Isolation and Characterization of a New *Lactobacillus delbrueckii* ssp. *bulgaricus* Temperate Bacteriophage

L. AUAD,* A.A.P. de RUIZ HOLGADO,* P. FORSMAN,† T. ALATOSSAVA,† and R. R. RAYA*,[1]

*Centro de Referencia para Lactobacilos (CERELA), Chacabuco 145, 4000, San Miguel de Tucumán, Argentina

†Department of Genetics, University of Oulu, FIN-90570 Oulu, Finland

ABSTRACT

Lactobacillus delbrueckii ssp. *bulgaricus* strain CRL 539 was shown to be lysogenic and inducible with mitomycin C. The conditions were determined for an optimal induction of temperate bacteriophage lb539 with mitomycin C as well as the sensitivity of lb539 to physical and chemical agents. Electron microscopy of lysates revealed bacteriophage particles with an isometric head of 47 nm and a noncontractile tail of 159 nm. Phage lb539 was classified within Bradley's B1 phage group and the Siphoviridae family. The host range of lb539 encompassed mainly *Lactobacillus delbrueckii* ssp. *lactis* strains; strain LKT (CNRZ 700) was the most sensitive for detection of lb539 lysates induced by mitomycin C. The lb539 genome is a linear, double-stranded DNA molecule of approximately 35 kbp. The presence of submolar fragments in restriction enzyme digests suggests that lb539 DNA may contain a *pac* site. Dot-blot experiments showed that the lb539 genome hybridized with the genomes of phages mv4 and LL-H, which are type phages of group *a* of *L. delbrueckii* ssp. phages. Restriction enzyme patterns and morphological features showed lb539 to be distinct from mv4 and LL-H.

(**Key words**: lactobacillus, bacteriophage)

Abbreviation key: **MC** = mitomycin C.

INTRODUCTION

Lactobacillus delbrueckii ssp. *bulgaricus* is used in the dairy industry as a starter culture for yogurt and cheese production. Despite its industrial importance, the genetics of this microorganism is poorly understood, partially because of the lack of an efficient gene transfer system in this species. An understanding of the biology of its bacteriophages represents an alternative approach to the study of the genetics of *L. delbrueckii* ssp. *bulgaricus*. In addition, phage genomes can be exploited to develop genetic tools, such as cloning, chromosomal integration vectors, and gene expression systems.

A large number of specific *L. delbrueckii* ssp. *bulgaricus* bacteriophages have been isolated and characterized. Phages of *L. delbrueckii* ssp. *bulgaricus* and *L. delbrueckii* ssp. *lactis* are closely related and have been classified into four groups (*a* to *d*) on the basis of immunoblotting tests and DNA-DNA hybridizations (13, 17). Most of the phages belong to the *Siphoviridae* family (14) corresponding to Bradley's group B1 (5). Group *a* is the largest and includes temperate and virulent phages of both subspecies. The type phages of group *a*, phages mv4 and LL-H, are presently the best characterized lactobacilli phages (3, 8, 10, 12, 20), and the site-specific integration functions of mv4 have been used to construct a site-specific integration vector for *Lactobacillus plantarum* (10). Group *b* is composed of the virulent phages of *L. delbrueckii* ssp. *bulgaricus*, and groups *c* and *d* contain temperate phages of *L. delbrueckii* ssp. *lactis*. Although the lytic spectra of group *c* and *d* phages are similar to those of group *a* phages, these groups are different morphologically, serologically, and molecularly.

In this study, the isolation and characterization of the new *L. delbrueckii* ssp. *bulgaricus* temperate bacteriophage lb539 are described.

MATERIALS AND METHODS

Bacteria and Phage

The bacteria used in this study are listed in Table 1. Strains from CNRZ culture collections and reference phages (Table 2) were kindly provided by J.-P. Accolas (Institut National Recherches Agronomique, Jouy-en-Josas, France). Stock cultures were maintained at –20°C in LATPg broth (15) with 15% glycerol. Bacterial cells were propagated at 37°C in LAPTg or MRS (9) broth. When appropriate, agar

Received April 16, 1997.
Accepted June 5, 1997.
[1]Corresponding author.

TABLE 1. Potential indicators tested for the temperate *Lactobacillus delbrueckii* ssp. *bulgaricus* phage lb539.

