Novel isolates of *Streptococcus thermophilus* bacteriophages from group 5093 identified with an improved multiplex PCR typing method

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ABSTRACT

Bacteriophages are a persistent problem in dairy plants. *Streptococcus thermophilus* is a component of thermophilic starter cultures used for cheese and yoghurt production. In this study, a multiplex PCR method was adapted to identify, in a single reaction, all known *S. thermophilus* phage groups. The method was compared with the previously published multiplex PCR typing method and showed higher specificity for classifying purified phage isolates. Three new representatives of phage group 5093, which were discovered with the developed PCR method, were genome sequenced and the genomes were compared with those available in GenBank. A validation of the two typing methods performed with whey revealed limitations of multiplex PCR assays when applied for the detection of phages in dairy samples. This study established an improved multiplex PCR method for classifying purified *S. thermophilus* phages, and expanded the genetic information of group 5093, both important steps to control phage infections in dairies.

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1. Introduction

Bacteriophages are the main cause of fermentation failures in dairy plants. *Streptococcus thermophilus* belongs to thermophilic starter cultures commonly used for cheese and yoghurt production (Binetti, Quiberoni, & Reinheimer, 2002). Control methods to detect *S. thermophilus* phages are therefore important to monitor and prevent phage infections during large-scale dairy fermentations.

Phage monitoring in dairy environment can be implemented through various microbiological and molecular methods. Microbiological assays, such as plaque assays and activity tests, are frequently used to confirm or exclude presence of phages in dairy samples (Magadán et al., 2009; Mahony, Murphy, & van Sinderen, 2012). Although these methods are accurate and sensitive, they are not optimal due to the long time required to obtain results (del Rio et al., 2007; Magadán et al., 2009). Moreover, microbiological assays do not provide information on the phage group (Labrie & Moineau, 2000). On the other hand, molecular approaches, such as amplification of a targeted phage sequence by polymerase chain reaction (PCR), are rapid and precise tools to detect and classify phages at different stages of the dairy product manufacture (Binetti, Del Río, Martin, & Álvarez, 2005; del Rio et al., 2007; Dupont, Vogensen, & Josephsen, 2005; Labrie & Moineau, 2000; Magadán et al., 2009). Molecular detection methods continuously evolve as they become more accessible and cost effective.

Characterising and grouping of dairy phages is essential for designing accurate PCR-based detection assays. *S. thermophilus* phages are currently divided into four groups: the two dominating groups *cos* and *pac*, as well as two more unique groups 5093 and 987 (McDonnell et al., 2017, 2016; Szymczak et al., 2017). Representatives of groups *cos* and *pac* can be detected with a *cos* and *pac* phage-specific multiplex PCR method (Quiberoni, Tremblay, Ackermann, Moineau, & Reinheimer, 2006). Some of the *cos*- and *pac*-group phages can be also detected by amplifying the variable region VR2 of the antireceptor gene (Binetti et al., 2005). In 2017, an improvement to the multiplex PCR assay was proposed by including two additional primer sets for the detection of 5093- and 987-group phages (McDonnell et al., 2017). However, the effectiveness of the updated method for typing phages in dairy samples has not been validated.

Genomic studies provide insights into the evolution and relatedness of phages and they aid in determining mechanisms of phage-host interactions, which is essential for the rational design of novel anti-phage strategies (Mahony, Bottacini, van Sinderen, &

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Fitzgerald, 2014; Mahony & van Sinderen, 2015). As of October 2018, there were 58 *cos*-group and 17 *pac*-group phage genomes available in GenBank, while groups 5093 and 987 had only six representatives each (Achigar, Magadán, Tremblay, Pianzzola, & Moineau, 2017; Ali et al., 2014; Desiere, Lucchini, & Brüssow, 1998; Deveau et al., 2008; Duarte et al., 2018; Guglielmotti et al., 2009; Hynes et al., 2018, 2017; Lavelle et al., 2018; Lévesque et al., 2005; Lucchini, Desiere, & Brüssow, 1999; McDonnell et al., 2016; Mills et al., 2011; Neve, Freudenberg, Diestel-Feddersen, Ehlert, & Heller, 2003; Stanley, Fitzgerald, Le Marrec, Fayard, & van Sinderen, 1997; Stanley, Walsh, van der Zwet, Fitzgerald, & van Sinderen, 2000; Szymczak et al., 2017; Tremblay & Moineau, 1999). Continuous expansion of the phage genomic database contributes to in-depth study of dairy phage biodiversity.

