Incidence of Inducible Bacteriophage in *Lactobacillus helveticus* Strains Isolated from Natural Whey Starter Cultures

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ABSTRACT

We studied the incidence of inducible *Lactobacillus helveticus* strains among 74 isolates from natural whey starter cultures that are used for the production of Italian Grana and Provolone cheeses. Mitomycin C was used for induction of cultures. Of the 77 strains tested, 35 strains (47%) released supernatant particles in the culture that were able to inhibit up to a maximum of 47 (61%) sensitive indicator strains. The presence of bacteriophages in culture supernatants of *L. helveticus* strains LH38 and LH60 was verified by electron microscopy. None of the positive culture supernatants formed plaques, and inhibition zones were observed only on agar plates that had been inoculated with the indicator strains. Fourteen of the 17 positive culture supernatants were able to propagate on some indicator strains, indicating the presence of completely infective virions in liquid medium. The particles were insensitive to proteolytic enzymes, were unable to pass through a microfiltration membrane, were sensitive to heat, and were sedimented by ultracentrifugation. Some of these particles showed killer activity against nonproliferating cells of indicator strains. The lysogenic state of the *L. helveticus* strains was related to a stronger resistance of these strains to the products of the induction; higher prevalence of indicator strains were found among the noninducible strains.
(**Key words**: *Lactobacillus helveticus*, lysogeny, bacteriophage, starter cultures)

Abbreviation key: $\mathbf{A_{650}}$ = absorbance 650 nm, **HC** = host strain culture, **ILC** = Istituto Lattiero Caseario, **INRA** = Institut National de la Recherche Agronomique, **MC** = mitomycin C.

INTRODUCTION

Lysogeny appears to be widespread in lactobacilli, and several studies on mesophilic and thermophilic lactobacilli, using mitomycin C (**MC**) as the induction agent, have demonstrated high frequencies of lysogenic strains. Inducible strains ranged from 10 to 80% (18, 22, 23), but complete temperate phages were present less frequently. All other active lysates from the strains that had been treated with MC contained defective phages or "killer" particles that were unable to propagate on suitable indicators (1, 4, 5, 9, 15, 22).

The incidence of lysogeny in lactobacilli, the DNA homology between some temperate and virulent phages, the transition from the temperate state to virulence, and the possible spontaneous induction indicate that lysogenic strains of lactobacilli are a potential source of virulent phages in dairy factories (4, 10, 11, 14, 15, 17). Phages from the lysogenic strains and from the environment can cause changes ranging from small fluctuations to a complete failure in acid production by the culture, depending on the type of starters used. For undefined starter cultures, only minor effects on the ability of these cultures to produce acid are normally observed (2). An example of undefined cultures is the natural whey starter cultures (also called artisan cultures) that are commonly used to manufacture many Italian hard and semi-hard cheeses (12).

The importance of phage infection during these traditional cheese-making technologies has not been studied. Recently, *Lactobacillus helveticus* phages have been found in natural whey starter cultures used for Grana cheese production that had previously exhibited acidification problems (7). Temperate and virulent phages of *L. helveticus* have also been studied in detail by Séchaud et al. (16), who found that 37% of the strains were inducible by MC and, among them, 57% harbored temperate phages that were active on indicator strains.

The objectives of this study were to attempt to determine the presence and function of lysogenic

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strains of *L. helveticus* in natural whey starter cultures.

MATERIALS AND METHODS

Bacteria, Bacteriophages, and Culture Conditions

Seventy-seven strains of *L. helveticus* were used, including 74 strains from the collection at **ILC** (Istituto Sperimentale Lattiero Caseario, Lodi, Italy); 32 were isolated from natural whey starter cultures used to manufacture Grana cheese, and 42 were isolated from natural whey starter cultures used in the production of Provolone cheese. Two strains (CNRZ 493 and CNRZ 892) came from the collection of the Station de Recherches Laitières of the Institut National de la Recherche Agronomique (**INRA**, Jouy-en-Josas, France). One strain, ATCC 15807, was obtained from the American Type Culture Collection (Rockville, MD). The temperate phages, designated by their corresponding parental strains, were isolated as described herein.

