Incidence of Lysogeny in Wild Lactococcal Strains

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ABSTRACT

The incidence of lysogeny among 172 lactococcal strains, isolated from natural dairy fermentations and selected by their high capacity to coagulate milk and to produce lactic acid, was studied. Lysis of 92 strains (53% of the strains tested) was observed after using mitomycin C for induction of cultures. Fifty-one of these 92 strains released phages that were able to propagate on indicator strains. Twenty-eight (16%) of the 172 strains tested acted as host for at least one phage, although some supported the development of more than one phage isolate. Spontaneous induction was insignificant in >50% of strains inducible by mitomycin C, but titers after mitomycin C induction varied between 10^1 and 10^6 pfu/ml. No plaque formation was observed at 37°C. The bacteriophages belonged to the family Siphoviridae, as revealed by electron microscopy. Head and tail dimensions ranged widely. Some temperate phages that were unable to form plaques of lysis appeared to be defective viral particles.

(**Key words**: lysogeny, lactococci, farmhouse cheese)

Abbreviation key: **SM** = buffer for storage of bacteriophages.

INTRODUCTION

Since 1935, bacteriophages have been recognized as one of the most important single causes of inhibition of cultures of lactococci (24). Consequently, continuous efforts have been made to control phages in order to avoid failed fermentations and the consequent economic losses. The role of lysogenic strains of lactic acid bacteria as a source of lytic phages is still unclear (8) despite several reports (2, 6, 11, 18) suggesting that many lactococcal strains used as starters in cheese making are potential reservoirs of phages. Thus, lysogeny has been included as one of the criteria to be used in the selection of new lactococcal strains isolated from natural dairy fermentations for use in cheese manufacture. The objective of this study was to determine the incidence of lysogeny among wild lactococci; this information would help in formulation of suitable combinations of strains for inclusion in a multiple-strain starter.

MATERIALS AND METHODS

Cultures and Culture Maintenance

Lactococcal strains were isolated by standard methods (7) from raw milk and from several batches of a farmhouse cheese throughout ripening. The identification of the strains was carried out according to the methods of Sharpe (21). Routinely, lactococci were propagated in M17 broth or agar (Biokar, Beauvais, France) at 30°C without agitation. Strains were stored frozen at –80°C in M17 broth with 50% glycerol.

Culture Activity

The acid-producing ability of strains was tested by the two methods described herein. Each strain was inoculated (1%) into sterile reconstituted 11% (wt/vol) NDM and incubated at 21°C for 18 h, at which time the formation of a coagulum was verified. Simultaneously, each strain was inoculated (3%) into sterile reconstituted NDM and incubated at 30°C for 6 h. The percentage of lactic acid was measured by titration to pH 8.2 with .1*N* NaOH.

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Strains that did not produce a coagulum in 18 h or that reached acidities of <.4% were considered to be unsuitable for cheese making.

Evaluation of Prophage Induction

Strains that passed the activity tests were used to determine their lysogenic status by measurement of the rate of spontaneous induction or induction mediated by mitomycin C. Overnight cultures were inoculated at 2% into 20 ml of M17 broth and incubated at 30°C until turbidity (absorbance at 600 nm) reached .1. Cultures were then divided into two 10-ml aliquots, and mitomycin C was added to one at a final concentration of .5 μg/ml. Incubation proceeded for 3 h, and the turbidity was continuously monitored in a spectrophotometer (Uvikon 930; Kontron Instruments SpA, Milan, Italy). Both tubes were centrifuged at 5000 rpm for 10 min, and the supernatants were filter-sterilized through a .45-μm nitrocellulose membrane (WCN type; Whatman, Kent, England) before being tested for the presence of phage by the double-layer method (23) using each strain as a potential indicator. When zones of lysis appeared on the indicator lawns, dilutions of the corresponding supernatants were plated to obtain individual plaques from which phages were isolated and propagated at least three times. Finally, the phages were resuspended in M17 broth, filtered, and stored at 4°C.

High Titer Bacteriophage Propagation

When indicator strains were available, phages were propagated on M17 agar supplemented with 10 m*M* $CaCl_2$ to obtain semiconfluent lysis (ca. 10^3 phage particles per plate). After overnight incubation at 30°C, the upper layer of each plate was gently scraped, submerged in 5-ml of buffer for storage of bacteriophages [(SM); 20 m*M* Tris·HCl, 100 m*M* NaCl, 10 m*M* $MgSO_4$, 10 m*M* $Ca(NO_3)_2$; pH 7.25] for 2 h (20), and then centrifuged at 5000 rpm for 10 min. The pellet was resuspended in SM again, and the procedure was repeated. Both supernatants were pooled, filter-sterilized, and ultracentrifuged at 35,000 rpm for 2.5 h at 8°C. The pelleted phages were resuspended in .2 ml of SM buffer, treated with RNAse A (40 μg/ml; Boehringer Mannheim, Mannheim, Germany) for 1 h at 30°C, pelleted, resuspended in SM, and stored at 4°C.