The purpose of this study is to propose an alternative multiplex PCR method for typing *S. thermophilus* phages and to compare it with the published method. Subsequently, we validate the usefulness of the assays for the detection of phages in whey. Moreover, we report complete genome sequences and comparison of three novel *S. thermophilus* phages from group 5093 that were identified with the method developed within this study.

2. Materials and methods

2.1. Phages, bacteria, and growth conditions

S. thermophilus and *Lactococcus lactis* strains and phages used in this study are listed in Table 1. Strains were stored at $-40\,^{\circ}\mathrm{C}$ in growth medium supplemented with 15% (w/v) glycerol and cultured overnight in appropriate growth conditions as described previously (Szymczak et al., 2017). Phages were propagated on their corresponding host and enumerated following the published

Table 1
List of phages and strains used in this study.[a]

Phage name	Phage group	Bacterial host strain
CHPC577	987	*S. thermophilus* STCH_01
CHPC926	987	*S. thermophilus* STCH_15
CHPC1151	5093	*S. thermophilus* STCH_16
CHPC951	*pac*	*S. thermophilus* STCH_12
CHPC1014	*cos*	*S. thermophilus* STCH_13
CHPC1198	5093	*S. thermophilus* STCH_17
CHPC1232	5093	*S. thermophilus* STCH_18
CHPC1282	5093	*S. thermophilus* STCH_16
—	—	*S. thermophilus* STCH_38
ul36	P335	*L. lactis* SMQ-86
P335	P335	*L. lactis* F7/2

[a] All *S. thermophilus* strains were from Chr. Hansen A/S Collection (Hørsholm, Denmark); *L. lactis* SMQ-86 and *L. lactis* F7/2 were as identified by Labrie and Moineau (2002) and Labrie et al. (2008), respectively.

procedure (Szymczak et al., 2017). Filtered phage lysates were stored at 4 °C.

2.2. Multiplex PCR

Primer sets for the detection of phage groups 987 and 5093 in method A were designed using CLC Main Workbench 7.7.3 (Invitrogen, USA). In silico analysis of specificity of primers listed in Table 2 was performed with CLC Main Workbench 7.7.3 (Invitrogen) including *S. thermophilus* and *L. lactis* strains and phages available in GenBank (as of October 2018) or in Chr. Hansen A/S Collection (unpublished data). The analysis was made using default settings for *Taq* polymerase and allowing mismatches.

In vivo testing of methods A and B was performed with selected *S. thermophilus* and *L. lactis* phages. One microlitre of purified phage lysate was used in a multiplex PCR reaction. The PCR reactions were prepared in a total volume of 50 μL using PCR Master Mix (Roche, Mannheim, Germany). Conditions for method A were: 94 °C × 5 min, followed by 35 cycles of 94 °C × 45s, 53 °C × 45s, 73 °C × 1 min, with a final extension of 73 °C × 5 min. Conditions for method B were: 95 °C × 2 min, followed by 30 cycles of 95 °C × 15s, 55 °C × 30s, 72 °C × 1 min, with a final extension of 72 °C × 10 min. PCR products were visualised on a 1.5% tris-acetate-EDTA (TAE) agarose gel.

2.3. Detection of phages in whey samples

Whey was obtained by inoculating boiled milk with *S. thermophilus* strain STCH_38, incubating overnight at 37 °C, and centrifuging at 5000×*g* for 10 min. The supernatant was filtered through 0.45-μm-pore-size filters (Sartorius, Göttingen, Germany), heat-treated at 95 °C for 15 min, and stored at 4 °C.