Bacteria and phages were propagated at 42°C in MRS medium (Biokar, Beauvais, France) that had been supplemented with 10 m*M* $CaCl_2$. The MRS agar (1.2% wt/vol) and soft agar (0.5% wt/vol) were used for the conventional double-layer plate test. Bacterial stocks were maintained at –80°C in MRS broth plus 15% glycerol (vol/vol). Phage stocks were obtained by the filtration of the induced broth cultures (Nalgene syringe filters, 0.45-μm pore size; Nalge Co., Rochester, NY) and stored at 4°C.

Strain Identification

The ILC strains used in this study had been previously classified by classic phenotypic tests and were confirmed as *L. helveticus* by DNA-DNA hybridization with the fragment *f*, a specific DNA probe for *L. helveticus* (6) (provided by P. Tailliez, INRA, Jouy-en-Josas, France). The probe was obtained by *Bss*HII-digested pBluescript plasmid (Stratagene, La Jolla, CA) that was cloned in *Escherichia coli* CNRZ 1814 (Station de Recherches Laitières, INRA) as described by Tailliez et al. (20). Dot blot hybridization between the total DNA that was extracted from bacterial strains and fragment *f* as a probe was performed with the enhanced chemiluminescence (ECL)-direct nucleic acid labeling and detection systems (Amersham Italia, Milan, Italy) were used according to the instructions of the supplier. Total DNA extraction, probe preparation, and dot blot hybridization techniques were performed as described by de los Reyes-Gavilán et al. (6).

Induction of Temperate Phages

Lysogenic strains of *L. helveticus* were carried out according to the methods of Séchaud et al. (16). After overnight growth, 30 μl of the cultures were inoculated in 3 ml of fresh MRS broth that had been supplemented with $CaCl_2$ and incubated at 42°C in a Multicell Transport unit (model HP 89075C), equipped with a six-sample holder with a thermostat and a water-driven magnetic stirrer (Hewlett Packard, Palo Alto, CA) and connected to the diode-array spectrophotometer (model HP 8452A). Every 30 min, absorbance at 650 nm (**A_{650}**) was measured automatically. At the beginning of the exponential growth phase (A_{650} between 0.15 and 0.20), MC was added to a final concentration of 0.2 μg/ml, and incubation was continued overnight. For each strain, a control culture without MC was also prepared.

The induction of a strain was detected either when a steady decrease occurred in the A_{650} of cultures treated with MC or when a drop of overnight MC culture supernatants (filter-sterilized and neutralized at pH 6.8 with 1 *M* NaOH) produced inhibition zones on double-layer MRS plates (19). All strains were also used as potential indicator strains, and the plates, each of which was inoculated with a single indicator strain, were incubated at 42°C for 24 h. The sensitivity of each indicator strain was also evaluated with respect to the dose of MC used for induction tests. The same quantity of fresh medium containing MC alone was added to agar or broth cultures as controls. When zones of inhibition occurred (positive samples), the presence and the activity (functionality) of the bacteriophage was further investigated; serial dilutions of the supernatants were plated to identify lysis plaques, or lysis was tested in MRS broth (inoculum 1%, vol/vol, of the indicator strain and 3%, vol/vol, of MC culture supernatant). If lysis or growth inhibition was observed, the lysate of the host strain culture (**HC**) was tested again in MRS broth on the same sensitive indicator strain. For each strain tested, a control without HC lysate was also incubated. The turbidity of each culture was regularly compared with that of the control by visual examination. Once growth of the control became clearly visible, both cultures were simultaneously transferred to fresh MRS broth. If no lysis occurred in the tube, three sequential subcultures were systematically done to detect a possible delayed lysis.

Characterization of Inhibitory Agents in Positive MC Culture Supernatants

Sensitivity to proteolytic enzymes. Positive MC culture supernatants were treated with 1 mg/ml of pronase E, proteinase K, ficin, trypsin, and α-chymotrypsin (Sigma Chemical Co., St. Louis, MO). The enzymes were prepared in sterile potassium phosphate buffer (10 m*M*, pH 7). Samples with and without enzymes were held at 37°C for 1 h. The activity remaining in both samples was detected by the double-layer plate diffusion method, as described, using highly sensitive strain LH4 as the indicator strain. Controls included buffer with and without the enzymes.

