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Multiplex Fast Real-Time PCR for Quantitative Detection and Identification of *cos*- and *pac*-Type *Streptococcus thermophilus* Bacteriophages▽†

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The fermentation of milk by *Streptococcus thermophilus* is a widespread industrial process that is susceptible to bacteriophage attack. In this work, a preventive fast real-time PCR method for the detection, quantification, and identification of types of *S. thermophilus* phages in 30 min is described.

Streptococcus thermophilus is a gram-positive thermophilic lactic acid bacterium used, along with *Lactobacillus* spp., as a starter culture for the manufacture of important fermented dairy foods, including yogurt and Swiss- or Italian-type hard cooked cheeses (5). Unfortunately, these bacteria are susceptible to infection by bacteriophages during the fermentation process, a phenomenon that ultimately results in fermentation failure. The common features of *S. thermophilus* phages include double-stranded DNA genomes that are 31 to 45 kb long, small isometric heads, long noncontractile tails, and affiliation with the *Siphoviridae* family, corresponding to Bradley's group B (3). They are currently divided into two groups (*cos* and *pac* types) based on the genome encapsidation machinery (11). In the case of yogurt isolates, these types are also related to host range and serotype (4).

Fast detection methods are an important tool to avoid phage attacks in dairy factories. Detection of bacteriophages in milk is normally carried out using standard microbiological methods (plaque assays, activity tests, etc.) (8), but these methods are time-consuming. To speed up the analysis, PCR techniques have been used to detect phages in different kinds of dairy samples (1, 4, 6, 7, 10, 12). Increasing demand for quantitative, more sensitive, and quicker procedures is prompting the development of real-time quantitative PCR (qPCR) methods. The objective of the present study was to develop a fast multiplex qPCR method that allows quantitative detection and identification of *cos*- and *pac*-type *S. thermophilus* bacteriophages in milk samples.

Primer and probe design. In the first step, databases were screened to select the most conserved genes of *cos*- and *pac*-type *S. thermophilus* phages. *orf1510* encoding the putative minor tail protein of the Sfi11 bacteriophage (*pac* type) and *orf18* encoding the antireceptor protein of the Sfi21 bacteriophage (*cos* type) were selected and aligned, using the CLUSTAL W algorithm (14), with the sequences of the orthologous genes available in the GenBank database. Highly similar sequences were selected to design primers qPac1, qPac2, qCos1, and qCos2 and probes mgbPac2 and mgbCos (Table 1) using Primer Express software (Applied Biosystems, Warrington, United Kingdom). The species specificity of the primers was assessed by using BLAST 2.2.15 (Basic Local Alignment Search Tool) to ensure that they amplify only the corresponding *S. thermophilus* bacteriophage sequences. Both the mgbPac2 and mgbCos probes were synthesized with a minor groove binder (MGB) nonfluorescent quencher attached to the 3′ end and with a different reporter dye attached to the 5′ end (VIC and 6-carboxyfluorescein [FAM], respectively) in order to combine them in the same sample.

IC. A general and important advantage of the qPCR based on fluorescent probes is the possibility of including an internal positive control (IC) in every reaction. pEM125, a plasmid containing an unrelated sequence (EMBL database accession no. X64695), was constructed as an IC. Primers IC-FW and IC-R and the TaqMan MGB probe mgbIC were selected (Table 1). The probe was NED labeled at the 5′ end and had an MGB nonfluorescent quencher attached to the 3′ end. A total of 10^6 copies of plasmid pEM125 (3 logarithmic units greater than the determined level of detection) was added to all the reaction mixtures as an IC. The reaction was considered to be inhibited if the cycle threshold (C_T) value increased more than 3 U. Correct amplification of the IC indicated that the whole biochemistry machinery worked properly and that there were no PCR inhibitors in the samples. Therefore, negative phage detection results were much more reliable than the results obtained using previous PCR methods.