Phages CHPC926, CHPC1151, CHPC951, CHPC1014 were added to whey to validate the usefulness of multiplex PCR method for the detection of phages in dairy samples. For the first test, an individual phage lysate was added to whey to obtain final concentrations ranging from 10^8 to 10^5 plaque forming units (pfu) mL^{-1}. Samples were 10-fold and 100-fold diluted in MilliQ water. For the second test, all four phage lysates were added to whey. Phages had equal final concentrations ranging from 10^8 to 10^5 pfu mL^{-1}. Samples were 10-fold and 100-fold diluted in MilliQ water. For the third test, all four phage lysates were added to whey, but the final concentration of one of the four tested phages was 10-fold or 100-fold lower compared with the others. Tested phage concentrations ranged from 10^8 to 10^6 pfu mL^{-1} (Supplementary material Table S1). Samples were 10-fold diluted in MilliQ water. Negative control, i.e., whey sample without phages added, was included in the tests. One microlitre of tested sample was used in a multiplex PCR reaction, methods A and B, with the conditions described above.

Table 2
List of primers used in the multiplex PCR methods A and B.

Phage group	Primer name	Sequence, 5′ − 3′	Amplicon, bp	Used in method(s)	Reference
cos	*cos* FOR	GGTTCACGTGTTTATGAAAAATGG	170	A & B	Quiberoni et al. (2006)
	cos REV	AGCAGAATCAGCAAGCAAGCTGTT			
pac	*pac* FOR	GAAGCTATGCGTATGCAAGT	427	A & B	Quiberoni et al. (2006)
	pac REV	TTAGGGATAAGAGTCAAGTG			
987	987_RBP600 FOR	TCTGTACCTGTCTTGTTG	641	A	This study
	987_RBP600 REV	GTTTGGGGAGATGGTATT			
5093	5093_MCP300 FOR	CTGATGCTGGACAAAACT	321	A	This study
	5093_MCP300 REV	CACCTCCGAATTTAACAAC			
987	987 FOR	CTAAGCGTTTGCCACTGTCAG	707	B	McDonnell et al. (2017)
	987 REV	GCTGCCGCTTGTTTGAAAAC			
5093	5093 FOR	CTGGCTCTTGGTGGTCTTGC	983	B	McDonnell et al. (2017)
	5093 REV	GCGGCAACCATCTTAGACCAG			

2.4. Phage genome sequencing and analysis

DNA was isolated from phages CHPC1198, CHPC1232, and CHPC1282 using Phage DNA Isolation Kit (Norgen Biotek Corp., Canada). Whole genome sequencing was performed using the Illumina MiSeq platform with 2 × 250 bp paired end sequencing. Sequencing data were processed using CLC Genomics Workbench 10.1.1 (Invitrogen). Reads were trimmed using the Trim Sequences tool and assembled using De Novo Assembly tool automatic setup and minimum contig length of 600. The assembled genomes were functionally annotated using RASTtk pipeline with default parameters (Brettin et al., 2015). Annotations were verified manually by comparative genomics with *S. thermophilus* phage genome entries available in GenBank. The similarities of phage genomes with other organisms available in GenBank (as of October 2018) were assessed by using the Basic Local Alignment Search Tool (BLAST), provided by the National Centre for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Genomic similarities were visualised using EasyFig (Sullivan, Petty, & Beatson, 2011).

2.5. Accession numbers

Phage genomes sequenced in this study were deposited in GenBank under the accession numbers MK202159 (CHPC1198), MK202160 (CHPC1232), and MK202161 (CHPC1282).

3. Results and discussion

3.1. Comparison of two multiplex PCR methods

Two multiplex PCR methods, which allow detecting all known groups of *S. thermophilus* phages, were tested in this study. Each method included four primer sets for typing four groups of *S.*

thermophilus phages: two previously published primer sets for the detection of *cos*- and *pac*-group phages (Quiberoni et al., 2006), and two different primer sets for the detection of 987- and 5093-group phages. For method A, primer sets targeting phages from groups 987 and 5093 were designed in this study. For method B, primer sets targeting these groups were published before (McDonnell et al., 2017). The predicted binding sites and amplicon lengths for groups 987 and 5093 varied between methods A and B. Sequences of all tested primers are listed in Table 2.

