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Restriction/Modification Systems and Restriction Endonucleases Are More Effective on Lactococcal Bacteriophages That Have Emerged Recently in the Dairy Industry†

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Recently, eight lytic small isometric-headed bacteriophages were isolated from cheese-manufacturing plants throughout North America. The eight phages were different, but all propagated on one strain, *Lactococcus lactis* NCK203. On the basis of DNA homology, they were classified in the P335 species. Digestion of their genomes in vitro with restriction enzymes resulted in an unusually high number of type II endonuclease sites compared with the more common lytic phages of the 936 (small isometric-headed) and c2 (prolate-headed) species. In vivo, the P335 phages were more sensitive to four distinct lactococcal restriction and modification (R/M) systems than phages belonging to the 936 and c2 species. A significant correlation was found between the number of restriction sites for endonucleases (purified from other bacterial genera) and the relative susceptibility of phages to lactococcal R/M systems. Comparisons among these three phage species indicate that the P335 species may have emerged most recently in the dairy industry.

The first report on cheese starter infection by bacteriophages was recorded over 55 years ago (49). These infections still occur today and are the major cause of starter culture failure in mesophilic milk fermentations (1, 11). Naturally occurring phage-insensitive lactococcal strains have been isolated and found to harbor multiple defense systems encoded by plasmids (27, 28). These defense mechanisms interfere with phage adsorption (9, 46), restrict incoming phage DNA (3, 41), and provoke the abortion of the phage infection (10, 18). The conjugative abilities of many of these plasmids have been exploited to construct lactococcal phage-insensitive strains (23, 42). After considerable industrial use of these strains, new phages have appeared which can overcome the innate and acquired bacterial defense mechanisms (1, 7).

Because of their extensive use in milk fermentation, the lactococci represent the most dynamic bacterial species for investigating phage-host interactions and studying cycles of bacterial defense and phage counterdefense (8, 29, 31). Phages can develop counterdefenses which provide escape from bacterial resistance mechanisms (30, 31). Point mutations and module exchanges in the phage genome can account for the genetic diversity and adaptability of lactococcal phages (16, 20). Lactococcal phages characterized at the DNA level have also shown a paucity of restriction sites for many endonucleases purified from other bacterial genera (4, 6, 24, 26, 35, 37–39, 45). Although many R/M systems have been identified in lactococci (for a review, see reference 5), only two type II restriction enzymes have been isolated and characterized thus far (12, 34), and these have nearly identical recognition sites (*Scr*F1, CCNGG where N is any nucleotide, *Lla*1, CCWGG where W is A or T). Presumably, elimination of restriction sites is an evolutionary response of the phages to pressures from their host restriction enzymes, which are likely isoschizomers to those of other genera (30, 43).

Lactococcal phages have been classified into 12 different species on the basis of their DNA relatedness (22). Strong DNA homology is observed among members of the same species, but little to no homology occurs between species (22). Phage species 936 (small isometric headed) and c2 (prolate headed) are by far the most troublesome lactococcal lytic phages worldwide (4, 35, 37, 39). However, a group of phages belonging to the lactococcal phage species P335 has recently emerged in some cheese plants across North America (1, 7, 35). Most of them were isolated following the introduction of pTR2030 transconjugants into the dairy industry in 1985. The emergence of these industrial phages may represent unique biological material for investigating phage evolution.

In this study, genomic characterization of North American industrial phages has shown an unusually high number of restriction endonuclease sites, and this correlated with increased sensitivity of the phages to lactococcal R/M systems in vivo. Phage DNAs refractory to the action of the same enzymes were less affected by the R/M systems. These observations prompt us to speculate that the P335 lactococcal phage species constitutes a newly emerging group relative to the lytic species that have long been known to disrupt milk fermentation.

