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Use of Real-Time Quantitative PCR for the Analysis of φLC3 Prophage Stability in Lactococci

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Bacteriophages are a common and constant threat to proper milk fermentation. It has become evident that lysogeny is widespread in lactic acid bacteria, and in this work the temperate lactococcal bacteriophage φLC3 was used as a model to study prophage stability in lactococci. The stability was analyzed in six φLC3 lysogenic *Lactococcus lactis* subsp. *cremoris* host strains when they were growing at 15 and 30°C. In order to perform these analyses, a real-time PCR assay was developed. The stability of the φLC3 prophage was found to vary with the growth phase of its host *L. lactis* IMN-C1814, in which the induction rate increased during the exponential growth phase and reached a maximum level when the strain was entering the stationary phase. The maximum spontaneous induction frequency of the φLC3 prophage varied between 0.32 and 9.1% (28-fold) in the six lysogenic strains. No correlation was observed between growth rates of the host cells and the spontaneous prophage induction frequencies. Furthermore, the level of extrachromosomal phage DNA after induction of the prophage varied between the strains (1.9 to 390%), and the estimated burst sizes varied up to eightfold. These results show that the host cells have a significant impact on the lytic and lysogenic life styles of temperate bacteriophages. The present study shows the power of the real-time PCR technique in the analysis of temperate phage biology and will be useful in work to reveal the impact of temperate phages and lysogenic bacteria in various ecological fields.

Lactic acid bacteria (LAB) have a long tradition of being used as starter cultures for the fermentation of a variety of food products. In the dairy industry, the species *Lactococcus lactis* has an important role as a starter for the production of various cheeses. Unfortunately, *L. lactis* is sensitive to bacteriophage attack, an event that may result in impaired quality of the product because of fermentation failure. Consequently, phages are of great economic significance (6, 10, 31, 36).

Studies have shown that lysogeny is common among lactococci (reviewed in references 11, 12, and 22), and recently the presence of six prophages in the genome of *L. lactis* IL1403 has been reported (7). The impact of lysogenic starter strains on fermentation processes is not fully understood. However, temperate phages and prophage-derived sequences of the P335 species have shown to give rise to new lytic phages (4, 13, 33), and the spontaneously induced temperate phages have the potential to infect and lyse or lysogenize sensitive strains in a mixed starter culture in cheese plants (11, 18, 23, 38). Furthermore, prophages of LAB are found to be involved in the lysis of starter cells during cheese ripening, in which release of desirable enzymes into the surroundings is an important step in flavor development (19, 24, 35).

Despite the increasing knowledge about the molecular basis for the lysis-lysogeny decision of temperate phages, there is still little information available on the influence of the host and environmental conditions (32, 37). Such studies would be beneficial for optimization of starter culture performance in the dairy industry. The growth temperature, multiplicity of infection, and nutritional resources are factors known to influence the choice of life cycle of the thoroughly studied *Escherichia coli* temperate bacteriophage λ, which is used as a paradigm for lysogeny in temperate bacteriophages. Low temperature, high multiplicity of infection, and starvation of the cells favor lysogenic development of λ (14, 37). With regard to LAB phages, studies have shown that a shift in temperature (from 30 to 40°C) leads to induction of prophages in several lysogenic *L. lactis* subsp. *cremoris* strains (15) and that the spontaneous induction rate varies greatly between lysogenic strains (from 10 to 10^6 PFU ml^{-1}) and depends on the indicator strain employed in the plaque assay (38). The low number of such studies of LAB might be due to the lack of suitable methods for identifying phages and monitoring phage numbers. In our laboratory an improved plaque assay technique has proven to be useful in identifying indicator strains (25), and we have reported a new method, based on quantitative analysis of specific DNA sites (i.e., the *attB*, *attP*, *attL*, *attR*, and *cos* DNA sites) involved in various cut-and-join events in the two life styles of the phage, that is useful for monitoring the lytic and lysogenic pathways of temperate phages (29).

The temperate phage φLC3 was obtained from fermented milk, and it belongs to the lactococcal P335 group of small isometric-headed phages (27). Characterized genetic elements of phage φLC3 include the *lysA* and *lysB* genes (1), the *int* gene and the attachment sites for site-specific integration and excision (the *attB*, *attP*, *attL*, and *attR* sites) (26, 28), the *cos* region (2), the repressor (*orf286*) gene (3), and the gene encoding the

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TABLE 1. Bacterial strains and phages

Bacterial strain or phage	Relevant characteristic(s)	Reference or source
L. lactis subsp. *cremoris* strains[a]		
IMN-C3	Original ϕLC3 lysogenic *L. lactis* subsp. *cremoris* strain	27
IMN-C18	Standard ϕLC3 host indicator strain	27
IMN-C17	Indicator for ϕLC3	26
IMN-C20	Indicator for ϕLC3	25
3107	Indicator for ϕLC3 and TP936-1	NCFB[b]
3107::TP936-1	TP936-1 lysogenic isolate of 3107	This work
IMN-C1814	ϕLC3 lysogenic isolate of IMN-C18	26
IMN-C17::ϕLC3	ϕLC3 lysogenic isolate of IMN-C17	This work
3107::ϕLC3	ϕLC3 lysogenic isolate of 3107	This work
3107::TP936-1::ϕLC3	ϕLC3 and TP936-1 double lysogenic isolate of 3107	This work
Bacteriophages		
ϕLC3	Induced from IMN-C3	27
TP936-1	Induced from *L. lactis* subsp. *cremoris* 936-1	5

[a] The strains designated IMN were isolated from cultured milk products at the Department of Food Science, Agricultural University of Norway.
[b] NCFB, National Collection of Food Bacteria, Agriculture and Food Research Council Institute of Food Research, Shinfield, Reading, United Kingdom.

Cro analogue (*orf76*) (J. M. Blatny, unpublished data). Sequence comparisons of the lysogeny module reveal that phage ϕLC3 shows homology to other P335 phages (3).