To verify the practical application of methods A and B for identifying the four groups of *S. thermophilus* phages, multiplex PCR was performed with selected phage representatives. For each method, the four primer sets (Table 2) were used together in one reaction and were tested with phages that belong to group *cos*, *pac*, 987 or 5093. *L. lactis* phages ul36 (Labrie & Moineau, 2002) and P335 (Labrie, Josephsen, Neve, Vogensen, & Moineau, 2008), which are related to dairy streptococcal phages from group 987 (Szymczak et al., 2017), were also used in the assay to verify the primer specificity for the detection of phages of one species. Additionally, three *S. thermophilus* phages of unknown groups, CHPC1198, CHPC1232, CHPC1282, were used in the assay to establish their identity. All phages used in the multiplex PCR are listed in Table 1.

Members of the four groups of *S. thermophilus* phages were successfully detected with method A (Fig. 1A, lanes 1–5). The two proposed primer sets, one specific for the 987-group phages and another for the 5093-group phages, were fully compatible with the previously published primers for the *cos* and *pac* grouping (Quiberoni et al., 2006). They had similar annealing temperature, so they could be used in a single PCR reaction. The DNA fragments amplified by the four primer sets had different lengths. Thus, the detected phages could be immediately assigned to the adequate phage group corresponding to the amplicon size visualised on the agarose gel. The proposed primers did not amplify a PCR fragment
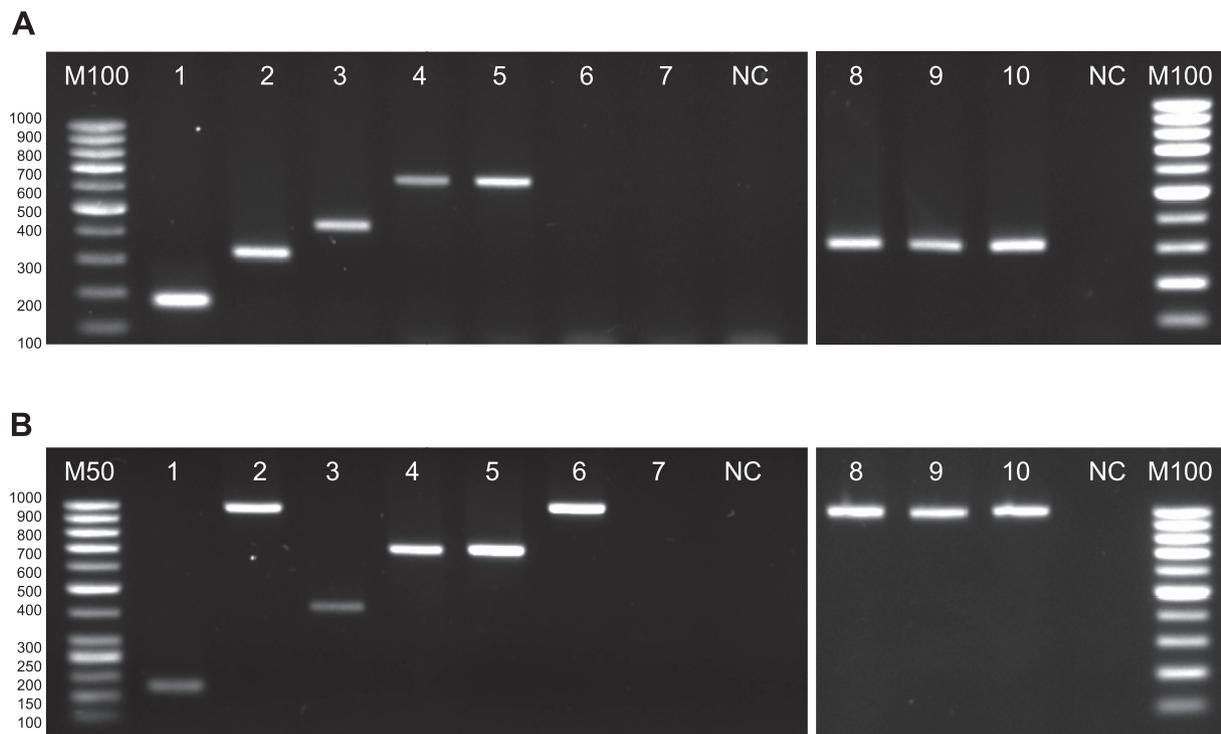
Fig. 1. Agarose gel with PCR fragments amplified from phage DNA with the multiplex PCR methods A (panel A) and B (panel B). Lanes 1–5 and 8–10, *S. thermophilus* phages: (1) CHPC1014, *cos* group; (2) CHPC1151, 5093 group; (3) CHPC951, *pac* group; (4) CHPC926, 987 group; (5) CHPC577, 987 group; (8) CHPC1198, (9) CHPC1232, (10) CHPC1282, investigated groups. Lanes 6 and 7, *L. lactis* phages from the P335 group: (6) ul36 and (7) P335. M100, GeneRuler 100 bp DNA Ladder (Thermo Scientific, Karlsruhe, Germany); M50, GeneRuler 50 bp DNA Ladder (Thermo Scientific); NC, negative control (MilliQ H$_2$O).