Strain[1]	Strain	Result[2]	
			(pfu/ml)[3]
L. delbrueckii ssp. *bulgaricus*			
CRL	65 strains	–	
ATCC	11842 (CRL 958)	–	
CNRZ	836	–	
	1004	+	ND[4]
	448 (LT4)	–	
L. delbrueckii ssp. *delbrueckii*			
ATCC	9649	–	
L. delbrueckii ssp. *lactis*			
CRL	702	–	
	655	–	
CNRZ	235	–	
	326	+	4.6×10^3
	327	+	ND
	700 (LKT)	+	3.0×10^6
	1006	+	ND
	1444	–	
ATCC	7830	–	
	8000 (CRL 934)	+	4.0×10^5
	12315	–	
	4797	–	

[1]CRL = Centro de Referencia para Lactobacilos; Tucumán, Argentina. Strains of *L. delbrueckii* ssp. *bulgaricus* used were 142, 401, 402, 403, 404, 405, 406, 407, 420, 421, 425, 426, 427, 445, 446, 447, 449, 450, 451, 452, 453, 454, 466, 468, 487, 492, 493, 496, 537, 539, 540, 541, 542, 545, 546, 547, 551, 552, 553, 554, 556, 558, 559, 562, 564, 566, 568, 569, 631, 632, 633, 658, 850, 853, 858, 859, 862, 863, 866, 867, 868, 871 and 872. ATCC = American Type Culture Collection (Rockville, MD); CNRZ = Centre National de Recherches Zootechniques (Jouy-en-Josas, France).

[2]+ = Sensitive; – = resistant.

[3]Plaque-forming units per milliliter of phage lb539 induced with mitomycin C.

[4]Not done.

was added at 0.6 and 1.5% to make a solid medium; sterile $CaCl_2 \cdot 6\ H_20$ was added to a final concentration of 10 m*M*.

Phage Induction

An overnight culture of *L. delbrueckii* ssp. *bulgaricus* CRL539 was transferred into 5 ml of fresh broth and incubated at 37 or 42°C for 30 min or until the absorbance at 600 nm was between 0.1 and 0.2, at which time mitomycin C (**MC**; Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 0.05 to 0.6 μg/ml. Absorbance at 600 nm was then measured for 6 h. After centrifugation at $1200 \times g$ (10 min at 20 to 24°C), the supernatant was evaluated for the presence of phage using standard overlay methods.

Phage stocks were prepared from lysates or by soaking plates showing near confluent lysis with LAPTg broth; the indicator strain was *L. delbrueckii* ssp. *lactis* CNRZ 326. Phage lysates (pH 4.5) were centrifuged, passed through a membrane filter (0.2-μm pore size), and stored at 4°C. Three successive plaque purifications were carried out on each phage isolate. All phage titrations were carried out at 37°C in LAPTg agar as described by Accolas et al. (1). Also studied were the effects of adenine (100 μg/ml), sodium formate (100 and 200 μg/ml), orotic acid (100 μg/ml), and glycine (12 and 18 mg/ml) on phage induction.

Host Range Determinations

Screening for indicator hosts of lb539 was carried out by spotting onto lawns of *Lactobacillus delbrueckii* ssp. *bulgaricus* (68 strains), ssp. *lactis* (12 strains), and ssp. *delbruekii* (1 strain) (Table 1).

Sensitivity to Physical and Chemical Agents

Phage sensitivity was determined according to the methods of Ackermann et al. (2). Agents tested included chloroform (Merck Chemical, Bueonos Aires, Argentina), Triton X-100 (Sigma Chemical Co.), and Nonidet P40 (Sigma Chemical Co.); pH ranged from 2 to 13, and temperatures tested were 50, 60, and 70°C. All tests, except those for the temperature variables, were performed at 25°C for 60 min using crude bacteria-free lysate from a LAPTg broth culture. Results are the means of three independent replicate assays and were expressed as the percentage of survival of lb539 compared with the titer of a lysate without treatment.

TABLE 2. The DNA homology among the temperate and virulent *Lactobacillus delbrueckii* phages used in this study.