The isolation of phages for which no indicator strains were found was undertaken from 500-ml cultures induced with mitomycin C as indicated. The lysed cultures were centrifuged at 10,000 rpm for 10 min at 4°C, and their supernatants were filter-sterilized. Then NaCl was added to a final concentration of 1 *M* and kept on ice for 1 h. Debris was removed by centrifugation, and polyethylene glycol 6000 was added to the supernatants to a final concentration of 10% wt/vol. The dissolved mixture was kept overnight at 4°C to allow phage particles to precipitate. Phages were recovered by centrifugation at 10,000 rpm for 15 min at 4°C and resuspended in 8 ml of SM. The polyethylene glycol supernatant was extracted with an equal volume of chloroform, and the aqueous phase containing the virions was ultracentrifuged, treated with RNAse, and stored as described.

The methods of lactococcal phage propagation have been adapted from those described for bacteriophage λ (20).

Electron Microscopy

A solution of 2% phosphotungstic acid and 2% ammonium molybdate, pH 5.0, was used for negative staining of phage particles. Concentrated phage suspensions were adsorbed for 1 min to copper grids coated with Formvar 15/95E (Sigma Chemical Co., St. Louis, MO), excess of liquid was wiped out, and the grids were immersed into the stain for 1 min. Phages were examined under an electron microscope (Jeol 2000 EX-II; Japan Electronics and Optics Laboratory, Tokyo, Japan).

RESULTS AND DISCUSSION

Afuega'l Pitu cheese is a popular dairy product manufactured in farmhouses from whole raw bovine milk in Asturias, northern Spain. Although for years this cheese has been consumed after a short ripening period (10 to 15 d), health authorities have recently become concerned with the potential public health risk and have advised the use of pasteurized milk for cheese manufacturing. For this reason, a multiple-strain starter is in development for this type of cheese.

One of the main features that could compromise the usefulness of a starter is phage development. Previous studies indicate that lysogeny is common among lactococcal strains from defined (4, 6, 14, 18) and mixed (16, 17) starter cultures in addition to those isolated from raw milk (5). Induction of resident prophages in these strains can harm other strains present in the starter and result in failed fermentations. Consequently, the knowledge of the lysogenic status of our strains was essential before a new and proficient starter could be developed to be used in manufacture of Afuega'l Pitu cheese.

The investigation was carried out on 172 lactococcal strains isolated from natural sources (milk, curd, and cheese throughout ripening). All of these strains passed the activity test and were able to coagulate milk within 18 h at 21°C and to produce >.4% of lactic acid. Of the strains tested, 158 were *Lactococcus lactis* ssp. *lactis*, and the remaining 14 strains were *L. lactis* ssp. *lactis* biovar. *diacetylactis*. No *L. lactis* ssp. *cremoris* were detected using this traditional identification method, possibly because this subspecies is present in very small numbers and its intrinsic sensitivity results in loss of viability (19). All of the strains were considered to be potential lysogens and indicators.

The induction of lysogenic strains was achieved with mitomycin C at a final concentration of .5 μg/ml because these conditions hardly affected the bacterial growth rate and because higher amounts did not increase the number of strains induced. The mitomycin C treatment induced different degrees of lysis in 92 strains, 53% of those tested, according to the optical density reduction of the cultures (Figure 1). No lytic effect was observed in the cultures of the remaining 80 strains after treatment with mitomycin C (Figure 2). The beginning of lysis varied, depending on the strain, between 60 and 120 min after mitomycin C