of the tested *L. lactis* phages from P335 group (Fig. 1A, lanes 6—7). These results highlighted the specificity of the multiplex PCR method A for typing *S. thermophilus* phages.

Method B also enabled detecting phages from the four groups (Fig. 1B, lanes 1—5), which confirmed the previous results (McDonnell et al., 2017). However, primer sets used in multiplex PCR method B gave an amplicon with *L. lactis* phage ul36 (Fig. 1B, lane 6). Further in silico and in vivo analyses showed, that primer set of method B, which was designed to detect 987-group phages, can attach to *L. lactis* phage ul36 and create a fragment of 1013 bp in length. This fragment size is only 30 bp longer from the amplicon length for group 5093 detected with method B. Thus, the study indicated that method B can give false positive results with phages from other dairy species and may lead to classifying phages into incorrect groups.

3.2. New representatives of group 5093

Methods A and B gave PCR products with the three phages of unknown groups used in the study. Based on the amplicon lengths, phages CHPC1198, CHPC1232, CHPC1282 were assigned to group 5093 (Fig. 1, lanes 8—9). To confirm those results, the three phages were subjected to genome sequencing and further genomic analyses.

Complete genome sequences were obtained for phages CHPC1198, CHPC1232, and CHPC1282. The studied phages had genome lengths of 33,245 bp, 31,974 bp, and 34,785 bp, respectively. The sequences had GC contents of approximately 38%. Using RASTtk (Brettin et al., 2015), 47 coding sequences (CDS) were annotated for phage CHPC1198, and 45 CDS were identified for phages CHPC1232 and CHPC1282.

A BLAST similarity search revealed that the three phages studied were closely related to *S. thermophilus* phages from group 5093. They displayed 98—99% nucleotide identity across at least 67% of the phage genomes that belong to this group. The nucleotide sequence similarities of the three investigated phages and phage 5093, which is the first described representative of the group (Mills et al., 2011),

are presented in Fig. 2. The highest genetic variation was observed in the replication and transcription modules, while the modules coding for DNA packaging and structural proteins were highly similar. The genomic analysis confirmed that phages CHPC1198, CHPC1232, CHPC1282 were correctly assigned to group 5093 based on the results of the PCR methods A and B (Fig. 1, lanes 8—10).

Phages CHPC1198, CHPC1232, CHPC1282 were originally purified on different strains (Table 1), however, as verified in a spot test, phages CHPC1198 and CHPC1232 formed plaques with each other hosts. These phages had high sequence similarity in the morphogenesis module, which includes the receptor binding protein, as well as in the replication module (Fig. 2). The observed similarity can explain the overlapping host range. Moreover, phages CHPC1198 and CHPC1232 were isolated from cheese fermentations located in the same geographic locations in USA, while phage CHPC1282 was obtained from a cheese fermentation in Argentina. As of October 2018, group 5093 contained six representatives, and all of them were isolated from dairy plants in Europe. Thus, genome sequences of the three new phages provide an important expansion of the diversity of group 5093.