Phage	Taxonomic group	Lysogenic strain of *Lactobacillus delbrueckii* subspecies	DNA Homology[1]
Temperate			
mv4	*a*	*bulgaricus* LT4	+++
0235	*c*	*lactis* CNRZ 235	+
1444 (7/11)	*c*	*lactis* CNRZ 1444	–
0252	*d*	*lactis* CNRZ 252	–
Virulent		Propagation host	
c5	*b*	*bulgaricus* LT4	–
LL-H	*a*	*lactis* LL-H	+++

[1]Results of dot-blot hybridization using lb539 DNA as probe: +++ = strong, + = weak, and – = negative.

DNA Isolation and Hybridization

Phage particles were purified by precipitation overnight at 4°C with 0.5 *M* NaCl and 10% polyethylene glycol (wt/vol) (23). The pellet, obtained by centrifugation for 20 min at 12,000 × *g* was resuspended in TE buffer (10 m*M* Tris·HCl and EDTA 1 m*M*; pH 8) and treated two to three times with an equal volume of Tris-saturated phenol and $CHCl_3$:isoamyl alcohol. The DNA was precipitated with absolute ethanol (16). Phage DNA was digested with restriction enzymes as recommended by the suppliers (New England Bio-Labs, Beverly, MA; Promega, Madison, WI; and Gibco BRL, Gaithersburg, MD). Gel electrophoresis in a 0.8% (wt/vol) agarose gel was performed as described by Sambrook et al. (16). The size of the lb539 DNA fragments were determined from comigrating *Hin*dIII-λ and *Bst*EII-λ phage DNA fragments. The dot-blot hybridization was carried out with an NE Blot Phototope kit (New England Bio-Labs), which incorporates biotin into hybridization probes through a random primer reaction. Nucleic acid was deposited on a nylon membrane (0.45 μm, Gibco BRL) as described by Sambrook et al. (16). Hybridization reactions were performed under highly stringent (68°C) conditions and in accordance with the specifications of the supplier (New England Bio-Labs).

Electron Microscopy

The sample preparation and negative staining of phages with aqueous 2% uranyl acetate were performed as described previously (11). Phages were analyzed in a Jeol Temscan 100 CX II electron microscope (Tokyo, Japan).

RESULTS

Induction of Prophage lb539 with MC

Lysis of *L. delbrueckii* ssp. *bulgaricus* CRL 539 that is induced by mitomycin C is shown in Figure 1. The lysogenic phage detected in CRL 539 supernatants was named lb539. Cultural conditions that were suitable for optimal MC induction of lb539 were studied. Factors such as temperature (37 and 42°C), age of the culture at the moment when MC was added (30 and 60 min), dose of MC (from 0.1 to 0.6 μg/ml), and percentage of initial inoculum (1, 2, and 4%) were evaluated. Figure 1 illustrates a successful induction experiment using strain CRL 539. Induction was optimal at 42°C when *L. delbrueckii* ssp. *bulgaricus* CRL 539 was inoculated at 1% in LAPTg broth and when MC was added 30 min after cells were inoculated. Optimal doses of MC ranged from 0.1 to 0.2 μg/ml. Higher MC concentrations were toxic for the cells.

Under our experimental conditions, CRL 539 cells propagated in LAPTg broth appeared as elongated rods. Cells became shorter when glycine was added to a final concentration of 12 mg/ml (data not shown). We investigated whether this morphological change affected induction of the lb539 prophage. Other compounds, such as adenine, orotic acid, and formic acid, were also included because they have been reported (18, 19) to act as purine precursors and growth stimulators, which reduced the cell length of *L. delbrueckii* ssp. *bulgaricus* cells (18, 19). (The latter