The aim of the present study was to use ϕLC3 as a model phage to analyze the effect of growth temperature and host strains on prophage stability in lactococci by the use of the method of Lunde et al. (29) and furthermore to develop a real-time PCR assay for quantification of the specific DNA sites involved in this method. The prophage stability of phage ϕLC3 was analyzed during growth in six *L. lactis* subsp. *cremoris* strains lysogenic for ϕLC3 at two different temperatures.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and culture conditions. The bacterial strains and bacteriophages used in this work are listed in Table 1. Isolation of the lysogens was done as described by Lillehaug (25). All strains were grown in M17 broth (Oxoid) containing 5 g of glucose per liter instead of lactose. Overnight cultures grown at 30°C were used as 1% inocula, and the cultures were further incubated at 15 and 30°C. To fully adjust and stabilize the bacterial metabolism to the two growth temperatures, the cultures were inoculated every day into new medium adjusted to 15 and 30°C, respectively, and analyses of the cultures were performed on the third day. Growth of the seven lysogenic strains was monitored by measuring optical density at 600 nm with a model 252 colorimeter (Ciba Corning Diagnostics Ltd., Suffolk, England). The study was repeated at least three times, and the data shown are from one representative experiment.

DNA template preparation. During growth of *L. lactis* subsp. *cremoris* IMN-C1814, samples were withdrawn at regular intervals to perform quantitative DNA analyses (see below) on the cell fraction. For the remaining five lysogenic strains, DNA was isolated from samples harvested at three chosen points at the end of the exponential growth phase and in the early stationary phase. For DNA isolation, samples of 1 ml were snap frozen in liquid nitrogen, centrifuged, and resuspended in lysis buffer (10 mM Tris [pH 8], 1 mM EDTA [pH 8], 1% Tween 80). Further isolation was performed by using liquid nitrogen and boiling water in a freeze-thaw method as described by to Utåker and Nes (39).

Plaque assay. The number of infectious phages present in the supernatant during growth of IMN-C1814 was determined as follows. One milliliter of bacterial culture were harvested at regular intervals and centrifuged at 7,000 × *g* for 15 min. The pellet was washed with 0.5 ml of saline containing 10 mM $MgCl_2$, and the cell suspension was centrifuged for 10 min. The pooled supernatant fraction was used for phage titration by a modified plaque assay as described by Lillehaug (25), using *L. lactis* IMN-C18 as a host.

PCR amplification. PCR amplifications of the repressor gene *orf286* in phage ϕLC3 (3) and the gene in phage TP936-1 that corresponds to *orf4* (repressor) in TP901-1 (30) were performed with the *Taq* DNA polymerase kit (Qiagen, Hilden, Germany) according to the instructions of the supplier. PCR amplifications were performed in a total volume of 50 μl and by the use of 30 cycles in a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany). The thermal cycling parameters used were a 3-min hot start at 97°C, followed by denaturation for 30 s at 94°C, annealing for 30 s at 58°C, and extension for 2 min at 72°C. The primers used for amplification of the repressor genes are shown in Table 2.

DNA sequencing. DNA sequencing of the *L. lactis* IMN-C18 region containing the *attB* site (see below) was performed by the cycle-sequencing dideoxynucleotide chain termination method (ABI Prism BigDYE kit; Applied Biosystems, Foster City, Calif.). Samples were analyzed with an ABI Prism 377 DNA sequencer (Applied Biosystems). The primers used for nucleotide sequence determination were as follows: attB-H1, 5′-TAGTCCTTTTCTTCATTCCTTTTGTTTG-3′; attB-H2, 5′-GACCATAAACCAAGCAAAAGC-3′; attB-H3, 5′-TGGGGTAGATGCCCAC-3′; attB-H4, 5′-CCTAGGCCTTCGTGTGCTTAGG-3′; attB-V1, 5′-GGAGCGGGGAATTATTATAGT-3′; attB-V2, 5′-TCCCATGAAGAATAACAAGGT-3′; attB-V3, 5′-TCCTTACGAACAGATAGAATGGC-3′; and attB-V4, 5′-GCGTGTCACATTCTCTTCGAC-3′.

TaqMan probe design. The primers and TaqMan probes for real-time amplification of the *attB*, *attP*, *attL*, and *chr* DNA sites were constructed by the use of Primer Express version 1.0 (Applied Biosystems). The sequences and localizations of the primers and TaqMan probes used are given in Table 2. The primer and probe sets for *attB*, *attL*, and *attP* were designed such that the respective amplification product spans the 9-bp core sequences (26) of the specific DNA attachment sites. The *chr* DNA site was located just upstream of the *attB*-core region; it was involved in this assay as an endogenous control to adjust for possible differences in DNA sampling and DNA isolation efficiency and was used to normalize the levels of the *attB*, *attP*, and *attL* DNA sites. The probes were labeled with a reporter dye (6-carboxyfluorescein) at the 3′ end, with a quencher dye (6-carboxytetramethyl-rhodamine) at the 5′ end, and with a phosphate blocking group at the 3′ end to prevent extension by AmpliTaq Gold DNA polymerase.

Detection and quantification of PCR products. Real-time quantitative PCR was performed by use of the ABI Prism 7700 sequence detection system (Applied Biosystems) on the *attB*, *attP*, and *attL* DNA sites and a chromosomal marker (*chr*) in DNA extracts obtained from the lysogenic cultures. All reagents used to perform real-time PCR detection were purchased from Applied Biosystems. The amplification reactions was carried out with TaqMan Universal PCR Master Mix containing AmpliTaq Gold DNA polymerase, AmpErase uracil-*N*-glycosylase, deoxynucleoside triphosphates with dUTP, and passive reference and optimized buffer components. The final concentrations of each oligonucleotide in the PCR mixture were as follows: *attB* and *chr*, 300 nM (each) forward and reverse primers; *attL* and *attP*, 900 nM (each) forward and reverse primers; all templates, 175 nM TaqMan probe. The total volume of the PCR mixtures was 25 μl, including 1 μl of DNA template, in GenAmp Optical tubes, and the PCR was carried out according to the instructions of the manufacturer. PCR assays for constructing standard curves were performed in triplicate, while the remaining PCR assays were performed in duplicates, both in three separate replicate runs. The concentrations of the primers and probes were optimized as described in the TaqMan Universal PCR Master Mix Protocol (Applied Biosystems).