### 3.3. Validation of the multiplex PCR methods for typing phages in dairy samples

Food components are known to interfere with PCR assays (Brussow et al., 1994; Labrie & Moineau, 2000). Therefore, methods A and B were validated for the detection of phages in whey samples. Phages CHPC926, CHPC1151, CHPC951, CHPC1014, representing phages from the four groups, were used in the study (Table 1). Whey samples spiked with each phage separately, mix of all four phages at equal concentrations, and mix of all four phages at different concentrations were used in tests.

Previously reported PCR assays for *L. lactis* phages enabled the detection of phages directly in whey samples (Brussow et al., 1994; Labrie & Moineau, 2000). In this study, inhibition of PCR assay was observed with whey samples (Fig. 3). Dilution of whey samples in
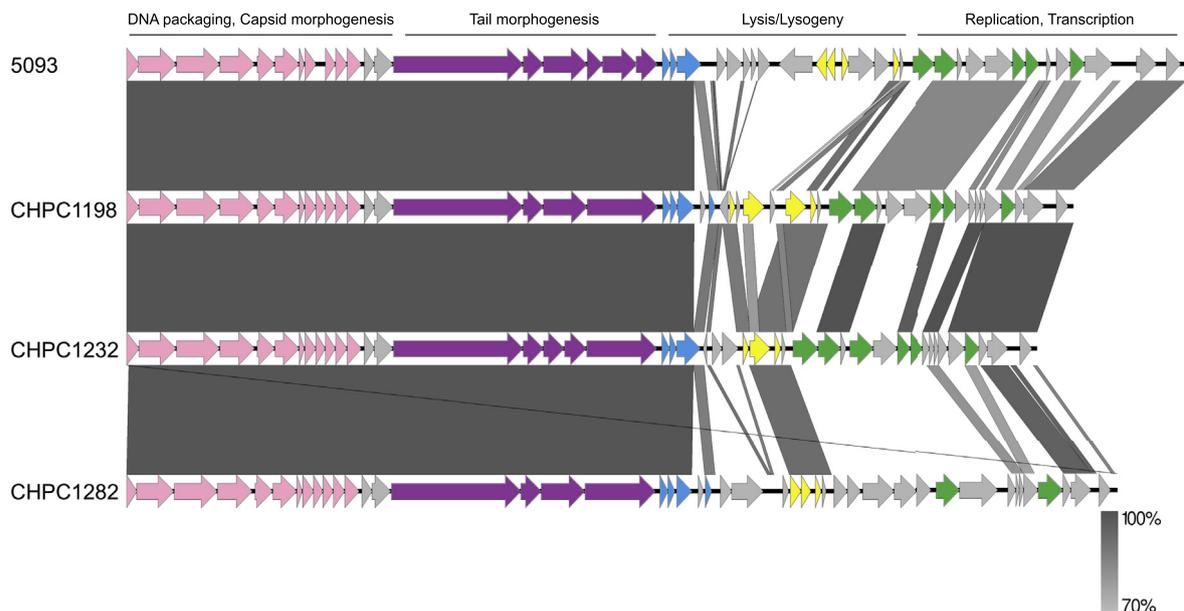