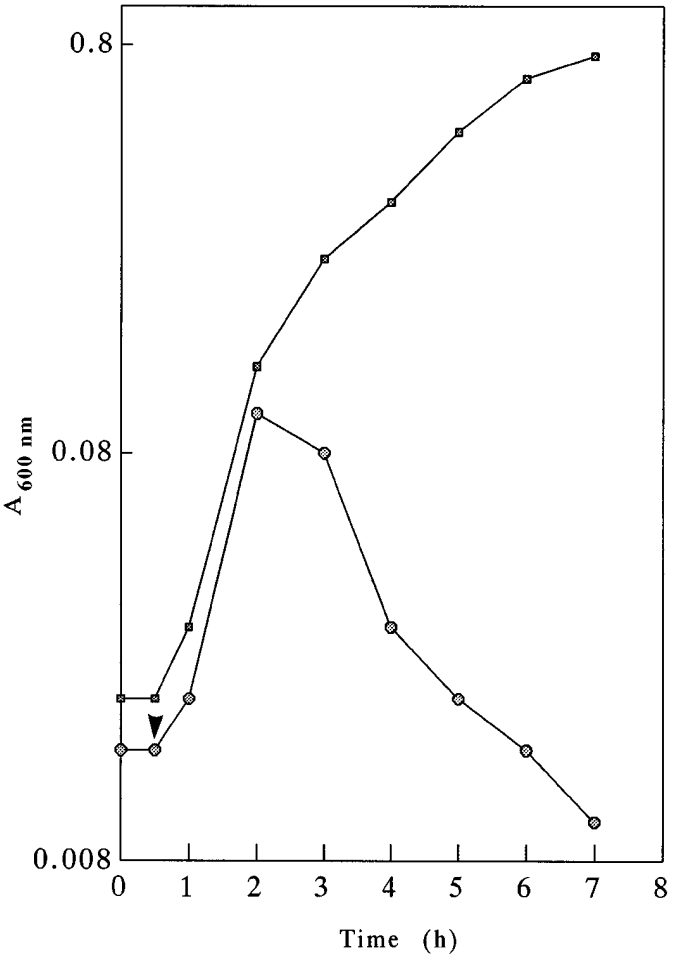

Figure 1. Mitomycin C induction of *Lactobacillus delbrueckii* ssp. *bulgaricus* CRL 539. Early log phase LAPTg broth cultures of *L. delbrueckii* ssp. *bulgaricus* CRL 539 were treated with mitomycin C 30 min after a 1% initial inoculum was made. Cultures were maintained at 42°C, and absorbance at 600 nm was monitored. Squares = control; circles = 0.1 μg/ml of mitomycin C. Arrow indicates addition of mitomycin C.

TABLE 3. Comparison of the *L. delbrueckii* phages LL-H, mv4, and lb539 group *a*.

	lb539	mv4[1]	LL-H[2]
Isolated from	*L. delbrueckii* ssp. *bulgaricus* CRL 539	*L. delbrueckii* ssp. *bulgaricus* LT4	
Type of action	Temperate	Temperate	Lytic
Morphology			
Head	47 nm	50 nm	55 nm
Tail	159 nm	180 nm	180 nm
Collar	no	triple collar	collar-like structure
Tail fibers	no	yes	yes
Base plate	no	no	yes
Host range[3]			
L. delbrueckii ssp. *lactis*			
CRL 934	+	+	+
CNRZ 326	+	+	+
CNRZ 700 (LKT)	+	+	+
L. delbrueckii ssp. *bulgaricus*			
CNRZ 1004	+	+	+
CNRZ 448 (LT4)	–	–	–
CRL 539	–	–	–

[1]See reference (8).

[2]See reference (3).

[3]See reference (7) for host range of LL-H and mv4.

effect was not observed during this study.) Addition of glycine, adenine, sodium formate, and orotic acid, however, did not affect the MC induction response of phage lb539.

Electron Microscopy

Examination of lb539 by electron microscopy showed phage particles with a hexagonal head of 47 nm and a long noncontractile tail of 159 nm (Figure 2). These characteristics placed phage lb539 in the *Siphoviridae* family (14), which correspond to the B group of Bradley (5).

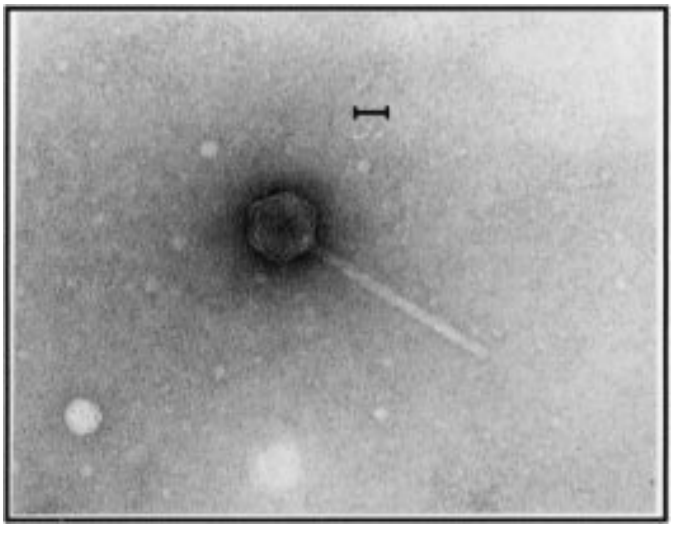

Figure 2. Electron micrograph of phage lb539 negatively stained with 2% aqueous uranyl acetate. Bar = 25 nm.