Sensitivity to heat. After heat treatment at 100°C for 10 min, the residual activity of positive MC culture supernatants was tested by the agar double-layer technique.

Passive microfiltration. Positive MC culture supernatants (20 μl) were placed on a membrane filter of 0.025-μm pore size (Millipore VSWP01300, Millipore Co., Bedford, MA) that was floating on sterile distilled water (10 ml). After 1 h at 20°C, the activity remaining in treated and untreated supernatants was tested as described.

Ultracentrifugation. Positive MC culture supernatants (3 ml) were ultracentrifuged at 100,000 $\times$ *g* for 20 min at 4°C (Centrikon T2100; Kontron Instruments SpA, Milan, Italy). A drop of the pellets, that had been previously resuspended in 30 μl of TMG (10 m*M* Tris·HCl, 10 m*M* $MgSO_4$, and 1% gelatin, pH 7.4), was then assayed for the presence of inhibitory activity by the double-layer method.

Electron microscopy. The pellets from the LH38 and LH60 MC culture supernatants, obtained by ultracentrifugation as previously described, were negatively stained using a solution of 1% phosphotungstic acid. Pellets were adsorbed for 1 min to copper grids coated with Formvar 15/95E (Sigma Chemical Co.), excess liquid was wiped off, and the grids were immersed into the stain for 1 min. Stained grids were examined by electron microscope (Philips EM300; Philips, Eindhoven, The Netherlands).

Determination of killing activity. The presence of killing activity in 7 MC culture supernatants was evaluated as described by Tohyama (21). Nine strains of *L. helveticus* with different patterns of sensitivity were chosen as indicators among inducible strains and strains that were not inducible. Strains were grown in MRS broth for 6 h at 42°C, and then cells were harvested, washed, and resuspended in phosphate buffer (0.1 *M*, pH 6.8). These cells were treated with the MC culture supernatants (3% vol/vol) and incubated at 42°C for 60 min. Then, the viable cells were counted on MRS agar. All plating was done in duplicate. The killing activity was determined by comparing the number of cells that survived in treated and control samples. Data are expressed as means ($\log_{10}$ colony-forming units per milliliter) plus or minus standard errors of two replicates. Student's *t* test was applied for comparison of the mean values.

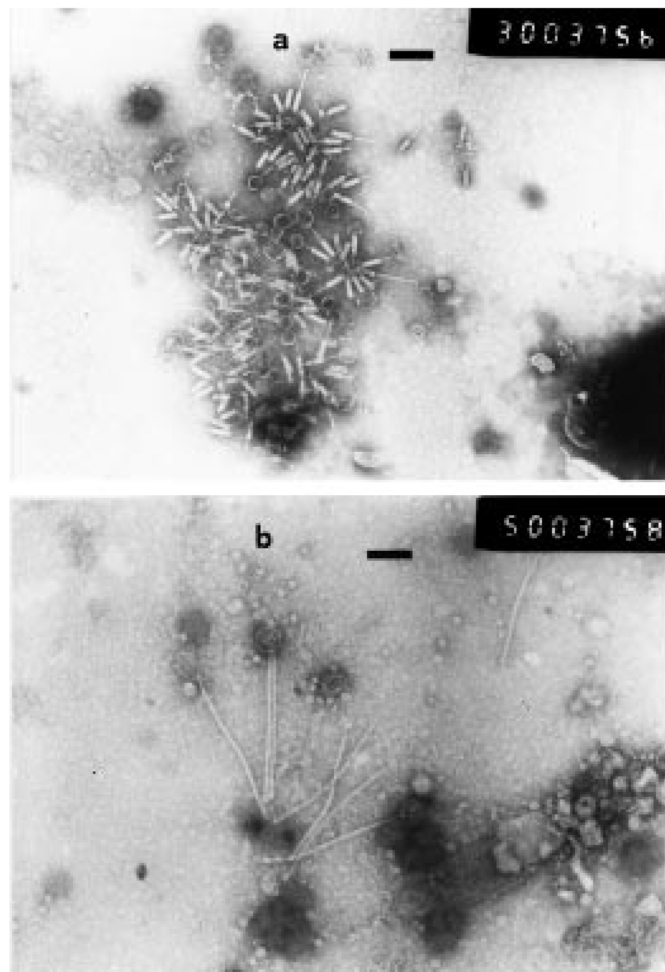

Figure 1. Electron micrographs of inducible bacteriophages of *Lactobacillus helveticus* strains LH38 and LH60. Phages Φlh38 (a) and Φlh60 (b) were shown at different magnifications: bar on part a = 160 nm, and bar on part b = 100 nm.