The increase in emission intensity was measured during the amplification

TABLE 2. Summary of PCR primers and TaqMan probes used in this study

Primer set	Amplified fragment	Sequence (5′→3′)[a]	Position (bases)	Accession no. (GenBank)	Denaturation temp (°C)
1	ϕLC3 *orf286*	TGCACAACGTGGTTTTTTCTTATAT (F)	387–411	AF242738	60.8
		CCTAGCTTTTAACGAGAATCAAGAC (R)	1323–1299	AF242738	59.0
2	TP901-1 *orf4*	CGCCTTTATCAATTTTTTCTTTAAGT (F)	2654–2679	AF304433	60.3
		TGTTCAAGATGCGCTATAATCTATTTAT (R)	3238–3211	AF304433	60.0
3	*attB*	CACCTATTCGCTTGAAAGTCC (F)	702–722	AY151819	58.0
		GCTTGTCTTTTATTCTTCTTGAGCT (R)	814–790	AY151819	58.1
		AACTAAAGAATTGCAAACCTTGTTATTCTTCATGG (P)	754–788	AY151819	66.8
4	*attP*	CCTTTACTATGTAGTTCTTCATGAACG (F)	1245–1271	L10286	56.9
		TGATATGTTGTAATTTTGTTGTACGAG (R)	1394–1368	L10286	56.6
		ACGGCTTAATATAGCCGTTTTTCCTGTTTTTAAAAC (P)	1307–1342	L10286	64.4
5	*attL*	CTTTCTTAACTTTGCACCTATTCG (F)	688–711	AY151819	57.8
		GTTTATTATCGTTCATGAAGAATAACAAG (R)[b]	1280–772	L10286/AY151819	57.7
		CTTGAAAGTCCCTTCAGATGTTGTTGTTCA (P)	712–741	AY151819	67.1
6	*chr*	CTTGGACGTGCGCTTCT (F)	446–462	AY151819	57.7
		GAATGCCATCATCTGTTTGAG (R)	560–540	AY151819	57.3
		CCTACGATATTTCTGGAAATCCACTAGTTGGAA (P)	507–539	AY151819	67.0

[a] F, R, and P, forward primer, reverse primer, and TaqManProbe, respectively. The 9-bp core sequence of the ϕLC3 attachment site is underlined.
[b] The phage-specific DNA sequence is in boldface. Base 1280 refers to the base number reported for ϕLC3 (GenBank accession no. L10286), and base 772 refers to the base number reported for *L. lactis* subsp. *cremoris* IMN-C18 (GenBank accession no. AY151819).

reaction by the ABI PRISM 7700 sequence detection system. Data acquisition and analysis were handled by Sequence Detector version 1.6 software (Applied Biosystems). Briefly, a threshold was assigned to the log phase of product accumulation. The point at which the threshold crosses the amplification curve is defined as a cycle threshold value, termed C_T. With increasing target quantity in the PCRs, the C_T value decrease linearly, and thus C_T values can be used as quantitative measurements of the input target amount (17).

The comparative C_T method (user bulletin 2, ABI PRISM 7700 sequence detection system; Applied Biosystems) was used for quantitative analyses, with the following modification: instead of using only one measurement to calculate the relative amounts of the *attB*, *attL*, and *attP* DNA sites compared to *chr*, we have used the regression functions described in "Statistical and mathematical analysis" below. Time point 8 during growth of *L. lactis* IMN-C1814 at 30°C (see Fig. 2A) was chosen as a calibrator in the comparative C_T method, in which the spontaneous induction frequencies at the other time points were decided relative to the induction frequency at this point. Standard curves were constructed with the DNA sample obtained from the calibrator.

Statistical and mathematical analysis. The statistical analyses were performed using the software package SAS 8.2 (SAS, 2001). In order to validate the comparative C_T method (user bulletin 2, ABI PRISM 7700 sequence detection system; Applied Biosystems), the amplification efficiencies of the amplicons (*attB*, *attP*, *attL*, and *chr*) were investigated by linear regression analysis of the C_T values of three replicate PCRs for each of three replicate 10-fold dilutions of DNA isolated at time point 8 during growth of *L. lactis* IMN-C1814.

In order to calculate the efficiency of the amplification reactions and the ratios between the different specific DNA markers and the chromosomal marker (*chr*), the following equations were used. Equation 1 describes the PCR in the exponential phase (40):

$$N_1 = N_0 \times (1 + E)^n \quad (1)$$

where N_1 is the amount of PCR product after n cycles of amplification, N_0 is the amount of DNA template prior to amplification, and E is the efficiency of the reaction per cycle. The overall efficiency of the exponential reaction can be calculated from equation 2 (resolved from equation 1):

$$E = (N_1/N_0)^{1/n} - 1 \quad (2)$$

where E is the efficiency of amplification per cycle and $N_1 - N_0$ is the number of amplified molecules produced in n cycles of exponential amplification.