Host Range Determination

On the basis of 16S rRNA sequence comparison, the *L. delbrueckii* species falls into three major subspecies: *lactis*, *delbrueckii*, and *bulgaricus*. Several strains of these three subspecies were tested for sensitivity to phage lb539 by spotting lysates of phage lb539. The lb539 was able to form plaques and propagate on *L. delbrueckii* ssp. *lactis* strains CNRZ 326 (ATCC 15808), CNRZ 327 (ATCC 10697), CNRZ 1006 (LL23), and CNRZ 700 (LKT); these indicator strains were defined previously for temperate bacteriophages mv1 and mv4 [(8); Table 1]. Strain LKT (CNRZ 700) was the most sensitive host for detection of lb539 lysates (3.0×10^6 pfu/ml). Phage lb539 also plaqued on CRL 934 (ATCC 8000; Table 1), a strain that had not been previously defined as an indicator for *L. delbrueckii* ssp. *bulgaricus* phages. The plaques produced by lb539 on *L. delbrueckii* ssp. *lactis* CNRZ 326 and CRL 934 were small with a clear center surrounded by a turbid area. However, CRL 934 is a better indicator strain for MC-induced lb539. Phage titer of induced lb539 lysates was 4.0×10^5 pfu/ml on CRL 934 versus 4.6×10^3 pfu/ml on CNRZ 326. *Lactobacillus delbrueckii* ssp. *lactis* CRL 934 was also useful as an indicator strain for other related phages from *L. delbrueckii* ssp. *lactis* and *L. delbrueckii* ssp. *bulgaricus*, such as mv4 and

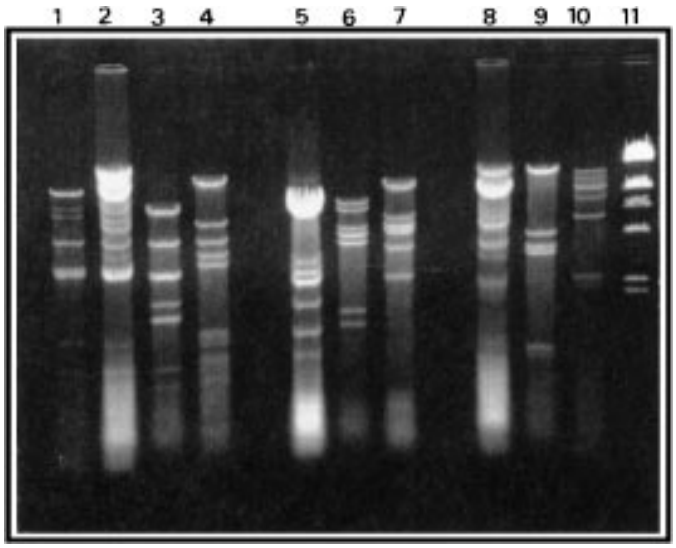

Figure 3. Gel electrophoresis of phages lb539 (lanes 1, 2, 5, and 8), LL-H (lanes 3, 6, and 9), and mv4 (lanes 4, 7, and 10) DNA after digestion with various enzymes. Lanes 1 to 4 were digested with *Bam*HI (lane 2 = DNA partially digested), lanes 5 to 7 were digested with *Pst*I, and lanes 8 to 10 were digested with *Sal*I. Lane 11 was digested with λ*Hin*dIII DNA.

LL-H (Table 3), as well as 1444 and 0235 (data not shown), but was otherwise resistant to the phages c5 and 0252 (data not shown).

With the exception of the *L. delbrueckii* ssp. *bulgaricus* CNRZ 1004 strain, no strains within the subspecies *delbrueckii* and *bulgaricus* were attacked by lb539 (Table 1).