RESULTS AND DISCUSSION

After treatment of the 74 ILC *L. helveticus* strains with MC, only 2 strains, LH38 and LH60, showed lysis of the cultures. The presence of phages in these lysates was confirmed by electron microscopy. Phage particles had hexagonal isometric heads and basal plates, but differed in the type of tail. Phages from strain LH38 (Figure 1a) showed contractile tails, and phages from strain LH60 (Figure 1b) had long non-

contractile tails. These 2 phages appeared to be defective because of the presence of empty heads and tail-like structures that were separated from the heads.

Each of the 74 supernatants from overnight MC cultures were tested using the double-layer technique against the 74 ILC strains and the 3 reference strains (CNRZ 493, CNRZ 892, and ATCC 15807), all of which were used as indicators. Supernatants from 35 of the 74 (47%) treated strains gave rise to inhibition zones on the agar plates; variation ranged from a minimum of 9 (12%) to a maximum of 47 (61%) sensitive strains (Table 1). Control samples (fresh MRS broth added with MC) never produced inhibition zones on any of the indicator strains.

TABLE 1. Incidence of strains of *Lactobacillus helveticus* induced by mitomycin C (MC) that were isolated from natural whey starter cultures.

Strain[1]	MC induction[2]	Activity of MC culture supernatant[3]	Strain sensitivity[4]	Strain	MC induction	Activity of MC culture supernatant	Strain sensitivity
		(no.)				(no.)	
LH1	–		1	LH43	+	27	27
LH2	–		28	LH44	–		3
LH3	–		11	LH45	+	41	8
LH4	–		34	LH46	–		5
LH5	–		17	LH47	+	43	11
LH6	+	18	29	LH48	–		1
LH7	–		11	LH49	–		12
LH8	–		19	LH50	+	28	10
LH9	–		18	LH51	+	41	1
LH10	–		32	LH52	+	21	31
LH11	–		22	LH53	+	31	6
LH12	–		13	LH54	–		26
LH13	–		20	LH58	–		0
LH14	–		4	LH59	–		0
LH15	–		13	LH60	+	25	0
LH16	–		14	LH61	+	9	0
LH17	–		19	LH62	+	33	0
LH18	–		14	LH63	+	18	0
LH19	–		30	LH64	+	23	0
LH20	–		24	LH65	+	15	0
LH21	+	36	2	LH66	+	20	0
LH22	–		19	LH67	+	20	0
LH23	–		16	LH68A	+	22	2
LH24	–		33	LH68B	+	29	0
LH25	+	34	6	LH69	+	11	0
LH26	+	35	0	LH70	–		30
LH27	–		33	LH71	+	17	0
LH28	+	18	0	LH72	+	15	0
LH29	–		33	LH73	+	24	0
LH30	–		21	LH74	+	25	0
LH31	–		14	LH75	+	17	0
LH32	–		16	LH76	+	20	0
LH33	+	16	0				
LH34	+	35	4				
LH35	+	47	0	ATCC15807	–		35
LH36	+	37	4	CNRZ892	–		34
LH37	–		5	CNRZ493	–		34
LH38	+	15	0				
LH39	–		1				
LH40	+	42	4				
LH41	–		5				
LH42	–		4				

[1]LH1 to LH32 strains were isolated from natural whey starter cultures that were used to manufacture Grana cheese. LH33 to LH76 strains were isolated from natural whey starter cultures that were used to manufacture Provolone cheese.

[2]Symbols: + = inhibition, – = lack of inhibition by supernate of the induced strain of indicator cultures on agar media.

[3]Number of strains inhibited by the positive MC culture supernatant.

[4]Number of MC culture supernatants active against the strain.