MATERIALS AND METHODS

Bacterial strains, plasmids, phages, and media. Bacterial strains, plasmids, and bacteriophages used in this study are shown in Table 1. Strains were propagated at 30°C in M17 broth (48) supplemented with 0.5% glucose. When required for plasmid selection or maintenance, erythromycin was

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TABLE 1. Bacterial strains, plasmids, and bacteriophages

Bacteria, plasmids, phages	Relevant characteristics	Source[a]
L. lactis		
LM0230	Lac$^-$, R/M$^-$, plasmid-free, host for ϕ c2, p2, jj50, sk1	L. L. McKay
NCK198	LM0230 (pTRK98), R/M$^+$	13
NCK302	LM0230 (pTRK302), R/M$^+$	47
NCK308	LM0230 (pTRK308), R/M$^+$	47
NCK317	LM0230 (pTRK317), R/M$^+$	47
NCK202	Lac$^-$, multiple plasmids including pTRK68 (R/M$^+$)	17
NCK203	Lac$^-$, R/M$^-$ derivative of NCK202, host for ϕ 31, 48, 50, a1, b1, cs, d1, ul36	18
NCK346	NCK203 (pTRK302), R/M$^+$	47
	NCK203 (pTRK98), R/M$^+$	This study
	NCK203 (pTRK308), R/M$^+$	This study
Plasmids		
pTRK68	Resident plasmid of NCK202, R/M$^+$	17
pTRK98	pSA34::pTN20, R/M$^+$, Erm^r, 34.0 kb	13
pTRK302	pSA34::pTRK12, R/M$^+$, Erm^r, 30.5 kb	47
pTRK308	pSA34::pTRK30, R/M$^+$, Erm^r, 28.0 kb	47
pTRK317	Resident plasmid of TDM1, R/M$^+$, 17.0 kb	47
Phages		
c2	Prolate-headed phage, c2 species	14
p2, sk1	Small isometric headed, 936 species	L. L. McKay
jj50	Small isometric headed, 936 species	J. Josephsen
31, 48, 50	Small isometric headed, P335 species	1
a1, b1, cs, d1	1990 isolate from Wisconsin, small isometric headed, P335 species	7
ul36	1990 isolate from Québec, Canada, small isometric headed, P335 species	35

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added at 5 μg/ml. Bacteriophages were propagated as described previously (35).

Phage assays. Phage adsorption and efficiency of plaquing (EOP) assays were conducted as described by Sanders and Klaenhammer (40).

Electroporation procedure. Electroporation was conducted as described by Holo and Nes (19). Electrocompetent *Lactococcus lactis* subsp. *lactis* NCK203 cells were grown in GM17 supplemented with 0.5 M sucrose and 1% glycine. The Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) was set at 25 μF and 2.45 kV, and the Pulse Controller was set at 200 Ω. Isolation of lactococcal plasmid DNA was performed as described by Anderson and McKay (2).

Thermal denaturation and G+C ratio of phage DNA. Phage DNA was isolated as described previously (36). Phage DNA thermal denaturation was performed with a Shimadzu UV-260 spectrophotometer (Shimadzu, Kyoto, Japan) connected to an Exacal EX-100DD circulating bath. The DNA samples were adjusted to an A_{260} of between 0.3 and 1.0 with 0.1× SSC (15 mM NaCl, 1.5 mM Na_2 citrate, pH 7.0). DNA samples and a reference blank were heated simultaneously, and the A_{260} was monitored from 25°C to the end of the melting transition. The observed absorbance was corrected for solvent expansion during thermal melting. The relative absorbance (corrected absorbance divided by the initial absorbance at 25°C) was plotted against temperature. The phage DNA melting temperature was determined as the midpoint of the exponential increase of the relative absorbance in the thermal denaturation curve. The moles percent G+C content of phage DNA was calculated by the method of Mandel and Marmur (32).

Expected number of endonucleases recognition sites. The expected number of endonuclease sites per phage genome was determined by multiplying the genome size by the probability of each base (on the basis of G+C content) occurring in the endonuclease recognition sites.

Restriction analysis and genome size. Restriction enzymes were used in accordance with the manufacturer's instructions (Boehringer Mannheim, Biochemicals Indianapolis, Ind.). DNA fragments were analyzed as described by Maniatis et al. (33) in 0.7 or 1.0% agarose gels in TAE. The genome size was estimated from the average of the sum of the DNA fragment lengths generated by five enzymes.

RESULTS

Phages. Members of the 936 species are very common in cheese wheys (4, 35, 37, 39). The small isometric-headed phages p2, sk1, and jj50 are members of this species, and they show a high degree of DNA homology to each other (data not shown). Phages p2 and sk1 have been used in many previous studies as reference phages (14, 18, 47). The prolate-headed phage c2 is a member of the c2 species, and it has also been used in numerous studies evaluating phage resistance mechanisms (14, 18, 40, 47). There is no DNA homology between phage c2 and the three small isometric-headed phages. These four phages propagate on *L. lactis* LM0230, but not on *L. lactis* NCK203.