Fig. 2. Schematic comparison of nucleotide sequences of phages CHPC1198, CHPC1232, CHPC1282, which were sequenced in this study, and phage 5093 (accession no. FJ965538), which is a representative of *S. thermophilus* phages from group 5093. The functions of the gene modules are indicated above the diagram. Each arrow represents an individual protein coding region with a corresponding colour coding: pink, DNA packaging and capsid morphogenesis encoding genes; purple, tail and receptor binding protein encoding genes; blue, holin and lysin encoding genes; yellow, repressor and antirepressor encoding genes; green, DNA replication encoding genes; grey, hypothetical proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
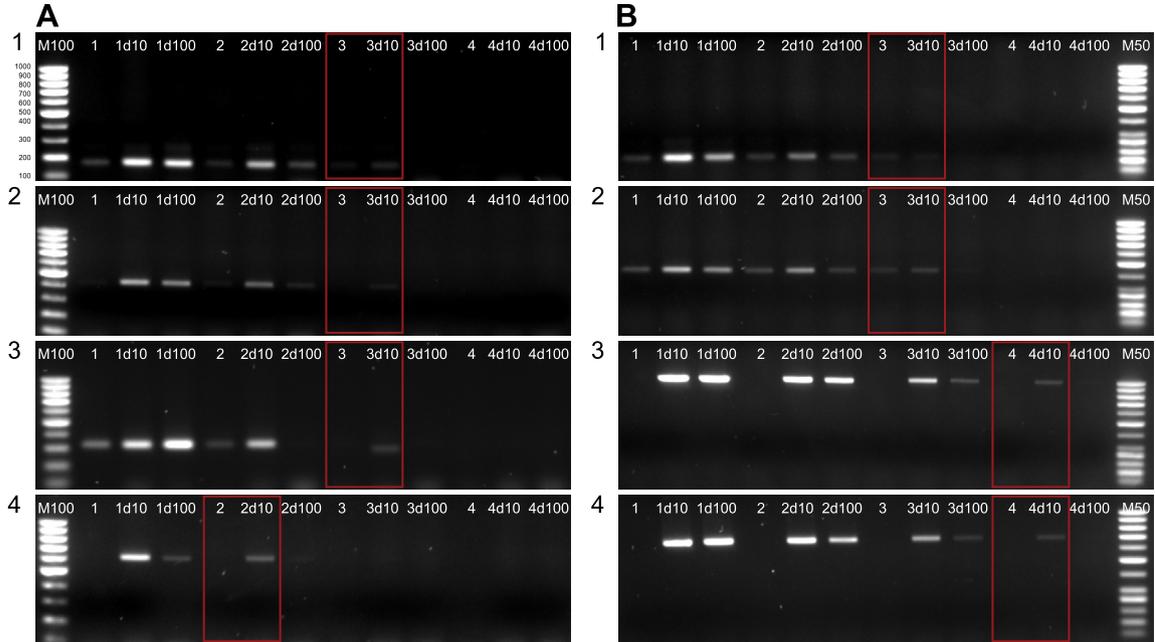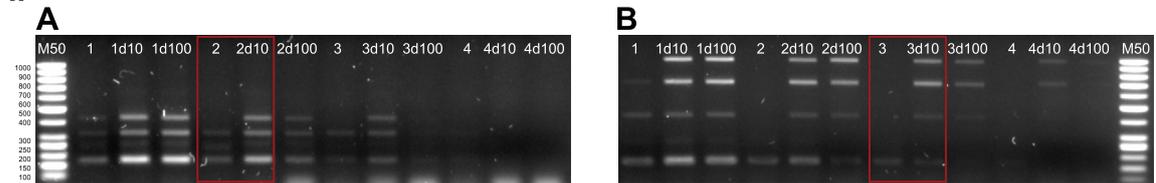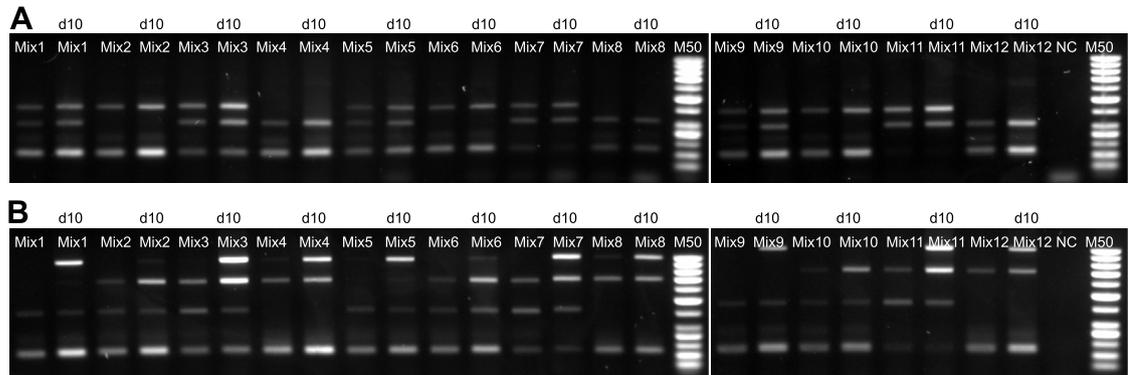
Fig. 3. Detection of phages in whey samples: agarose gels with PCR fragments amplified with multiplex PCR methods A (panels A) and B (panels B). Test I: whey samples spiked with individual *S. thermophilus* phages: CHPC1014, *cos* group (gels no. 1); CHPC951, *pac* group (gels no. 2); CHPC1151, 5093 group (gels no. 3); CHPC926, 987 group (gels no. 4). Test II: whey samples spiked with mix of four phages, CHPC1014, CHPC951, CHPC1151, CHPC926, at equal concentrations. Test III: whey samples spiked with mix of four phages, CHPC1014, CHPC951, CHPC1151, CHPC926, at different concentrations. Final concentrations of phages used in test III are listed in Supplementary material Table S1. Final concentrations of phages used in tests I and II: samples 1, 2, 3, 4 represent 108, 107, 106, 105 pfu mL^{-1}, respectively. d10, 10-fold dilution of a given sample in MilliQ water; d100, 100-fold dilution of a given sample in MilliQ water; M100, GeneRuler 100 bp DNA Ladder (Thermo Scientific); M50, GeneRuler 50 bp DNA Ladder (Thermo Scientific); NC, negative control (whey without phages added). Detection limits are marked with red frames. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