Quantification range and sensitivity. pEM212, the plasmid used as a standard and a positive control in the qPCR for *pac*-type bacteriophages, was constructed by cloning a 1,196-bp fragment of the *orf1510* gene from the Sfi11 bacteriophage into the pCR-2.1 TOPO vector (Invitrogen, Carlsbad, CA). pEM213, the plasmid used as a standard and a positive control in the qPCR for *cos*-type bacteriophages, was constructed by cloning a 147-bp fragment of the *orf18* gene from the Sfi21 bacteriophage into the same vector. Triplicate experiments with serial 10-fold dilutions ranging from 10^{11} to 1 copy of plasmid per ml of milk were performed to generate the standard curves. The qPCR conditions are described in the supplemental material. A linear function between the average C_T values and the logarithm of the gene copy number was established (Fig. 1A and 1B for plasmids pEM212 and

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TABLE 1. PCR primers and TaqMan MGB probes used in this study

Specificity	Primer or probe	Sequence (5′–3′)[a]	Position (bases)
orf1510[b]	mgbPac2	VIC-ACATGGCTGCATCTCT-MGB (P)	13105–13120
	qPac1	CGGGTGCTGGTTTCAATCA (F)	13041–13060
	qPac2	CTGCTGAGTTATCACTAATCGAACC (R)	13143–13167
orf18[c]	mgbCos	FAM-TTGGTCGTTCTACTGTTAA-MGB (P)	15874–15892
	qCos1	TGCCATATCATGTTGAGATAAGGAC (F)	15820–15844
	qCos2	TGCATCAACAATTTTATCGCCTTG (R)	15906–15929
pEM125	mgbIC	NED-CAAGCTCGAAATTAACCCTCACTAA-MGB (P)	
	IC-FW	GAGTAGGTCATTTAAGTTGAGCATAATAGG (F)	
	IC-R	CAAGCTCGAAATTAACCCTCACTAA (R)	

[a] (F), forward primer; (R), reverse primer; (P), TaqMan MGB probe.
[b] *orf1510* is the putative minor tail protein gene of the Sfi11 bacteriophage (accession no. AF158600).
[c] *orf18* is the antireceptor gene of the Sf21 bacteriophage (accession no. AF115103).

pEM213, respectively). The results showed that the detection limit was one plasmid molecule in 33.22 cycles with a standard deviation (SD) of ±0.7 for plasmid pEM212 and one plasmid molecule in 33.23 cycles with an SD of ±1.0 for plasmid pEM213. The assay variability increased when less than 100 copies were present. However, the dynamic range of the qPCR assay was wide (from 1 to 10^8 copies of the standard plasmids).

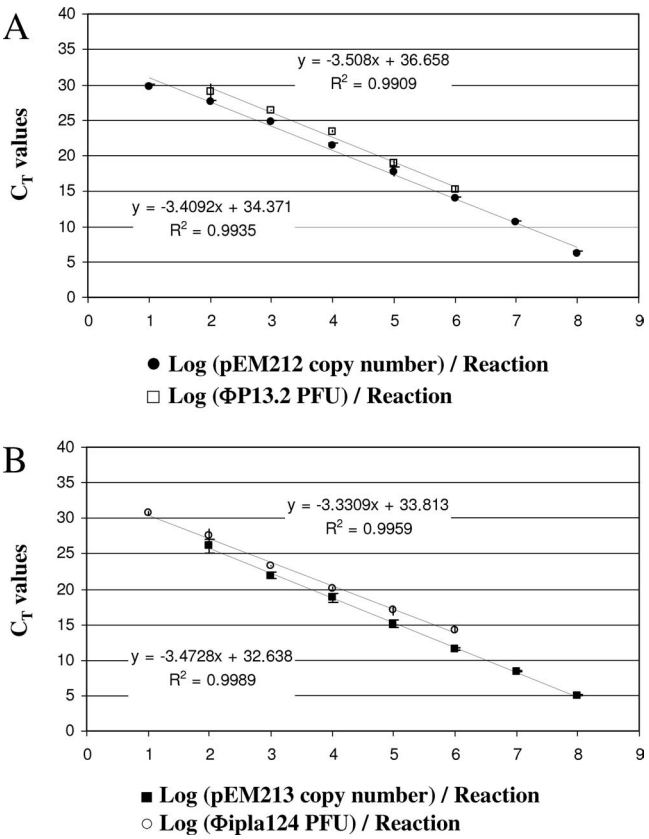

FIG. 1. Real-time qPCR analysis of 10-fold serial dilutions in skim milk of (A) plasmid pEM212 DNA digested with BglII and bacteriophage ϕP13.2 and (B) plasmid pEM213 DNA digested with BglII and bacteriophage ϕipla124. C_T values were plotted against the logarithm of the calculated plasmid copy number or the number of PFU included in each reaction mixture.