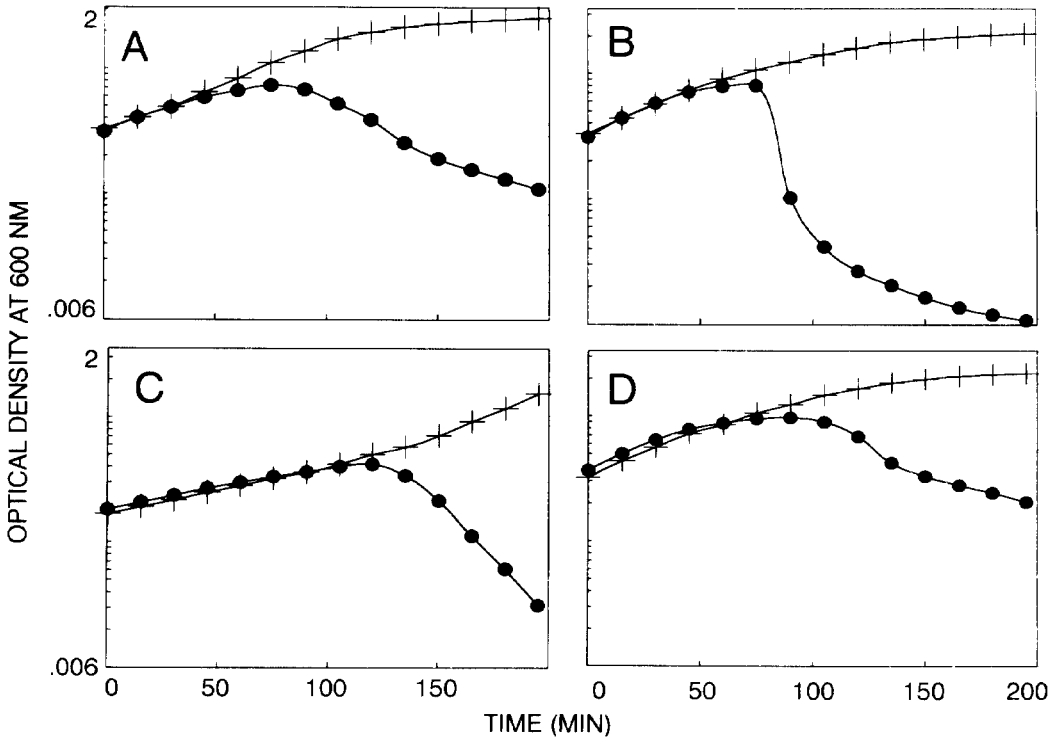

Figure 1. Optical density changes shown by cultures of representative lysogenic strains of wild lactococci after treatment with mitomycin C (●) or controls (+) for *Lactococcus lactis* ssp. *lactis* IPLA 628 (A), *L. lactis* ssp. *lactis* IPLA

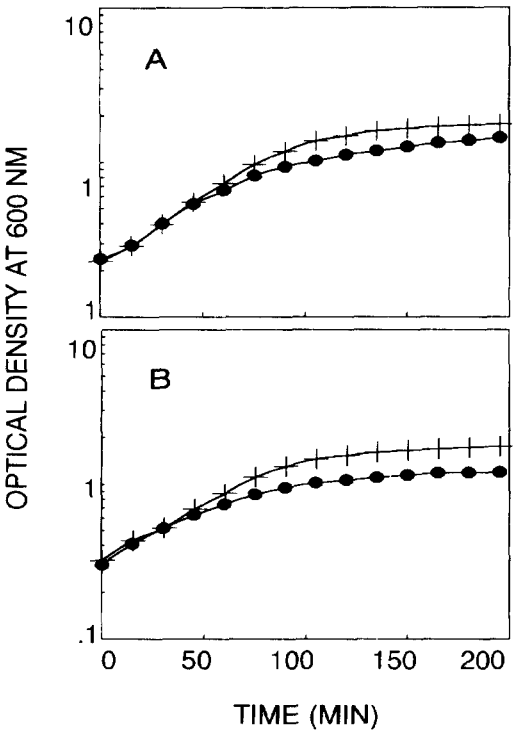

Figure 2. Optical density changes shown by cultures of representative nonlysogenic strains of wild lactococci after treatment with mitomycin C (•) or controls (+) for *Lactococcus lactis* ssp. *lactis* IPLA 785 (A) and *L. lactis* ssp. *lactis* IPLA 1029 (B).

addition. This result is similar to the 52 and the 60% induction obtained by Park and McKay (16) and Huggins and Sandine (6), respectively, after UV or mitomycin C treatment of starter strains. However, lower percentages of induction also have been reported: 37% indicated by Heap et al. (5) and 20% by Davies and Gasson (3).

The lysates originating from the 92 induced cultures were tested for plaque-forming ability on lawns of the 172 strains studied. Only 51 of the lysates were able to form plaques of lysis on some of the indicator strains tested (Table 1). This value indicates that ca. 30% of the strains tested liberated viable phages. This percentage is clearly higher than the range of 8 to 11% reported (6, 10, 23). Nevertheless, this percentage is remarkably lower than the 43% detected by Reyrolle et al. (18). As expected, none of the inducible strains were sensitive to phages in their supernatants. Of the 172 strains isolated in this work, only 28 strains were sensitive to phage infection, of which 20 were lysogenic, which indicates that phages belonged to several incompatibility groups. The frequency of indicator strains, 16%, is higher than those described by others (3, 5, 13, 23) except for the 25% obtained by Reyrolle et al. (18). The best indicator was the strain IPLA 1066, which was sensitive to 18 different phages.