water lowered the concentration of potential PCR inhibitors below the interfering threshold, resulting in a positive fragment amplification.

The individual phages present in whey samples, which were 10- and 100-fold diluted in water, were detected with methods A and B. The detection limit for method A was between 10^6 and 10^7 pfu mL^{-1}, while for the method B it was between 10^5 and 10^6 pfu mL^{-1} (Fig. 3, Test I). Both methods showed lower sensitivity compared with the multiplex PCR typing methods of other dairy phages reported previously (Brussow et al., 1994; del Rio et al., 2007; Labrie & Moineau, 2000).

All four phages added simultaneously to whey, followed by a 10- fold dilution in water, were successfully detected with methods A and B, if phages were present in a sample at equal concentrations of

minimum 10^7 pfu mL^{-1} and 10^6 pfu mL^{-1}, respectively (Fig. 3, Test II). However, phages present in concentrations 10-fold or 100-fold lower than other phages in the mixture, were not detected with the multiplex PCR methods A and B (Fig. 3, Test III). Phages in dairy samples would likely be present at different concentrations. Therefore, the results question the usefulness of multiplex PCR for detecting phages in whey samples.

The additional in silico analysis showed, that the primer set designed to detect 987-group phages in method A could attach to the DNA of *L. lactis* strain holding a prophage. If tested in the presence of these bacteria, the proposed multiplex PCR could give a false positive result. This should be considered as another limitation of applying multiplex PCR methods for typing phages in dairy samples that contain starter cultures. Moreover, PCR assays cannot establish whether the detected phages are viable or whether they can infect a starter culture. For this purpose, the microbiological assays, such as spot tests, would be compulsory (Magadán et al., 2009).

The proposed multiplex PCR method A can be used for a rapid grouping of phages purified from fermentation samples based on different PCR fragment lengths. The proper phage classification could be useful for the selection of strains for a rotation scheme (Derkx et al., 2014). Since the genetic diversity of dairy streptococcal phages may be greater than currently known, improvements to the PCR-based typing system will likely be developed in the future.

4. Conclusions

In this report, we delivered a molecular tool for the rapid classification of *S. thermophilus* phages purified from dairy fermentations and provide genome sequences of three novel representatives of group 5093, which were identified with the proposed method. As opposed to the previously published multiplex PCR method for the detection of dairy streptococcal phages (McDonnell et al., 2017), the new PCR method was specific for classifying purified *S. thermophilus* phages and did not amplify fragments from *L. lactis* phages. Additionally, the results of this study exposed limitations of multiplex PCR systems for the detection of phages in dairy samples, since different phages can only be detected, if they are present in whey samples at equal concentrations. Thus, these methods should be used with scepticism for the detection of phages in dairy plants.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2018.12.001.

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