The ratio between the attachment sites and the chromosomal control was estimated by using the calculated amplification efficiency (E) and the ΔC_T (see above) between the attachment sites to the *chr* DNA site in the following equation:

$$\text{Ratio (attachment site}_i/chr) = (1 + E)^{\Delta C_T} \quad (3)$$

where i is the DNA attachment site *attB*, *attP*, or *attL*; *chr* is the chromosomal target DNA; E is the efficiency of amplification per cycle; and ΔC_T is the distance (number of cycles) between the intercepts (see below) of the regression functions of the specific attachment sites and the chromosomal control (*chr*). The intercept (β_0) is the value of y when x equals zero in the regression function (equation 4) in which a straight line relating two quantities y and x can be described as follows:

$$Y = \beta_0 + \beta_1 x \quad (4)$$

where y is the response variable, x is the predictor variable, β_0 is the intercept, and β_1 is the regression coefficient (34).

Nucleotide sequence accession number. The nucleotide sequence of the *L. lactis* subsp. *cremoris* IMN-C18 region that contains the ϕLC3 integration site (*attB*) has been deposited in the GenBank database under accession no. AY151819.

RESULTS AND DISCUSSION

We have studied the effect of growth temperature on the prophage stability of the temperate lactococcal phage ϕLC3 in six lysogenic hosts by using the recently reported method of Lunde et al. (29). This method is based on the quantitative analysis of specific DNA sites involved in various cut-and-join events during the lytic and lysogenic life styles of the ϕLC3 phage, such as the attachment sites for site-specific integration and excision (i.e., the *attB*, *attP*, *attR*, and *attL* DNA sites) and the phage cohesive ends (*cos* DNA site). A real-time PCR assay was developed to perform the quantitative analysis of the specific DNA sites used in this work.

Validation of the real-time PCR assay. Relative quantification of the *attB, attP*, and *attL* DNA attachment sites by real-time PCR was performed with reference to the total amount of bacterial DNA represented by a chromosomal DNA fragment here called *chr.* A previously reported comparative C_T method (see Methods and Materials) was used for this analysis. In order to apply this method, it is important that the templates to be compared have equal amplification efficiencies, and in order

TABLE 3. Average C_T values from three standard series of $\log_{10}$ dilutions of a DNA sample used as a calibrator in the comparative C_T method

Site and dilution factor	C_T for the following replicate of the standard curve:			Mean C_T (SD)
	1	2	3	
attB				
0	20.13	20.11	20.23	20.16 (0.06)
1	23.97	24.13	23.93	24 (0.10)
2	27.49	27.64	27.58	27.57 (0.07)
3	30.73	31.01	31.08	30.94 (0.19)
4	32.96	32.52	33.32	34.21 (0.24)
5	35.93	36.45	36	37.73 (0.18)
attP				
1	18.65	18.41	18.44	18.5 (0.13)
2	22.23	22.09	22.13	22.15 (0.07)
3	25.96	25.71	25.82	25.83 (0.13)
4	29.09	29.01	29.06	29.05 (0.04)
5	33.15	33.08	32.85	33.03 (0.16)
6	36.11	NA[a]	36.08	36.1 (0.02)
attL				
1	17.32	17.33	17.34	17.33 (0.01)
2	20.8	20.68	20.88	20.79 (0.1)
3	24.26	24.33	24.3	24.3 (0.03)
4	27.55	27.58	27.86	27.66 (0.17)
5	31.12	31.18	31.38	31.23 (0.13)
6	34.68	34.72	34.74	34.71 (0.03)
chr				
1	17.05	17.06	17.04	17.05 (0.01)
2	20.37	20.36	20.44	20.39 (0.05)
3	24	24.04	24.1	24.05 (0.05)
4	27.63	27.36	27.46	27.48 (0.14)
5	31.04	31.13	31.3	31.16 (0.13)
6	34.51	NA[a]	34.39	34.45 (0.09)

[a] NA, not analyzed.

to verify that this condition was fulfilled, standard curves for the *attB*, *attP*, *attL*, and *chr* DNA sites were constructed by the use of regression analysis of three replicate 10-fold serial dilutions of a DNA sample. The C_T measurements (see Materials and Methods) were performed on different days, and they were highly reproducible, with a standard deviation of less than 0.24 (Table 3). Therefore, the mean C_T value of the three replicates for each dilution factor for each template was used in the analysis below, in which two statistical tests were carried out. When comparing the regression functions of the four templates in the first test, no significant differences between the regression coefficients ($P = 0.64$) were found, but the intercepts differed significantly ($P < 0.001$). By the use of multiple comparisons in the second test, it was shown that all templates differed in the intercept except for that of *attL* and *chr* ($P = 0.06$). Therefore, a common regression coefficient (-3.502) but different intercepts (*attB*, 20.35; *attP*, 15.19; *attL*, 13.75; and *chr*, 13.51) (*attL* and *chr* were not significantly different) were used for construction of the four standard curves in Fig. 1. The regression functions had a coefficient of determination of greater than 0.98. These results indicated that the efficiencies of the amplification reactions of the four templates were equal, and by using equation 2 (see Materials and Methods), where n was replaced by the common regression coefficient of the regression lines, the amplification efficiency was calculated to 0.93. Furthermore, the standard curves in Fig. 1 showed a linear relationship between the amount of input DNA and the C_T values for the four templates over a range of 5 to 6 log units. The large dynamic range is one of the advantages of the real-time quantitative PCR method over other methods for quantitative analysis of DNA fragments, along with the facts that the method involves no post-PCR step, offers high throughput of samples, avoids the use of radioactivity, and requires relatively little manual input.

ϕLC3 prophage stability varies during growth of the host cell. The spontaneous induction of prophages in lysogenic bacteria, including lactococci, has traditionally been determined by lysis of bacterial cultures and enumeration of the phages by the use of plaque assays (38). However, the plaque assay has several limitations. The efficiency of plaque formation is highly influenced by the physical and chemical conditions applied with the double-agar plate technique (25). Furthermore, the lack of suitable indicator strains has been a major barrier for studying the temperate bacteriophages. In this work the spontaneous induction frequency of the lactococcal prophage ϕLC3 was determined directly on genomic DNA obtained from the lysogenic bacterium by measuring the level of reestablished *attB* sites on the bacterial chromosome. Furthermore, the phage DNA propagation in the lytic state was elucidated by measuring the level of the *attP* DNA sites on the extrachromosomal phage genomes in either the linear, circular, or concatamer configuration. The amount of integrated prophages was detected by amplification of the attachment site *attL* located in the junction between the phage and host DNA, and hence the level of *attL* DNA sites represented the amount of the ϕLC3 lysogenic bacteria in the cultures.

