* genus (Klaenhammer and Kleeman, 1981; Vescovo et al., 1990). A large cell variability leading the clonal multiplication of sub-populations with differences in the phenotypic expression of lysozyme resistance was described in *Lactobacillus helveticus* (Veaux et al., 1991).

Colony heterogeneity of *L. delbrueckii* subsp. *lactis* Ab1 was variable according to the culture media used, but it was more evident on MRS agar (Fig. 1). Two clearly different colony morphologies were observed on this medium: the C-type, which appeared as circular opaque colonies and the T-type, which were transparent and rough colonies. These results were similar to those found in *L. delbrueckii* subsp. *bulgaricus* LB6 (Vescovo et al., 1990) and *Lactobacillus acidophilus* RL8K (Klaenhammer and Kleeman, 1981).The C variants constituted approximately 85% of total cell population, whereas the T variants accounted for the remaining 15%.

The two variants observed under microscopy showed different cellular morphologies (Fig. 2), since a typical C variant showed short curved bacilli in chains whereas the T variants showed perfectly defined, long rods. The phage-resistance of these variants was then studied.

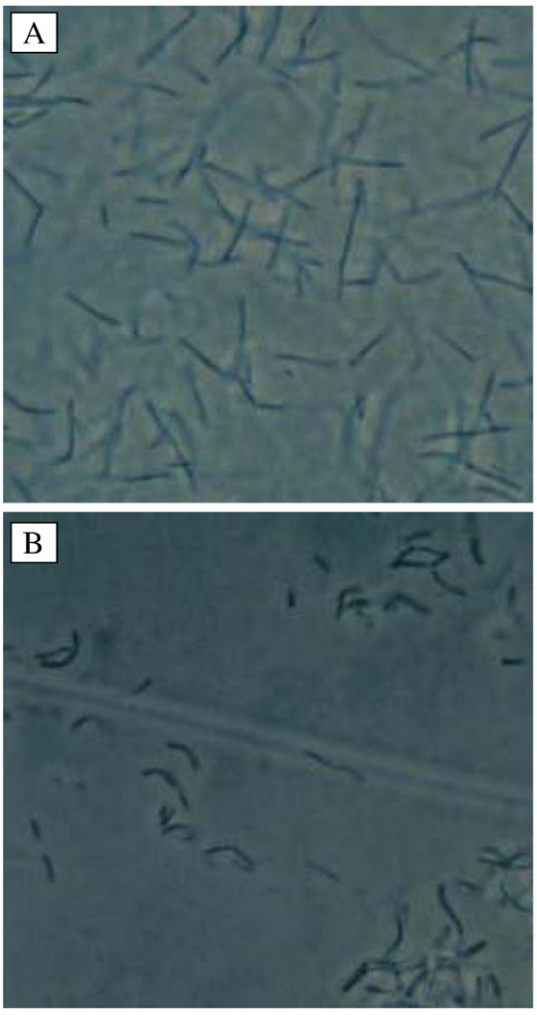

Fig. 2. Cell morphology of Ab1 T (A) and Ab1 C variant (B) of *L. delbrueckii* subsp. *lactis* Ab1, observed from MRS broth.

3.2. Phage-resistant derivatives

The lysis of *L. delbrueckii* subsp. *lactis* Ab1 with phage YAB was incomplete. This unlysed cell remnant was counted and this constituted approximately 0.01% of the total cell count obtained in a control culture (without phages). A total of 19 isolates from each variant of these presumptive phage-resistant cells were infected with the phage, but only 4 of them (10.5% of the total presumptive resistant mutants) were resistant after three subcultures. Within these confirmed phage-resistant derivatives, there were three T variants named Ab1 T(b), Ab1 T(b1) and Ab1 T(b2). The fourth phage-resistant derivative belonged to C variant (Ab1 C(a)). Similar values were reported for *L. helveticus* CNRZ 328 (Reinheimer et al., 1993) and *Streptococcus thermophilus* (Binetti et al., 2007). Capra (2006) failed to isolate stable phage-resistant derivatives from *Lactobacillus paracasei*. Carminati et al. (1993) isolated 32 phage-resistant derivatives from nine *L. helveticus* strains. These derivatives constituted 35% of the presumptive phage-resistant population. Another study on *L. helveticus* reported a similar percentage (37.5%) of phage-resistant derivatives among 176 presumptive phage-resistant isolates (Quiberoni et al., 1998a), whereas a study on *S. thermophilus* (Binetti et al., 2007) reported a bit lower frequency (22.6%).