Phage 31 is also a small isometric-headed phage, but it does not share DNA homology with phage p2, sk1, or jj50 (data not shown). On the basis of DNA hybridization studies, it is classified as a member of the P335 species (1, 7). Phages 48, 50, a1, b1, cs, and d1 are also members of this species, and each has been isolated since 1989 from different U.S. cheese plants (1, 7; unpublished data). Their genomic organization suggests that all, except phage 50, are derivatives of phage 31, isolated from a cheese plant prior to 1985 (1, 7). The other phage used in this study, ul36, was isolated

TABLE 2. Adsorption of lactococcal phages

Phage	Adsorption (%)	
	L. lactis NCK203	*L. lactis* LM0230
p2	0	83.9
sk1	0	98.5
jj50	0	91.2
c2	0	95.0
31	87.1	0
ul36	99.1	0
48	92.5	0
50	68.6	0
a1	98.6	0
b1	89.8	0

in 1990, but from a Canadian cheese plant (35). All of these phages are distinct strains, but they share DNA homology with each other and with the P335 type phage (data not shown). Except for phage 31 and ul36, these phages can overcome the defense mechanisms coded by pTR2030 (1, 7). Finally, they all propagate on NCK203, but not on LM0230.

Adsorption. The adsorption of 10 phages was determined on the two lactococcal hosts used in this study (Table 2). Phages p2, sk1, jj50, and c2 adsorb to LM0230 very efficiently but do not adsorb to NCK203. The reverse situation occurred with the P335 phages in that they adsorbed strongly to NCK203 but not to LM0230. Phage 50 adsorbed least efficiency of all phages examined. It also appears somewhat unstable, and titers can drop upon storage of phage lysates (1).

G+C content. The 12 phages used in this study were characterized at the DNA level, and the number of fragments was determined following digestion with endonucleases (Table 3). To determine whether these phages showed a paucity of restriction sites, we estimated the expected number of sites on the basis of the enzyme recognition site, the phage genome size, and G+C content. The genome size was determined by the sum of the DNA fragment sizes generated by cutting with each of the endonucleases shown in Table 3.

The G+C content was estimated from DNA thermal denaturation curves (Table 3). Few data are available on the G+C content of lactococcal phages. Most of the phage genomes studied so far are A+T rich. The data obtained herein are in agreement with previous results (22). The G+C content of the prolate phage c2 was estimated at 36.2 mol%. Powell and Davidson (38) have previously reported 36.7 mol% G+C content for the prolate phage c6A. Jarvis (21) reported a G+C content of 39.9 mol% for prolate phages. Recently, the lysin gene from the prolate phage vML3 was sequenced (1.2 kb), and the G+C content of this gene was 37.6 mol% (44). The G+C contents of the small isometric-headed phages p2, jj50, and sk1 (936 species) were estimated in the range of 39 to 40 mol%. These values are slightly higher than that of phage P008, estimated to be 37.5 mol% (22). However, Jarvis (21) previously has reported a G+C content of 40.7 mol% for members of the 936 species. The G+C content of the eight members of the small isometric-headed P335 species used in this study ranged from 34 to 38 mol%, suggesting more diversity in this species. To our knowledge, the G+C content of phages within the P335 species has not been published previously. However, we recently sequenced 3 kb of phage 50, and a G+C content of 35.4 mol% was determined in this region (15, 16). Therefore, it appears that the small isometric-headed phages of the 936 species have a slightly higher G+C content (39 to 40 mol%) than the small isometric-headed phages of the P335 species (34 to 38 mol%).