Determination of the spontaneous induction frequency of ϕLC3 (*attB*) and the level of newly synthesized phage DNA (*attP*) during growth of the ϕLC3 lysogenic *L. lactis* IMN-C1814 strain at 30 and 15°C was carried out by the use of the comparative C_T method as described in Materials and Methods, and the results are shown in Fig. 2. Time point 8 at 30°C (Fig. 2A, 8 h) was chosen as the calibrator, and genomic DNA

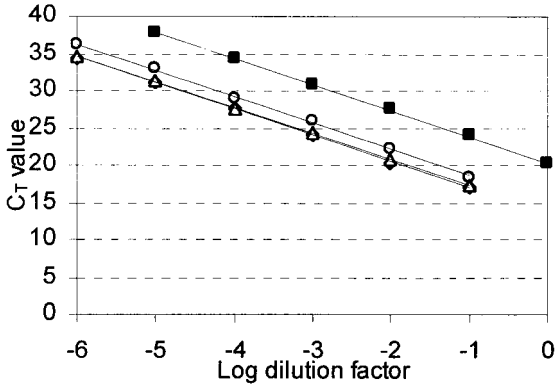

FIG. 1. Evaluation of amplification efficiency and range of dynamic amplification of the *attB*-, *attP*-, *attL*-, and *chr*-specific DNA templates. The mean C_T values of *attB* (■), *attP* (○), *attL* (△), and *chr* (◆) from three serial $\log_{10}$ dilutions of DNA extracted in the early stationary phase during growth of the ϕLC3 lysogenic *L. lactis* IMN-C1814 were used to construct standard curves. Note that the symbols for the *chr* and *attL* data overlap.

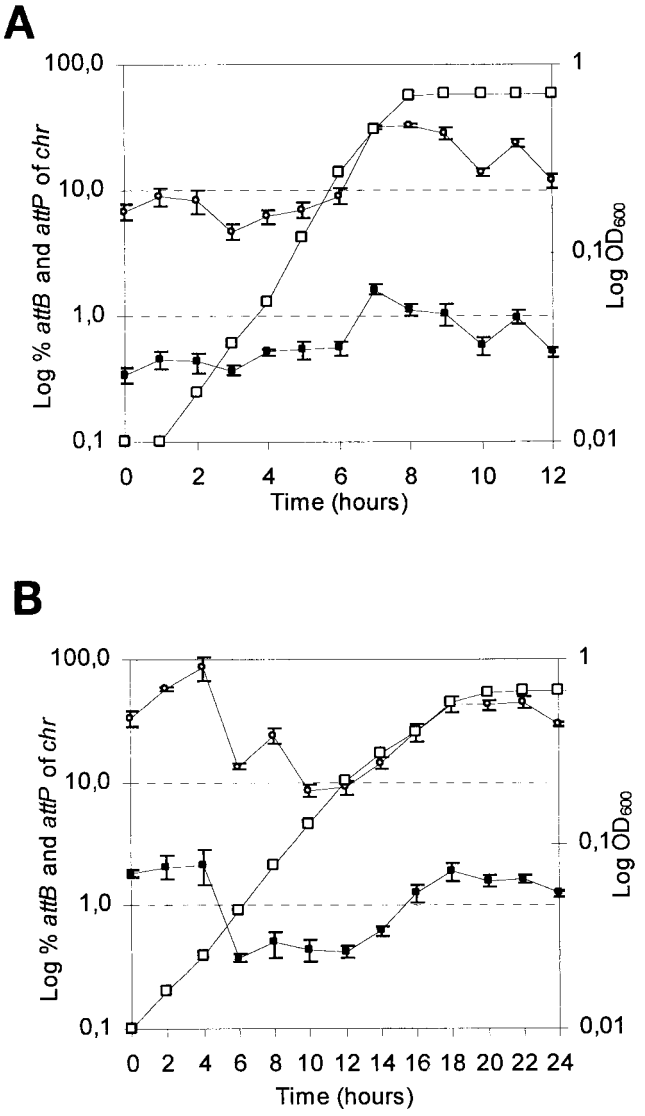

FIG. 2. Spontaneous prophage induction frequency (*attB*) and level of extrachromosomal phage DNA (*attP*) monitored during growth of the lysogenic *L. lactis* subsp. *cremoris* IMN-C1814 at 30°C (A) and 15°C (B) by a real-time quantitative PCR method. Samples were withdrawn at regular intervals for 12 and 24 h at 30 and 15°C, respectively, as indicated on the growth curves (□). The levels of the *attB* (■) and *attP* (○) ϕLC3 attachment sites were normalized to the chromosomal marker *chr* and plotted as percent attachment sites with respect to the total amount of cells, represented by *chr* (100%) (not shown). Error bars show the standard deviations of the means. OD_{600}, optical density at 600 nm.

obtained from this point was used to analyze the amplification efficiencies of the various genetic markers (see above). The spontaneous induction rate and the level of the extrachromosomal phage DNA in the calibrator were determined from the ratio of *attB* and *attP* DNA sites to the total amount of bacterial *chr* DNA, respectively, by using the intercepts of their respective regression functions and equation 3 (see Materials and Methods). These ratios (*attB*/*chr* and *attP*/*chr*) were estimated to 0.0112 and 0.331, respectively. This implies that 1.12% of the cells in the bacterial culture at time point 8 had induced their ϕLC3 prophage and reestablished their *attB* site and that 33.1% of phage genomes (*attP*) were present extrachromosomally compared to the total amount of the bacterial chromosomal DNA (100%) (*chr*). According to the 1.12% of *attB* sites formed, only a small reduction of *attL* sites (from 100% to 98.9%) should have taken place, and such a small decrease in *attL* sites could not be determined significantly by our technology.