Quantitative measurement of phage production, when a sensitive host was available, indicated the following: 1) in >50% of the strains inducible by mitomycin C, no spontaneous induction was observed, although values were as high as 10^4 pfu/ml in the other inducible strains; 2) the titers varied from about 10^1 to 10^6 pfu/ml after induction by mitomycin C, depending on the strain tested; and 3) the titers varied up to one log unit for a single supernatant, depending on the host used for propagation (Table 1). Finally, plaques of lysis were detected at 30°C (the standard temperature of incubation), 25°C, and 21°C, but not at 37°C, although their hosts showed active growth even at this last temperature. Temperature sensitivity has been detected before in phages infecting lactic acid bacteria (15, 22), which could be one reason why some starter strains are less susceptible than others to phage attack during dairy fermentations (12).

The dimensions of the phages obtained after induction of the lysogenic cultures are shown in Table 2. All phages showed isometric heads and noncontractile tails, corresponding to the morphological type B1 of Ackermann et al. (1), and were consequently classified into the family Siphoviridae. However, head and tail dimensions ranged widely. According to the head size, four groups of phages were established (Table 2). The first one (I), with a head diameter of about 13 to 14 nm, showed very different tail lengths: phage 1026 had a tail of 60 to 66 nm, and phage 732 had a tail of 29 to 30 nm; these phages are representatives of this group. The second group (II) had a head size of 17 to 18 nm. Representatives of this group were phage 664, with a tail of 37 to 40 nm, and phage 552, with a longer tail of 62 to 68 nm. The third group (III), represented by phage 1066, had larger dimensions: head diameter ranged from 43 to 50 nm, and a tail length was 128 to 134 nm, ended by a baseplate with at

TABLE 1. Groups of temperate phages according to the number of indicator strains on which they were able to propagate and according to the phage titers obtained by spontaneous and mitomycin C induction of their lysogenic hosts.

Group	Phage isolates	Indicators	Induction	
			Spontaneous	Mitomycin C
	(no.)	(no.)	(pfu/ml)[1]	
1	12	1	<1	$4.0 \times 10^1 - 3.8 \times 10^4$
2	3	1	$1.2 \times 10^1 - 3.2 \times 10^2$	$4.0 \times 10^3 - 6.9 \times 10^6$
3	3	1	$3.9 \times 10^3 - 2.1 \times 10^4$	$2.2 \times 10^4 - 2.3 \times 10^6$
4	6	2	<1	$6.4 \times 10^2 - 3.2 \times 10^4$
5	3	2	$1.2 \times 10^1 - 3.6 \times 10^2$	$6.8 \times 10^3 - 6.0 \times 10^5$
6	2	2	$1.3 \times 10^3 - 2.1 \times 10^4$	$1.0 \times 10^6 - 3.7 \times 10^6$
7	9	3	<1	$2.1 \times 10^2 - 6.8 \times 10^5$
8	4	3	$1.2 \times 10^2 - 7.3 \times 10^3$	$4.6 \times 10^4 - 1.0 \times 10^6$
9	4	4	<1	$2.9 \times 10^4 - 4.2 \times 10^5$
10	5	4	$2.1 \times 10^1 - 1.2 \times 10^3$	$1.6 \times 10^4 - 7.8 \times 10^5$

[1]pfu = Plaque-forming units.

least three tail pins. Phage 1009, representative of the fourth group (IV), had a head of 58 to 60 nm and a tail between 160 to 165 nm. Different studies on lactococcal bacteriophages, reviewed by Jarvis (8) and Jarvis et al. (9), indicated that most phages described herein have a head diameter ranging from 52 to 55 nm and a tail length from 109 to 159 nm. Thus, the phages included in groups III and IV of this study have these dimensions, but phages of groups I and II are clearly smaller.

Finally, the visualization of the supernatants of lysed cultures that did not form plaques of lysis on any of the indicators revealed the presence of viral particles, although they generally appeared to be defective, as for phages 504 and 728, which showed empty heads and particles without DNA, respectively. An extreme situation was that of the supernatants of the induced *L. lactis* ssp. *lactis* IPLA 659 cultures, which showed exclusively tail-like structures with cross striations ended by fibers. Thus, the apparent lack of sensitive indicators for these phages could be partially due to the presence of defective virions.

CONCLUSIONS

These results corroborate the high incidence of lysogeny among lactococci, independent of their origin. Consequently, it is necessary to verify the lysogenic status of any strain before its inclusion in a starter, and the sensitivity and resistance of the others, for mixed starters, with respect to the virions derived from resident prophages.

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TABLE 2. Dimensions of representative phages induced from wild lactococcal strains.

Group	Dimensions		Phages
	Head	Tail length	
	(nm)[1]		
I	13–14	29–30	732
		60–66	1026
II	17–18	37–40	664
		62–68	552
III	43–50	128–134	1066
IV	58–60	160–165	1009

[1]At least 25 particles were measured for each phage.

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