Observed and expected number of sites for restriction endonucleases. The small isometric-headed phages of the 936 species and the prolate-headed phage c2 showed far fewer restriction sites for *Eco*RI, *Hin*dIII, and *Eco*RV than expected on the basis of G+C content, genome size, and specific recognition sites of the endonucleases. Alternatively, the number of observed *Pvu*II sites was slightly higher than expected. This could suggest a rarity of *Pvu*II isoschizomers among the lactococci. For the lactococcal endonuclease *Scr*F1, two values were calculated for the number of restriction sites (Table 3) because of the nature of the recognition site (CCNGG, where N = any nucleotide). Since lactococcal phages are A+T rich, the first number calculated (where N = A or T) is probably more accurate. The observed number of sites for this enzyme was also significantly less than expected for the 936 and c2 phage

TABLE 3. Restriction endonuclease sites in lactococcal bacteriophage genomes

Phage	Species	Shape of head	Genome size (kb)	G+C content (mol%)[a]	No. of restriction sites detected (no. expected)[b]				
					*Eco*RI	*Hin*dIII	*Pvu*II	*Eco*RV	*Scr*F1[c]
p2	936	Isometric	30.5	40.1 ± 0.7	1 (10)	3 (10)	7 (4)	4 (10)	5 (15/10)
sk1	936	Isometric	28.1	39.7 ± 1.4	1 (9)	0 (9)	5 (4)	5 (9)	3 (13/8)
jj50	936	Isometric	30.5	39.3 ± 1.7	0 (10)	3 (9)	7 (4)	4 (10)	3 (14/9)
c2	c2	Prolate	20.7	36.2 ± 0.9	3 (7)	0 (7)	2 (5)	5 (7)	5 (7/4)
31	P335	Isometric	31.9	34.4 ± 1.7	8 (11)	13 (11)	8 (3)	7 (11)	9 (9/5)
ul36	P335	Isometric	28.8	38.1 ± 2.4	12 (10)	13 (10)	6 (4)	7 (10)	11 (12/7)
48	P335	Isometric	31.1	35.0 ± 0.8	8 (11)	12 (11)	9 (3)	13 (11)	14 (9/5)
50	P335	Isometric	29.8	37.4 ± 0.9	14 (10)	ND (10)	5 (4)	14 (10)	12 (12/7)
a1	P335	Isometric	31.8	36.4 ± 1.4	10 (11)	10 (11)	6 (4)	11 (11)	13 (11/6)
b1	P335	Isometric	30.5	36.8 ± 1.7	9 (10)	10 (10)	5 (4)	10 (10)	11 (11/6)
cs	P335	Isometric	32.2	36.8 ± 1.7	10 (11)	9 (11)	5 (4)	10 (11)	13 (12/7)
d1	P335	Isometric	32.9	36.2 ± 0.9	9 (11)	10 (11)	5 (4)	11 (11)	13 (11/6)

[a] Genome size was estimated by averaging the sum of restriction fragments for the five enzymes.
[b] Recognition site: *Eco*RI (GAATTC), *Hin*dIII (AAGCTT), *Pvu*II (CAGCTG), *Eco*RV (GATATC), *Scr*F1 (CCNGG, with N being any nucleotide). ND, not determined.
[c] Expected number of sites if N = A or T/expected number of sites if N = G or C.

TABLE 4. EOP of lactococcal phages on hosts bearing R/M systems

Phage	EOP of given plasmid host				
	pTRK302	pTRK308	pTRK68	pTRK198	pTRK317
p2	1.1×10^{-2}	5.0×10^{-3}	NT[a]	1.2×10^{-3}	3.3×10^{-1}
sk1	3.1×10^{-2}	1.5×10^{-2}	NT	1.2×10^{-3}	1.5×10^{-1}
jj50	1.0×10^{-3}	2.2×10^{-4}	NT	1.0×10^{-2}	2.0×10^{-2}
c2	3.4×10^{-2}	7.5×10^{-2}	NT	2.3×10^{-1}	3.1×10^{-1}
31	1.6×10^{-3}	5.1×10^{-5}	4.0×10^{-4}	1.9×10^{-5}	NT
ul36	3.3×10^{-3}	8.9×10^{-6}	4.0×10^{-4}	6.5×10^{-4}	NT
48	2.0×10^{-4}	3.7×10^{-6}	9.8×10^{-4}	1.0×10^{-5}	NT
50	8.0×10^{-5}	7.0×10^{-5}	4.5×10^{-3}	1.5×10^{-5}	NT
a1	2.8×10^{-4}	1.6×10^{-5}	2.3×10^{-4}	2.1×10^{-5}	NT
b1	8.8×10^{-4}	6.6×10^{-5}	8.8×10^{-4}	4.0×10^{-5}	NT

[a] NT, not tested.

species evaluated. In contrast, for all of the P335 members evaluated in this study, the number of observed restriction sites was similar to the number of sites expected. Therefore, the P335 phage genomes did not exhibit the paucity of restriction sites that was apparent in the other lactococcal phages examined in this and previous studies (4, 6, 24, 26, 35, 37–39, 45).