Consequently, the quantification limit was determined to be 10 copies per reaction. Another important parameter, the reaction efficiency (9), was obtained from the standard curves. In both cases the amplification efficiency was high (0.96 and 0.94, respectively).

To test the precision of the standard curves using phages as templates, two new curves were generated using milk artificially contaminated with known titers of ϕP13.2 (*pac* type) and ϕipla124 (*cos* type) ranging from 1 to 10^6 PFU per PCR mixture (Fig. 1A and 1B). As expected, the results revealed that the slopes of the curves were similar to the slopes of curves previously generated with the pEM212 and pEM213 control plasmids. Thus, the *S. thermophilus* bacteriophage titer of a milk sample could be determined by means of the pEM212 and pEM213 regression functions.

Reproducibility and specificity of primers and probes. To determine the reproducibility of the proposed method, quadruplicate reactions with two independent ϕP13.2 and ϕipla124 suspensions were performed. Tenfold milk dilutions containing from 10^3 to 10^9 PFU ml^{-1} were used as templates. The SD of the C_T values obtained were calculated and ranged from a minimum of ±0.07 to a maximum of ±0.6 for ϕP13.2 (*pac* type) and from a minimum of ±0.04 to a maximum of ±0.92 for ϕipla124 (*cos* type). C_T values obtained for the same dilutions on three different days were used to determine the interassay variability.

The specificity was assessed by testing 27 different *S. thermophilus* bacteriophages previously isolated in Europe from failed industrial fermentations and characterized in our laboratory (unpublished results), 15 different *S. thermophilus* phages isolated in America (1, 13), and the type phages Sfi11 and Sfi21. Four different bacteriophages infecting *Lactobacillus delbrueckii* and nine bacteriophages infecting *Lactococcus lactis* were also tested. All these bacteriophages are listed in Table S2 in the supplemental material. The qPCR method designed in this work was extremely specific since only the *S. thermophilus* phages were detected. Moreover, using the function of the fluorescent dye detected, VIC or FAM, it was possible to identify the type of phage (*pac* and *cos*, respectively).

Although milk is unlikely to be contaminated with different phages in practice (2), the method was used successfully to detect both *S. thermophilus* phage types in the same sample.

Milk samples simultaneously contaminated with titrated suspensions of ϕP13.2 (*pac* type) and ϕipla124 (*cos* type) were used as template sources. No interference was observed in these multiple qPCR assays.

Phage detection is still mainly done by the plaque assay, which depends on knowledge of the identity of the starter strain. PCR methodology is an interesting alternative since it does not depend on the starter strain that is being used. On the other hand, PCR cannot distinguish viable phage particles from DNA, but the presence of viral DNA is an indication of the potential presence of infective virions. Moreover, soluble DNA is rapidly degraded in milk and dairy products. To our knowledge, this is the first phage detection method based on fast real-time PCR technology. The most important advantages compared to previously described methods (plaque assays, activity tests, conventional PCR, etc.) are probably the considerable time reduction and simplicity of the analysis, since it is possible to detect phage in no more than 30 min without previous or subsequent sample treatments. In addition, this method is able to classify *S. thermophilus* phages in one of the two groups that were established based on the DNA packaging mechanism (11). Fast and simple classification techniques are useful for obtaining epidemiological data for the industrial environment.

In conclusion, the proposed qPCR procedure is easy, sensitive, and specific and allows detection, quantification, and identification of the type of *S. thermophilus* phages in a short period of time and thus is suitable for routine use in factory-associated laboratories. Since milk storage time plays an important strategic role with economic implications, fast qPCR detection of phages would be profitable for dairy industries. Correct and rapid identification of bacteriophages potentially able to attack starter cultures allows speedy decisions concerning the destination of contaminated milk. Such milk might be earmarked for use in processes in which phages are deactivated, processes that do not require starters, or processes that employ starter bacteria not sensitive to the detected phage. In addition, qPCR would also be useful for detection and characterization of phages at all stages of milk product manufacture and in all the niches of the dairy industries.

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