This phage-resistant cell population was calculated as the 0.001% of the cell count reached in the control culture of the strain Ab1.

Table 2
Characterization of phage-resistance phenotype of morphological variants obtained from *L. delbrueckii* subsp. *lactis* Ab1

Strain/variant	EOP[a]	Phage sensitivity (in broth)	Phage-resistance stability[b]	Adsorption rate[c] (%)	R/M system
Ab1		+		99	
Ab1 C	1.07	+		95	
Ab1 T	0.80	+		99	
Ab1 C(a)	$<10^{-7}$	−	+ (4°)	93	Yes
Ab1 T(b)	$<10^{-7}$	−	−	15	−
Ab1 T(b1)	$<10^{-7}$	−	−	13	−
Ab1 T(b2)	$<10^{-7}$	−	−	0	−

[a] Phage count on variant/phage count on parental strain.
[b] Lysis (+) or lysis absence (−) until 7° subculture. (*n*): lysis subculture number.
[c] Adsorbed phages after 30 min at 42 °C.

Table 3
EOP of phage YAB on its host strain *L. delbrueckii* subsp. *lactis* Ab1 (*L.l* Ab1) and of the propagate phages on the restrictive variant Ab1 C(a)

Phage	Host strains[a]	EOP[b] on *L.l* Ab1 C(a)
YAB	*L.l* Ab1	10^{-7}
	L.l Ab1; *L.l* Ab1 C(a)	$8.9\ 10^{-1}$
	L.l Ab1; *L.l* Ab1 C(a); *L.l* Ab1	10^{-7}

[a] Strain sequence on which the phage was propagated before plate count.
[b] EOP defined as a ratio between phage count on restrictive strain and phage count on host strain.

3.3. Phage-resistance phenotype

Table 2 shows the characteristics of phage-resistance phenotype for the parental strain, the morphological variants and phage-resistant derivatives. EOP values were near 1 and lower than 10^{-7} for the variants and the four phage-resistant derivatives, respectively, indicating a high level of resistance (Moineau, 1999). Similar results were reported for derivatives isolated from *L. helveticus* (Carminati et al., 1993; Reinheimer et al., 1995a; Quiberoni et al., 1998a), *S. thermophilus* (Binetti et al., 2007) and *L. delbrueckii* (Guglielmotti et al., 2006).

Phage-resistance for T derivatives was more stable (they resist at least seven subcultures) than for the C derivative (four subcultures).

Adsorption rate values were high (>95%) for both morphological variants (T and C). For T phage-resistant derivatives (Ab1 T(b), Ab1 T (b1), Ab1 T(b2)), values were ≤15%. Adsorption interference is a very common phage-resistance mechanism in all LAB species (Quiberoni et al., 1998b; Guglielmotti et al., 2006; Binetti et al., 2007) and it is related to a loss or modification of phage receptors due to mutations in corresponding genes (Riipinen et al., 2007).

For phage-resistant derivative Ab1 C(a), the adsorption rate was similar (93%) to that found for the parental strain. Table 3 shows EOP values for phage YAB after propagation on the mutant Ab1 C(a) and after a new propagation on the host strain Ab1. After this last infection, the EOP value returned to that observed for phage YAB after propagation on its host strain. This result combined with the stability

Table 4
Technological characterization of morphological variants and phage-resistant derivatives of *L. delbrueckii* subsp. *lactis* Ab1 (values are media of three determinations)

Strain/variant	pH	Acidifying activity[a]	Proteolytic activity[b]
Ab1	3.82±0.05	1.26±0.10	0.387±0.038
Ab1 C	4.00±0.08	1.26±0.11	0.346±0.041
Ab1 T	3.96±0.04	1.26±0.08	0.387±0.032
Ab1 C (a)	4.50±0.05	0.84±0.05	0.301±0.020
Ab1 T (b)	4.92±0.04	0.82±0.08	0.318±0.031
Ab1 T (b1)	5.39±0.07	0.67±0.10	0.191±0.023
Ab1 T (b2)	3.92±0.05	1.30±0.09	0.474±0.029

[a] % lactic acid after 24 h at 42 °C, in RSM (10%, w/v).
[b] Absorbance value (340 nm), after 24 h at 42 °C, in RSM (10%, w/v).