Sensitivity to Physical and Chemical Agents

Phage lb539 was not inactivated by chloroform, Triton X-100, or Nonidet P40, suggesting that this phage is not composed of lipids (6). Phage lb539 tolerates pH values of 4 to 10 (Table 4). The differences in phage survival within the pH range were not significant ($P > 0.01$). The degree of infectivity of phage lb539 declined at pH 3 and was inhibited at extreme pH values (pH 2 and 13) (Table 4). Phage particles were very sensitive to temperature. After heat treatment of the phage suspensions at 50, 60, and 70°C for 60 min, plaque-forming units were reduced to 10, 0.1, and 0.01% of the initial population.

Genetic Characterization

The lb539 phage genome consists of a linear, double-stranded DNA. Genome size was estimated as approximately 35 ± 3.0 kbp by summing the size of the DNA fragments generated by the restriction enzymes *Sal*I, *Eco*RV, *Bam*HI, *Bss*HI, and *Sac*II. Figure 3 shows that the lb539 DNA restriction pattern differed from other *Lactobacillus* phages DNA of group *a*, specifically LL-H and mv4.

The lb539 genome did not appear to contain cohesive ends because heating 10 min at 60 or 75°C (4) before electrophoresis did not alter the restriction patterns of *Eco*RI, *Bam*HI, *Eco*RV, *Sal*I, or *Pst*I. Furthermore, submolar fragments were usually observed as a consequence of DNA digestions with several restriction endonucleases, which would be consistent with the absence of a *cos* site and the presence of a *pac* site in the lb539 DNA molecules.

DNA-DNA Hybridization

Table 2 includes reference phages of the *L. delbrueckii* ssp. *bulgaricus* and *L. delbrueckii* ssp. *lactis* bacteriophages, according to the classification established by Sechaud et al. (17). Virulent phage LL-H and temperate phage mv4 share 45% of DNA homology (13) and belong to group *a*; phages c5, 0235 and 1444, and 0252 belong to the groups *b*, *c*, and *d*, respectively. Biotinylated lb539 DNA was used as a probe for dot-blot hybridization to DNA from all the reference *Lactobacillus* phage groups. Phage lb539 DNA hybridized strongly with mv4 and LL-H phage DNA (Figure 4 and Table 2). A slight hybridization signal was observed with DNA from phage 0235 (Figure 4 and Table 2). In contrast, no homology was detected between lb539 and c5 (group *b*), 1444 (group *c*), or 0252 (group *d*) DNA (Figure 4 and Table 2).

DISCUSSION

The isolation and characterization of the new *L. delbrueckii* ssp. *bulgaricus* phage lb539 are described. Phage lb539 belongs to the most common *L. del-*

TABLE 4. Effect of pH on survival of *Lactobacillus delbrueckii* phage lb539.[1]

pH	(pfu/ml)[1]	(log pfu/ml)	
		$\bar{X}$	SD
2	$<3.9 \times 10^6$	ND	
3	1.1×10^8	7.95[a]	0.44
4	3.9×10^9	9.58[b]	0.10
5	2.1×10^9	9.31[b]	0.15
7	1.6×10^9	9.15[b]	0.30
10	1.6×10^9	9.22[b]	0.06
13	$<3.9 \times 10^6$	ND	

[a,b]Means with different superscripts differ ($P < 0.01$) by Tukey's multiple comparisons test for honestly significant difference (22).

[1]Residual after treatment. Values were determined on LKT cells and represent means of at least three experiments.

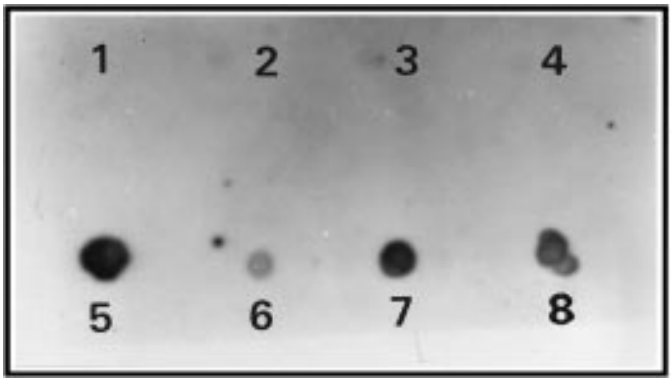

Figure 4. Dot-blot hybridization of biotinylated lb539 DNA with 1) DNA of plasmid pACYC184 (negative control) and DNA of phages 2) 1444, 3) 0252, 4) c5, 5) lb539 (positive control), 6) 0235, 7) LL-H, and 8) mv4.