Effect of R/M systems. The in vivo effects of five different lactococcal R/M systems of distinct specificity on 12 phages representing three species were determined (Table 4). The prolate phage c2 was least susceptible to restriction by the different R/M systems; EOPs ranged from 10^{-1} to 10^{-3}. For the three members of the 936 species (phage p2, jj50, and sk1), the EOPs ranged from 10^{-1} to 10^{-4}, roughly the same as for phage c2. It was further noted that, although the phages within the 936 species share strong DNA homology, their responses to the different R/M systems varied considerably. For example, with pTRK308, a 2-log difference in the EOP was observed between phages sk1 and jj50. The six members of the P335 species tested had lower EOPs (10^{-3} to 10^{-6}) in four R/M systems. These phages, which have high numbers of restriction sites for endonucleases from other genera, were far more susceptible to restriction by lactococcal R/M systems than the reference phages from the 936 and c2 species. Figure 1 shows that a significant correlation exists between the number of actual restriction endonuclease sites in lactococcal phage genomes and their in vivo susceptibility to R/M systems.

DISCUSSION

Lactococcal phages have been classified recently into 12 species in which the lytic phages of the 936 (small isometric-headed) and c2 (prolate-headed) species are the most common types isolated from cheese wheys throughout the world (22). In a 1990 survey in Quebec cheese plants, 28 of 30 phage isolates belonged to these two species (35). Reports denoting isolation of phages in the P335 species are scarce (4, 22). Interestingly, this species includes both temperate and lytic phages which share DNA homology (22).

L. lactis LM0230, as well as numerous other lactococcal strains, adsorbs and replicates members of the 936 and c2 species (Table 2). However, no phages of the P335 species tested in this study could adsorb to this strain. *L. lactis* NCK203 is a Lac$^-$ derivative of the industrial strain LMA12, which has been used extensively in cheesemaking because of its resistance to phages commonly encountered in cheese-manufacturing plants (1). This industrial strain may have been successful because the most common phages do not adsorb or propagate on this host (Table 2). It was shown in this study that *L. lactis* NCK203 possesses receptors for the P335 phage species (Table 2). Despite extensive use of this host background, it is curious that the more common phage species such as 936 and c2 have not appeared for NCK203. One possible explanation could be the absence of receptors for these phage species on NCK203. Two phages of the P335 species (ul36 and ul39) we have isolated from Canadian cheese plants also limit their adsorption and propagation to one or two hosts (35), suggesting that phage receptors for the P335 species are not widely distributed among lactococcal strains. That P335 phage-specific strains are not commonly used in the industry may explain the lower occurrence of this phage species relative to the 936 and c2 species (22).

Because starter rotation systems employ many different strains, lactococcal phages (especially 936 and c2 species) have the opportunity to propagate on numerous hosts if compatible receptors are available. Many R/M systems have been identified in lactococci, and some strains harbor two or

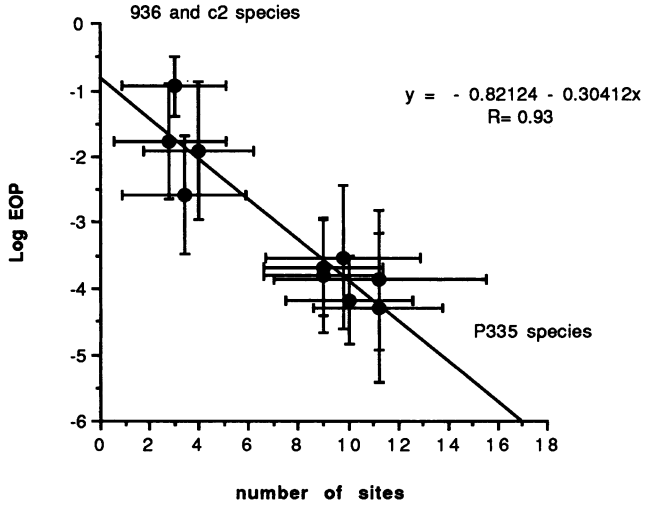

FIG. 1. Correlation between number of restriction sites per phage genome and EOP of phage on hosts harboring different R/M systems. Each point represents the average number of restriction sites for five enzymes (horizontal bar is the standard deviation) correlated with the average EOPs for the four R/M systems (vertical bar is the standard deviation). Each point represents one phage.