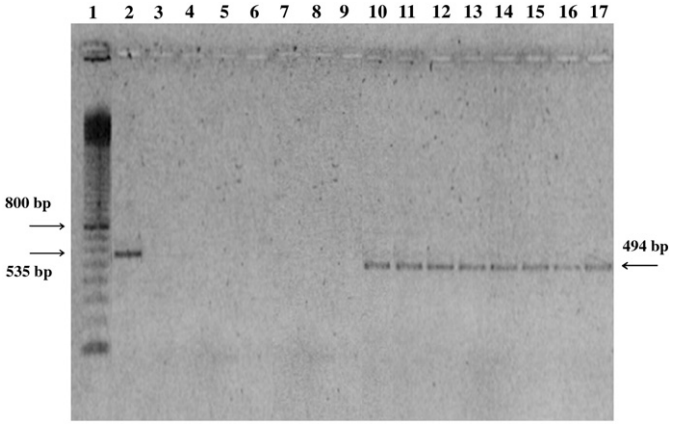

Fig. 3. PCR of *L. delbrueckii* subsp. *lactis* Ab1 and their morphological variants and phage-resistant derivatives, with specific primers for amplification of *hsdS* (Lines 3, 4, 5, 6, 7, 8 and 9) and *hsdR* (Lines 11, 12, 13, 14, 15, 16 and 17) subunits. Line 1: molecular weight marker 1 kbp (Amersham Biosciences UK limited, UK), Lines 2 and 10: *L. delbrueckii* subsp. *lactis* 204 as positive control, Lines 3 and 11: Ab1, Lines 4 and 12: Ab1 C, Lines 5 and 13: Ab1 T, Lines 6 and 14: Ab1 C(a), Lines 7 and 15: Ab1 T(b), Lines 8 and 16: Ab1 T(b1), Lines 9 and 17: Ab1 T(b2).

assay, suggest the existence of an active intracellular phage-resistance mechanism.

3.4. Technological characterization

Sugar fermentation patterns for the parental strain, morphological variants and phage-resistant derivatives were obtained (data not shown). Acidifying and proteolytic activity values for the morphological variants were similar to those obtained for the parental strain *L. delbrueckii* subsp. *lactis* Ab1 (Table 4). On the contrary, phage-resistant derivatives (with exception of Ab1 T(b2)), showed poor performance in milk, revealed by their proteolytic ($A_{340\ nm}$ from 0.191 to 0.318) and acidifying activities (from 0.67 to 0.84% of lactic acid). Additionally, the final pH value reached after an incubation of 24 h in milk (from 4.50 to 5.39) were lower than those obtained for the parental strain ($A_{340\ nm}$=0.387, 1.26% of lactic acid and pH=3.82). Strain Ab1 T(b2) was the most interesting phage-resistant derivative obtained since it showed the same technological characteristics than the parental strain. This variability in the results was also reported for *L. helveticus* (Carminati et al., 1993; Reinheimer et al., 1995a; Quiberoni et al., 1998a). Binetti et al. (2007) isolated phage-resistant mutants from 12 commercial *S. thermophilus* strains, which showed similar or even improved technological characteristics (acidifying rates, acidifying and proteolytic activities) with respect to the parent strains. For *L. delbrueckii*, Guglielmotti et al. (2006) reported the isolation of phage-resistant mutants both with similar and different technological properties with respect to their parental strain.

3.5. Genetic analysis

PCR-ARDRA profiles and the dendrogram obtained after RAPD-PCR confirmed the identity of the parental strain, its variants and derivatives as belonging to *L. delbrueckii* subsp. *lactis* (data not shown).

Fig. 3 shows the results for PCR amplification of genes for R/M systems. For both parental strain and variants/derivatives, a fragment of 494 bp corresponding to the amplification of the *hsdR* subunit of Type I R/M system was visualized. However, R/M phenotype was only demonstrated for Ab1 C(a) derivative, since this mutant was the only one that showed a normal adsorption. Amplification products for *hsdS* subunit of Type I R/M system (Fig. 3, lines 3, 4, 5, 6, 7, 8 and 9) were not obtained neither parental, morphological variants nor phage-resistance derivatives. The absence of *hsdS* fragment could be explained considering that R/M Type I system is the only defense mechanism that presents point mutations (Coffey and Ross, 2002), which could change the target points of the R/M complex.

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