brueckii ssp. *bulgaricus* type of phages, group *a*, according to the classification of Sechaud et al. (17) and Mata et al. (13). As Table 3 shows, the reference type *a* phages, mv4 and LL-H, and the temperate bacteriophage lb539 have a genome of double-stranded DNA of about 35 kbp containing a *pac* site; the ultrastructural morphology of their phages particles are comparable, having an isometric head and a noncontractile tail, and their host ranges are identical. However, dimensions of lb539 phage particles are shorter (47-nm head and 159-nm tail) than the mv4 and LL-H particles (50-nm head and 180-nm tail and 55-nm head and 180-nm tail, respectively). The triple collar and tail fibers of mv4 or the 30-nm tail fiber described in LL-H was not observed in electron micrographs of negatively stained lb539 particles. Furthermore, restriction analysis confirmed that lb539 DNA was distinct from mv4 and LL-H DNA and that lb539 had not been identified previously.

Cell length variants of *L. delbrueckii* ssp. *bulgaricus* display different susceptibilities to environmental stress. Vescovo et al. (21) have reported a noteworthy difference in the sensitivity of two cell morphological variants of *L. delbrueckii* ssp. *bulgaricus* LB6 to their homologous phage ϕ-LB6. Phage ϕ-LB6 had better plaquing efficiency, larger plaque size, and higher burst size in elongated rod-shaped cells than those of spiral or curved rod variants of LB6 cells. However, addition of adenine, glycine, orotic acid, and formic acid, which act as purine precursors and growth stimulants of *L. delbrueckii* ssp. *bulgaricus* (18, 19), have no effect on MC-mediated induction of lb539, even though short cells were produced by the presence of glycine. Induction of temperate phage lb539 showed that the most important parameter was the addition of MC 30 min after the cells were inoculated in fresh broth.

Bacteriophages of *L. delbrueckii* ssp. *bulgaricus* and *L. delbrueckii* ssp. *lactis*, which belong to group *a* are very closely related, and indicator strains that are sensitive to phage attack by both types are common. In a previous report (8), 5 indicator strains (4 *L. delbrueckii* ssp. *lactis* strains and 1 *L. delbrueckii* ssp. *bulgaricus* strain) were found for the temperate phages mv4 and mv1. Here, we report that *L. delbrueckii* ssp. *lactis* ATCC 8000 (CRL 934) may also be useful as an indicator strain for *L. delbrueckii* ssp. *bulgaricus* and *L. delbrueckii* ssp. *lactis* phages. In addition to lb539, CRL 934 cells were also attacked by phages mv4, 1444, 0235, and LL-H, but CRL 934 was resistant to lytic phage c5 and temperate phage 0252. The difference in the sensitivity patterns of phage lb539 observed between the CNRZ 326 and CRL 934 strains was not further investigated, although the difference cannot be correlated with the presence of a plasmid because both cells are free of plasmids (data not shown). Among the *L. delbrueckii* ssp. *bulgaricus* strains tested as potential lb539 indicators, only the mv4 prophage-cured derivative, CNRZ1004, was found to be sensitive. Therefore, resistance of LT4 cells, the mv4 lysogenic strain, to the lb539 attack suggests either that both phages may possess a homoimmune system to superinfection or that phenotypic expression of the lysogenic conversion phenomenon occurred as described for LT4 cells (7).

ACKNOWLEDGMENTS

This work was supported by a grant from the Consejo de Investigaciones de la Universidad Nacional de Tucuman (CIUNT; Argentina). A special thanks to J.-P. Accolas and Patrick Tailliez for providing the strains of the CNRZ collection and reference phages. We also thank Graciela Savoy and Fernando Sesma for critical reading of the manuscript, Elena Bru for her helpful advice on statistical analysis, and Gladys Martos and María Luisa Ragout for their technical support.

REFERENCES

1 Accolas, J.-P., and H. Spillmann. 1979. Morphology of bacteriophages of *Lactobacillus bulgaricus, L. lactis* and *L. helveticus*. J. Appl. Bacteriol. 47:309.

2 Ackermann, H. W., A. Audurier, L. Berthiaume, L. A. Jones, J. A. Mayo, and A. K. Vidaver. 1978. Guidelines for bacteriophage characterization. Adv. Virus Res. 23:1.