When MC culture supernatants were serially diluted and plated on the same sensitive indicators, none was able to form individual plaques, and the inhibition zones disappeared after one or two decimal dilutions. The use of liquid medium, however, showed that some indicator strains were able to support the infective propagation of some of these temperate phages. Seventeen of the 35 positive MC culture supernatants were tested in this manner using 4 of the most sensitive indicator strains (ATCC 15807, CNRZ 892, CNRZ 493, and LH29). Fourteen of these supernatants were able to propagate on 1 or 2 (ATCC 15807 and CNRZ 493) indicator strains. After lysis with MC culture supernatants, the HC lysates that were obtained from these strains maintained the ability to lyse the strains after reinfection, indicating the presence of completely infective virions (Table 2).

The induction of prophages and the presence of infective or defective viral particles, as well as the possible induction of other inhibitory compounds in the MC culture supernatants, were investigated further. After treatment of the 35 positive MC culture supernatants with the proteolytic enzymes, only 4 strains (LH33, LH61, LH69, and LH72) showed a partial sensitivity. The inhibition zones on agar plates after enzymatic action were smaller and not clear as were those obtained with the other 31 MC supernatants. This sensitivity demonstrated the possible presence of proteinaceous inhibitory substances other than the inducible phages. Furthermore, after microfiltration of all the 35 MC culture supernatants through membrane sizes that were small enough to be able to retain phage particles, only supernatants from strains LH33, LH61, LH69, LH72, and LH63 showed a partial failure of their ability to produce inhibition zones. Therefore, these inhibitory substances were sufficiently small to diffuse through the pores of the membrane. Other tests of characterization showed that the inhibitory activity of all of the MC culture supernatants was destroyed by heat treatment at 100°C and sedimented by ultracentrifugation. These results demonstrated that inhibitory activity of most of the MC culture supernatants was related to the presence of viral particles.

The inability of many MC culture supernatants to propagate on sensitive indicator strains, together with the morphology of the two representative phages observed, led us to consider these phages as defective phage or killer particles (15). Killer particles from *Lactobacillus salivarius* killed sensitive cells without forming plaques and without producing newly synthesized particles (22). The presence of killing activity toward nonproliferating cells of 9 indicator strains that were found in 7 MC culture supernatants (Table 3) was related to the indicator strains used. The number of viable cells of LH4 and LH29 decreased markedly after contact with all 7 MC culture supernatants. Strains LH23 and LH70 were less sensitive, and the remaining 5 indicator strains tested were not affected by any of the 7 MC culture supernatants (Table 3). Notably, LH38 and LH60 MC culture supernatants, when phages were observed, showed the same behavior as the other induced supernatants.

When used as indicators, most (91%) of the inducible strains (47% of all the strains studied) were resistant to the other MC culture supernatants. Conversely, 67% of the noninducible strains (53% of all the strains studied) were sensitive to the MC culture supernatants. The three reference strains (CNRZ 493, CNRZ 892, and ATCC 15807) were confirmed as not being induced by MC and as being sensitive indicators (Table 1).

For the ILC strains, the results of MC induction trials confirmed a frequent presence (84%) of noninducible strains within the group that was isolated from Grana cheese starter cultures (85% of which were highly sensitive to MC culture supernatants), and a low incidence (28.5%) within the group from

TABLE 2. Lytic activity of 17 host strain culture (HC) lysates on 4 sensitive strains.[1]

	Lysis after treatment with HC lysates[2]																
Strain	HC25	HC26	HC28	HC33	HC34	HC35	HC36	HC38	HC40	HC43	HC45	HC47	HC50	HC51	HC52	HC53	HC60
LH29	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CNRZ493	+[3]	+[4]	–	–	–	+[3]	+[4]	+[3]	+[4]	+[4]	+[3]	+[4]	–	+[4]	–	+[3]	+[3]
CNRZ892	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
ATCC15807	+[3]	+[3]	–	–	+[3]	+[3]	+[3]	+[3]	+[3]	+[3]	+[3]	+[3]	+[4]	+[3]	–	+[4]	+[3]

[1]The HC lysates obtained by the infection of the 4 sensitive strains with the respective 17 MC culture supernatants.

[2]Symbols: – = absence of lysis of the infected strain after four sequential subcultures in fresh medium, + = lysis of the infected strains.