more R/M systems (3, 5, 40, 41, 47). Therefore, phages are continuously exposed to R/M pressure. Phages have evolved multiple mechanisms to avoid the DNA restriction systems of their hosts (for a review, see reference 30). Antirestriction mechanisms of phages include inhibition of restriction enzymes by viral proteins, self-methylation, and destruction of endonuclease cofactors. The self-methylation observed thus far in a lactococcal phage has been due to acquisition of an active portion of a bacterial modification enzyme (16).

Another possibility is the lack of recognition sites in the phage genome for a particular endonuclease (30, 37, 43). During the course of phage evolution, selective pressure will tend to eliminate, as much as possible, some DNA recognition sites for restriction enzymes encountered in replicative hosts (30, 43). Previous studies have shown that the number of restriction endonuclease sites for numerous phage genomes from other bacterial genera was significantly lower than expected (25, 30, 46). Thus, it is not surprising that lactococcal phages of the 936 and c2 species also exhibit this phenomenon (4, 6, 24, 26, 35, 37–39, 45) (Table 3). Either the DNA lacks recognition sites for many enzymes or the sequences are masked by the presence of unusual or modified bases (30). On the basis of thermal denaturation studies and sequencing, no atypical bases have been implicated in lactococcal phages (15, 16, 36, 38). To our knowledge, cloning of lactococcal phage DNA fragments and subsequent propagation in *Escherichia coli* has not created additional cleavage sites (15, 16, 38). Such propagation would liberate the phage DNA fragment of any phage-specific DNA modifications or unusual bases (38).

In this study it was shown that the DNA of eight phages from the P335 species was cut in vitro much more frequently by endonucleases than that of most lactococcal phages. Interestingly, Braun et al. (4) showed that some members of the P335 species, isolated in France and Germany, were highly refractory to the action of many endonucleases. Therefore, the high number of restriction sites reported herein may not be a general characteristic of the P335 species. Another characteristic of the P335 phages was their higher susceptibility in vivo to various lactococcal R/M systems (Table 4). The phages used in this study represent three distinct species. When the number of sites per phage genome was plotted against the average susceptibility of phage to R/M systems (Fig. 1), two distinct clusters were defined. One group represented phages from the 936 and c2 species, while the other represented the phages in the P335 species. A significant correlation (0.93) was found between the number of restriction sites and the relative sensitivity of phage to R/M systems in vivo. Phages with more restriction sites in their genome (for endonucleases from other bacterial species as well as *Scr*F1) were more susceptible to R/M systems and vice versa. This suggests that the paucity of restriction sites may represent a very important and universal lactococcal phage counterselection defense again R/M systems.

Consequently, we suggest that because of the high number of sites these eight phages have emerged recently in the dairy industry, and they may represent "young" members of the P335 species. Although their exact origin and progenitor are unknown, it is noteworthy that they share DNA homology with the temperate phage present in *L. lactis* NCK203 (7). These phages may be lytic derivatives of recent recombination events between lytic and temperate phage DNA. The diversity in G+C contents could be readily explained given the potential combinations which could occur within a species composed of both lytic and temperate phages.

Some of the phages used in this study, 50, a1, b1, cs, and d1, have already exhibited genetic recombination events with the lactococcal plasmid pTR2030 (1, 7). These phages have become problematic in cheese plants because they have naturally acquired part of the methylase gene from pTR2030, a 46.3-kb plasmid bearing an R/M system and an abortive infection mechanism (16, 28). Thus, they are capable of self-methylating and are totally resistant to restriction by hosts bearing pTR2030. They remain, however, quite susceptible to other lactococcal R/M systems (see reference 1 and Table 4). The counterdefense developed by these phages is an excellent example of the adaptive capabilities of lactococcal phages in the dynamics of a cheesemaking environment.

Most of the lactococcal phage defense mechanisms studied to date have been tested and evaluated against two lactococcal phage species, 936 and c2. The use of strains which do not replicate these two predominant species should be expected to lead to the appearance of other novel phage species, despite the introduction of selected defense systems.

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