3 Alatossava, T., and M. J. Pyhtilá. 1980. Characterization of a new *Lactobacillus lactis* bacteriophage. IRCS Med. Sci. 8:297.

4 Boyce, J. D., B. E. Davidson, and A. J. Hillier. 1995. Sequence analysis of the *Lactococcus lactis* temperate bacteriophage BK5-T and demonstration that the phage DNA has cohesive ends. Appl. Environ. Microbiol. 61:4089.
5 Bradley, D. E. 1967. Ultrastructure of bacteriophages and bacteriocins. Bacteriol. Rev. 31:230.
6 Bradley, D. E., and E. L. Rutherford. 1975. Basic characterization of a lipid containing bacteriophage specific for plasmids of the P, N, and W compatibility groups. Can. J. Microbiol. 21:152.
7 Cluzel, P.-J., J. Serio, and J.-P. Accolas. 1987. Interactions of *Lactobacillus bulgaricus* temperate bacteriophage 0448 with host strains. Appl. Environ. Microbiol. 53:1850.
8 Cluzel, P.-J., M. Veaux, M. Rousseau, and J.-P. Accolas. 1987. Evidence for temperate bacteriophages in two strains of *Lactobacillus bulgaricus*. J. Dairy Res. 54:397.
9 de Man, J. C., M. Rogosa, and M. E. Sharpe. 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. 23:130.
10 Dupont, L., B. Boizet, M. Coddeville, F. Auvray, and P. Ritzenthaler. 1995. Characterization of genetic elements required for site-specific integration of *Lactobacillus delbrueckii* subsp. *bulgaricus* bacteriophage mv4 and construction of an integration-proficient vector for *Lactobacillus plantarum*. J. Bacteriol. 177: 586.
11 Forsman, P., and T. Alatossava. 1991. Genetic characterization of *Lactobacillus delbrueckii* subsp. *lactis* bacteriophages isolated from cheese processing plants in Finland. Appl. Environ. Microbiol. 57:1805.
12 Lahbib-Mansais, Y., M. Mata, and P. Ritzenthaler. 1988. Molecular taxonomy of *Lactobacillus* phages. Biochimie 70:429.
13 Mata, M., A. Trautwetter, G. Luthaud, and P. Ritzenthaler. 1986. Thirteen virulent and temperate bacteriophages of *Lactobacillus bulgaricus* and *Lactobacillus lactis* belong to a single DNA homology group. Appl. Environ. Microbiol. 52:812.
14 Mathews, R.E.F. 1982. Classification and nomenclature of viruses. Fourth report of the International Committee on Taxonomy of viruses. Intervirology 17:1.
15 Raibaud, P., M. Caulet, and G. Mocquot. 1961. Studies in bacterial flora of the alimentary tract of pigs. II. Selective enumeration and differentiation of dominant group. J. Appl. Microbiol. 24:285.
16 Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
17 Sechaud, L., P.-J. Cluzel, M. Rousseau, A. Baumgartner, and J.-P. Accolas. 1988. Bacteriophages of lactobacilli. Biochimie 70: 401.
18 Suzuki, I., S. Kato, T. Kitada, N. Yano, and T. Morichi. 1985. Growth of *Lactobacillus bulgaricus* in milk. 1. Cell elongation and the role of formic acid in boiled milk. J. Dairy Sci. 69:311.
19 Suzuki, I., S. Kato, T. Kitada, N. Yano, and T. Morichi. 1985. Growth of *Lactobacillus bulgaricus* in milk. 2. Characteristics of purine nucleotides, pyrimidine nucleotides, and nucleic acid synthesis. J. Dairy Sci. 69:971.
20 Trautwetter, A., P. Ritzenthaler, T. Alatossava, and M. Mata. 1986. Physical and genetic characterization of the genome of *Lactobacillus lactis* bacteriophage LL-H. J. Virol. 59:551.
21 Vescovo, M., V. Bottazi, and P. A. Prestini. 1990. Different phage sensitivity in morphological variants of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB6. Ann. Microbiol. 40:197.
22 Wilkinson, L. 1990. SYSTAT: The system for statistic. SYSTAT, Inc., Evanston, IL.
23 Yamamoto, K. R., B. M. Alberts, R. Benzinger, L. Lawhorne, and G. Treiber. 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. Virology 40:737.