[3]Lysis observed after two subcultures.

[4]Lysis observed after three subcultures.

TABLE 3. Killing activity of 7 mitomycin C (MC) culture supernatants on nonproliferating cells of 9 sensitive strains.[1]

Strain	Untreated cells (control)		Viable cells after treatment with MC culture supernatants													
			MC25		MC33		MC35		MC38		MC47		MC51		MC60	
	(log$_{10}$ cfu/ml)															
	$\overline{X}$	SE	$\overline{X}$	SE	$\overline{X}$	SE	$\overline{X}$	SE	$\overline{X}$	SE	$\overline{X}$	SE	$\overline{X}$	SE	$\overline{X}$	SE
LH4	6.14	0.10	4.61[b]	0.08	4.02[b]	0.09	4.14[b]	0.10	3.87[b]	0.09	3.78[b]	0.18	3.50[b]	0.20	4.31[b]	0.05
LH8	5.71	0.01	5.77	0.23	5.67	0.27	5.90	0.12	5.91	0.16	5.73	0.03	5.43	0.43	ND[2]	
LH23	6.04	0.04	4.97	0.08	5.89	0.05	5.73[a]	0.10	5.92[a]	0.01	5.75[a]	0.07	5.79[a]	0.07	5.64[b]	0.08
LH29	5.31	0.02	4.38[b]	0.08	4.19[b]	0.06	4.24[b]	0.05	4.33[b]	0.15	4.39[b]	0.06	4.48[b]	0.05	4.87[a]	0.13
LH51	4.65	0.05	4.92	0.18	4.89	0.04	5.06	0.15	5.10	0.22	4.89	0.11	4.89	0.04	5.27	0.27
LH52	4.71	0.23	5.25	0.01	5.39	0.05	5.56	0.04	5.33	0.07	5.63	0.12	5.49	0.04	5.37	0.06
LH68A	3.67	0.07	3.67	0.05	3.92	0.10	3.88	0.10	3.81	0.05	3.57	0.03	3.85	0.07	3.72	0.04
LH70	5.07	0.04	4.47[b]	0.07	5.44	0.02	5.44	0.01	5.65	0.04	5.46	0.04	5.30	0.02	5.45	0.15
CNRZ892	5.36	0.10	5.18	0.08	ND		ND		ND		ND		ND		5.41	0.14

[a]Different from the respective control ($P < 0.05$).

[b]Different from the respective control ($P < 0.01$).

[1]Killing activity was evaluated by a decrease of viable cells after treatment with MC culture supernatants for 60 min at 42°C compared with the decrease of untreated cells (control) incubated at the same conditions and with the same concentration of MC. Data are log$_{10}$ means (± SE) of two replicates.

[2]Not determined.

Provolone cheese cultures (25% of which were highly sensitive). In this study, of the 35 strains that were inducible by MC, 30 were isolated from the Provolone whey starter cultures.

The difference in sensitivity between cultures that were used to manufacture the two types of cheese could have evolved from differences in cheese manufacturing technology and in the handling of these natural starter cultures. Temperatures of cheese making and the degree of acidification of the cultures that are propagated in whey could have created differences in natural starter composition (12, 13).

In the natural whey starter cultures, the high prevalence of inducible *L. helveticus* strains (shown to have stronger resistance to the products of the induction) and of indicator strains (mainly detected among the noninducible strains) contributed the understanding of the complex phenomena that control the relationships between phage and host and the delicate equilibrium among the strains in these natural thermophilic starter cultures.

CONCLUSIONS

The role of lysogenic lactobacilli in the undefined microflora of natural whey starter cultures still needs to be resolved. Some studies (3, 4, 10, 11, 17) suggest that temperate phages may be involved in the development of the lytic phages attacking lactobacilli. These phages could perturb fermentation if the starters contain sensitive strains. The presence in the natural whey starter cultures of an unknown number of strains of thermophilic lactobacilli (mainly *L. helveticus*) probably represents a natural barrier against phage infection. The relatively stable balance of strains that are susceptible or resistant to phage presumably makes possible the replacement of phage-sensitive strains, thus avoiding strong variations of starter activity and, consequently, serious failures during cheese making (8, 12).

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