During growth at both temperatures (Fig. 2), most of the lysogenic bacterial population remained lysogenic, while a fraction of the prophages were spontaneously induced into the lytic pathway. Remarkably, the spontaneous induction frequency did not remain at the same level during the course of cell growth but increased in the exponential growth phase and reached a maximum level in the early stationary phase, as implied by the fact that the development of the *attB* and *attP* DNA sites followed the kinetics of the growth curve (Fig. 2). At 30°C (Fig. 2A) the levels of the genetic markers were 0.4 to 0.6% *attB* and 5 to 9% *attP* in the mid-exponential growth phase and increased to averages of 1.3% *attB* and 30% *attP* in the end of the exponential growth phase and the early stationary phase. Correspondingly, at 15°C (Fig. 2B) the levels of the *attB* and *attP* DNA sites were 0.4 to 0.6 and 8 to 15%, respectively, in the mid-exponential growth phase and increased to averages of 1.6 and 40%, respectively, in the early stationary phase. At 30°C (Fig. 2A) a slow decline in the relative concentrations of both *attP* and *attB* was observed in the stationary phase, ending at 0.3% *attB* and 7% *attP*. This decline in spontaneous induction frequency is consistent with early studies of phage λ that showed that the lysogenic response is favored if the cells are grown past the exponential growth phase (14). The decline in the spontaneous induction rate and the level of extrachromosomal phage DNA was not seen in a 24-h-old culture grown at 15°C (Fig. 2B). This was probably due to the fact that the slow-growing culture at 15°C did not enter the stationary phase before 18 to 20 h and was still in its early stationary phase after 24 h. Consequently, the overnight culture used as an inoculum consisted of cells with a relatively high spontaneous induction frequency. Reinitiation of cell growth in fresh medium led to the expected degradation of the induced cells, seen as a decrease in the levels of the *attP* and *attB* DNA sites (Fig. 2B) due to phage burst and lysis of the induced stationary-phase cells.

During growth, the level of the *attL* DNA sites was not significantly different from the level of the *chr* DNA sites as shown by the statistical tests described above, confirming that most of the *L. lactis* IMN-C1814 cells remained lysogenic. These findings were consistent with the calculated prophage induction rates of between 0.4 and 1.3% at 30°C and between 0.4 and 1.6% at 15°C, depending on the growth stage of the lysogenic bacterium.

The level of the *attP* DNA sites reflects the level of extrachromosomal ϕLC3 phage DNA in a population of cells, in which the phages may be in different stages in their development due to different time points of induction. Hence, the ratio between the ϕLC3 *attP* and *attB* DNA sites gives an estimate of the average amount of phage genomes in the induced cells, and consequently the *attP*/*attB* ratio will indirectly reflect the burst size. In the stationary phase during growth of IMN-C1814 (Fig. 2), the *attP*/*attB* ratio remained at approximately 30 at both 15 and 30°C. This *attP*/*attB* ratio agrees with a burst

size of about 60 phages (26). Furthermore, these results indicated that the phage DNA propagation efficiency seemed to be unaffected by temperature.

In the stationary phase during growth of IMN-C1814 (Fig. 2) the number of φLC3 phages at 30°C was about 10^6 to 10^7 ml^{-1}, while the culture grown at 15°C contained 10- to 100-fold fewer PFU. These levels of free phage particles are consistent with the levels reported for other temperate lactococcal phages in overnight cultures of lysogenic strains (8, 38). A reduction in the number of infectious phage particles in cultures with lower growth rates was also observed in studies on lambdoid prophages in *E. coli* cultures (9). However, Czyz et al. (9) could not determine if the reduction was caused either by a lower frequency of prophage induction or by less efficient lytic development after excision of the phage from the host chromosome.

From the results obtained in this study, it can be concluded that the lower number of infectious φLC3 phages in cultures grown at 15°C compared to 30°C was obviously not due to a lower frequency of prophage induction in the slowly growing culture (Fig. 2) or to a less efficient propagation of excised phage DNA (see above). It seemed more likely that the maturation process of phage particles, including packaging, was less efficient at low temperature. Comparative results have been obtained for the *E. coli* bacteriophage T4, in which the burst size of the phage is not limited by cell size or DNA composition, or directly by the rate of metabolism, but rather is limited by the rate of synthesis and assembly of phage components and by lysis time (16).

φLC3 prophage stability is dependent on host strain and growth temperature. The decision of the temperate phage to enter either a lytic or lysogenic life cycle is believed to be affected by phage- and host-encoded factors in addition to environmental conditions (37). Studies regarding this lysis-lysogeny decision of temperate phages have been hampered by the lack of good techniques. In this work we have analyzed the spontaneous rate of induction of φLC3 in six lysogenic strains when grown at 15 and 30°C by the new method of Lunde et al. (29).

The strains used in this study were the parental strain of φLC3, IMN-C3, and five additional constructed φLC3 lysogenic lactococcal strains (Table 1). The new lysogenic derivates were still inducible and tested to be resistant to superinfection by φLC3 after more than 10 rounds of single-colony isolations. The results strongly suggested that the strains were true φLC3 lysogens. Furthermore, the use of sequence specific primers for amplification of the phage φLC3 regulator gene, *orf286* (3), verified that the strains in use were φLC3 lysogenic at the time point of analysis. The presence of TP936-1 in the double lysogenic strain 3107::TP936-1::φLC3 was verified by PCR with primers specific to the TP901-1 repressor gene (*orf4*) (30) (results not shown).

The previous experiments with the lysogenic strain IMN-C1814 (Fig. 2) showed that the highest frequency of φLC3 prophage induction was obtained when the cells were about to enter the stationary phase. This result indicated that the induction seemed to depend on the cell growth stage. Consequently, it was important to obtain the growth kinetics of the six lysogenic strains at both growth temperatures (15 and 30°C) in order to compare the spontaneous induction frequency at the same growth stage when the prophage was most unstable. At 30°C the lysogenic cultures entered the stationary phase after approximately 6 to 8 h, while at 15°C they entered the stationary phase after approximately 19 to 23 h, depending on the individual strain. The generation times for the six strains at 30 and 15°C, respectively, were as follows: C1814, 1.08 and 2.2 h; C3, 1.05 and 2.8 h; C17::φLC3, 0.99 and 2.95 h; C20::φLC3, 1.26 and 2.65 h; 3107::φLC3, 0.73 and 3.89 h; and 3107::TP901-1::φLC3, 1.03 and 3.99 h. DNA samples were isolated from the cultures at three time points, one at the end of the exponential growth phase and two in the early stationary phase. The average ratio of *attB* to *chr* at these three time points was used to determine the φLC3 prophage stability of each strain, and the results are presented and discussed below.

The strains showed considerable variability in prophage stability, as shown in Fig. 3A. Between the lowest (IMN-C17::φLC3 at 15°C, 0.32% *attB*) and the highest (IMN-C20::φLC3 at 15°C, 9.1% *attB*) spontaneous prophage induction frequency, a 28-fold difference was observed. Growth at high and low temperatures did not affect the φLC3 prophage stability in the lysogenic hosts in one direction (Fig. 3A). The spontaneous induction rates were higher at 30°C than at 15°C for the three strains IMN-C3, 3107::φLC3, and 3107::TP936-1::φLC3 (1.7-, 2.9-,and 1.8-fold, respectively), while the two strains IMN-C1814 and IMN-C20::φLC3 showed the highest spontaneous induction rates at 15°C (1.2-and 2.1-fold, respectively). Growth at two different temperatures did not significantly alter the induction rate in IMN-C17::φLC3 even though the generation times varied from 1 to 2.9 h at 15 and 30°C, respectively. Compared to the stability in the single lysogenic 3107::φLC3 strain, the φLC3 prophage was more stable in the double lysogenic strain 3107::TP936-1::φLC3 at 30°C, while no significant difference in prophage stability was seen at 15°C. The fact that the growth rate of the double lysogenic strain was lower than that of the single lysogenic strain at 30°C but did not differ at 15°C may explain the lower induction rate of 3107::TP936-1::φLC3 at 30°C. However, the IMN-C1814, IMN-C3, IMN-C17::φLC3, and 3107::TP936-1::φLC3 strains showed approximately the same generation time of 1 h at 30°C, but their prophage induction frequencies varied between 1 and 6.3%. From the results obtained from this study, it can be concluded that the different growth rates alone certainly cannot explain the differences in prophage stability between the different strains and that changes in growth temperature affect the induction rates in different directions.

The different spontaneous induction rates of φLC3 in the single and double lysogenic derivatives of the 3107 strain at 30°C may indicate that some kind of cross-communication between the two P335 phages (20) may play a role in φLC3 prophage stability; however, this has to be investigated further. Furthermore, DNA from phage φLC3 hybridized to genomic DNA obtained from the lactococcal φLC3 host strains, except for IMN-C18 (Table 1), before they were lysogenized with φLC3 (data not shown). Hence, it cannot be ruled out that putative phage factors other than from φLC3 played a role in the regulation of the lysis-lysogeny decision of φLC3. However, the *attB* and *attP* DNA sites detected by real-time PCR in this study were specific to φLC3, since the level of the *attB* sites in the non-φLC3 lysogenic strains was equivalent to the amount of cells (*chr* marker) in DNA samples obtained from the cultures. Hence, no other phage occupied the attachment site of phage φLC3. Furthermore, no *attP* DNA was detected

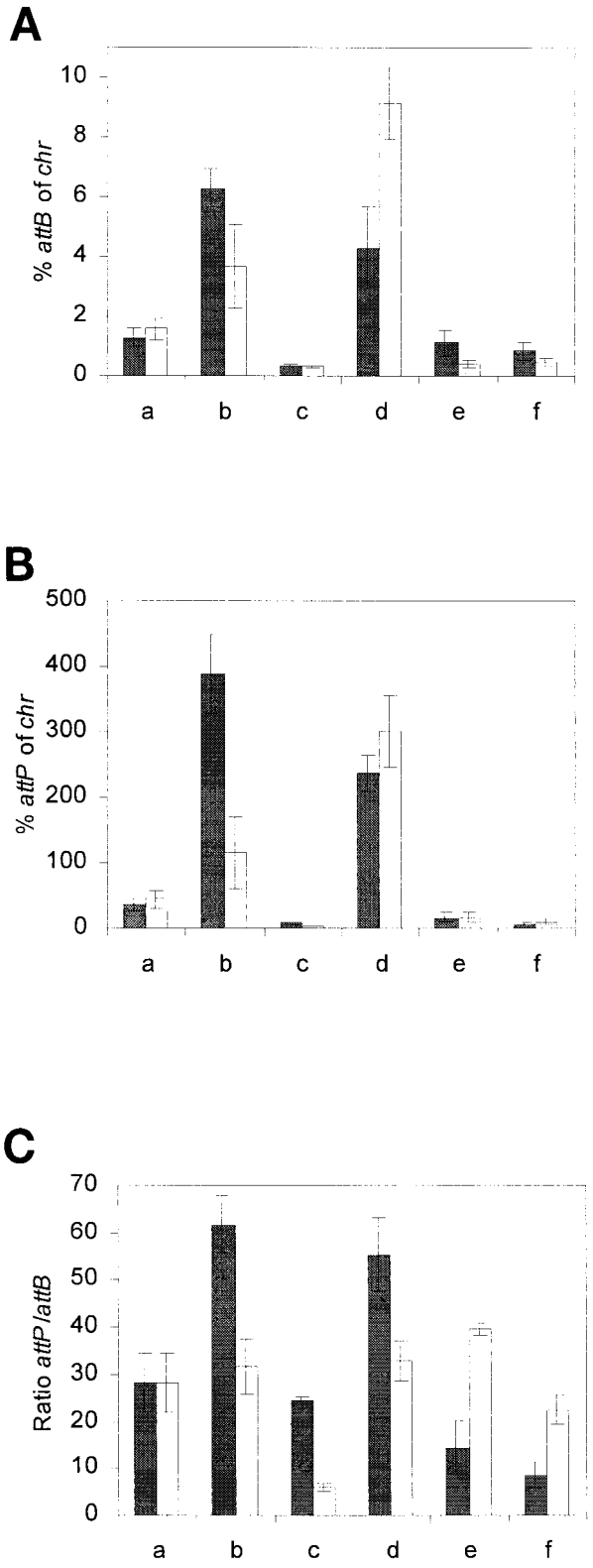

FIG. 3. Spontaneous induction of the ϕLC3 prophage into the lytic life cycle and the following phage DNA propagation in the *Lactococcus lactis* subsp. *cremoris* strains IMN-C1814 (bars a), IMN-C3 (bars b), IMN-C17::ϕLC3 (bars c), IMN-C20::ϕLC3 (bars d), 3107::ϕLC3 (bars e), and 3107::TP936-1::ϕLC3 (bars f) as measured by the real-time quantitative PCR method. The lysogenic strains were grown at 30°C (gray bars) and 15°C (white bars). (A) The level of *attB*, given as the percentage of that of *chr*, reflects the spontaneous prophage induction frequency. (B) The level of *attP*, given as the percentage of that of *chr*, reflects the level of extrachromosomal phage DNA compared to the level of host DNA in a sample. (C) The *attP*/*attB* ratio reflects the level of extrachromosomal phage DNA in the cells where the prophage is induced into the lytic life cycle. Error bars show the standard deviations of the means.

in the host strains before they were lysogenized with ϕLC3 (results not shown).

ϕLC3 DNA propagation is unique for each host strain and depends on the growth temperature. After prophage induction, the phage follows the lytic pathway, which involves DNA replication, expression of phage-encoded genes, packaging, maturation, and lysis of the host cell (37). We have studied the effect of growth temperature on the ϕLC3 phage DNA propagation after induction of the phage from its lysogenic to its lytic state in six different hosts by determination of the ratio between the *attP* and *attB* DNA sites in genomic DNA samples.

As stated above, the *attP*/*attB* ratio gives an indirect estimate of the burst size of ϕLC3 in the induced cells, and the results shown in Fig. 3C indicated that the theoretical burst sizes of the six lysogenic strains varied between 16 and 120 phage particles. These burst sizes are similar to values documented in the literature for other lactococcal phages (21). However, to our knowledge this is the first study that shows that the burst size of one particular temperate phage varies between different hosts (up to eightfold) after being induced from the lysogenic to the lytic pathway. The formation of the *attB* and *attP* DNA sites in the lysogenic strains was generally within expected values, since when large amounts of *attB* sites were formed (Fig. 3A), correspondingly high levels of *attP* sites were produced (Fig. 3B). However, the quantitative relationship between *attB* and *attP* was not consistent between the strains and between temperatures, as shown in Fig. 3C. As described above, the *attP*/*attB* ratio in IMN-C1814 was approximately 30 at both 15 and 30°C, and this result indicated that the phage DNA propagation in the lytic state was not affected by growth temperature. For the three strains IMN-C3, IMN-C17::ϕLC3, and IMN-C20::ϕLC3, propagation of the ϕLC3 DNA genome seemed to be more efficient at 30 than at 15°C, since the *attP*/*attB* ratio was approximately twofold higher at the highest temperature. In contrast, for both the single and double lysogenic strains of 3107, the *attP*/*attB* ratio was about 2.5-fold higher at 15 than at 30°C, which indicated that the phage DNA propagation was more efficient at the lower temperature for these two lysogenic strains. Furthermore, the *attP*/*attB* ratio was 1.7-fold higher in the single lysogenic strain than in the double lysogenic strain of 3107 at both temperatures. The results obtained here indicated that the propagation of ϕLC3 DNA after induction of the prophage into the lytic life style differs between different hosts and that growth temperature affects the propagation step differently in the host strains.

Conclusions. The temperate lactococcal phage ϕLC3 originally induced from the industrial strain *L. lactis* subsp. *cremoris* IMN-C3 was able to exist as a lysogen in several hosts. The results from this study showed that the spontaneous frequency of induction of the ϕLC3 prophage was dependent on the growth phase of the host cell. Furthermore, the frequency of

spontaneous prophage induction and the estimated burst sizes varied considerably (28- and 8-fold, respectively) between the lysogenic hosts regardless of the growth rate. Changes in growth temperature did not lead to a uniform response with regard to the spontaneous prophage induction and extrachromosomal phage DNA propagation in the lytic pathway. The present results add to the well-established knowledge that the genetic switch between the lytic and lysogenic pathways is subjected to complex regulation and that the host contributes significantly to prophage stability.

We believe that this work, with the use of phage ϕLC3 and its host as a model system, has provided important knowledge about the complex interaction between a temperate phage and its host. Currently we are exploring how dairy-related environmental stress, such as variations in temperature, pH, osmolarity, and nutritional resources, influences both the prophage stability and the lysogenization process of ϕLC3. Such information will be useful to reveal the impact of lysogeny in bacterial fermentation processes as well as in other ecological systems. This study also shows the innovative use of a new technology in the analysis of temperate-phage biology. We believe that this method is also a valuable tool for studying the effects of various phage- and host-encoded gene products on phage development and phage-host interactions.

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