GENETIC DIVERSITY OF BACTERIOPHAGES HIGHLY SPECIFIC FOR STREPTOCOCCUS THERMOPHILUS STRAIN LBB.A

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ABSTRACT

Eight virulent bacteriophages fA1, fA2, fA5, fA6, fDmA, fSfA, fPtA and fA7, that showed high specificity for Streptococcus thermophilus strain LBB.A, the streptococcal component of an industrial yogurt starter, were isolated. The comparison of the HindIII and EcoRV restriction patterns of the phage DNA revealed limited relatedness between the isolates, clustering together only fA5 with fA6 and indicating similarity between fSfA, fPtA and fA7. All eight bacteriophages were classified as cos-type phages. An internal region within a conserved 2.2 kbp EcoRI DNA fragment typical for the majority of S. thermophilus bacteriophages was present in six of the phage isolates and was absent in fDmA and fA1. PCR amplification of the VR2 region of the antireceptor gene yielded an 800 bp product for seven of the phages and a 700 bp product for fA2. The genome size of the eight phage isolates was in the range of 33.4-37.3 kbp. Of a total of 62 other S. thermophilus cultures fA1, fDmA and fPtA were able to infect only two strains with reduced effectiveness of plaquing. Despite the broad genetic diversity of phages of S. thermophilus strain LBB.A, a two-step selection procedure, involving successive challenge of the bacterial culture with fA1 and fA7, was sufficient to obtain mutants resistant to all eight phages.

Keywords: bacteriophage, bacteriophage insensitive mutant, genetic diversity, restriction polymorphism, Streptococcus thermophilus

Introduction

In yogurt production phage activity results in prolonged fermentation process leading to economic loss or product quality deterioration. Bacteriophages that infect the two principle bacterial species of yogurt Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus thermophilus have been isolated and well characterized (2, 4, 15, 20, 22, 23, 29, 31). Different phage defence systems have been described and/or introduced in S. thermophilus cultures to combat phage attack (8, 14, 18, 21, 25, 26). However, the effectiveness of a phage resistance mechanism is challenged by the high genetic variability among S. thermophilus phages. It is proposed that the phage genome has a modular evolutionary structure (17), what makes it difficult to estimate the relatedness between phage species. Presently S. thermophilus phages can be classified mainly according to their cos- or pac-type packaging mechanism (15, 23).

S. thermophilus strain LBB.A was used to produce an industrial yogurt starter with 30-years old history of application, preferred in the production of traditional Bulgarian yogurt with typical aroma and free amino-acid profile (10, 12, 30). The aim of the present study was to isolate and perform a comparative characterization of bacteriophages infecting S. thermophilus strain LBB.A as a starting point in the selection of phage protection strategy for this culture. For this purpose phage DNA restriction profiles analysis and PCR amplification of characteristic phage DNA sequences were applied.

Materials and Methods

Bacterial strains, isolation, propagation and enumeration of bacteriophages

S. thermophilus strains LBB.A, LBB.T12/14 and LBB.K9/2 and another 60 strains originate from home-made yogurt and are maintained in the LBB culture collection (LB Bulgaricum PLC, Sofia, Bulgaria). Strain LBB.A is used as the streptococcal component of an industrial yogurt starter. Bacterial strains were grown on sterile 10% reconstituted skim milk powder or M17 broth (28).

Samples of yogurt or raw milk were initially collected in the South-Western part of Bulgaria in the period of 2002-2007. Bacteriophages infecting S. thermophilus LBB.A were isolated from the samples from single plaques obtained with the double layer plaque method (27), using M17-Ca medium (M17 supplemented with 10 mM CaCl2). Single plaques were then transferred to M17-Ca broth and propagated on S. thermophilus LBB.A. For long-term preservation phages were also propagated in milk medium and whey preparations were stored at -18°C in 30% glycerol. A list of isolated phage cultures is given in Table 1. The double layer plaque method was also used for titration of bacteriophages in 10-fold serial dilutions.

All incubations were performed overnight at 37°C.

Determination of the host range of the phage cultures

The host range of the isolated bacteriophages was determined by the spot test as described by Svensson & Christiansson (27). Briefly, 10 µl undiluted phage lysates, obtained with strain LBB.A, were spotted on a double-layer M17-Ca agar with BIOTECHNOL. & BIOTECHNOL. EQ. 23/2009/3
the upper soft agar mixed with overnight M17 culture of a *S. thermophilus* strain. In parallel, 50 µl of each *S. thermophilus* culture were inoculated into 5 ml M17-Ca broth and tested for the occurrence of lysis at 37°C for 24 hours after the addition of 100 µl phage lysate containing not less than 10^6 pfu ml⁻¹ of a particular phage isolate. Strains which gave clear zones with the spot test and/or were lysed by any of the tested phage cultures were considered sensitive and their effectiveness of plaquing (EOP) was determined with the double layer plaque method. The EOP was calculated from the ratio between the number of plaques formed on an alternative host strain and the number of plaques formed on the original host strain *S. thermophilus* LBB.A.

**Bacteriophage insensitive mutant (BIM) isolation**

Two-step selection procedure of BIMs was applied using successive challenging the parental strain with two phage cultures. The remaining viable cells of *S. thermophilus* strain LBB.A in M17-Ca broth lysate, obtained with φA1, were collected by centrifugation at 3000 g for 10 min. The pellet was resuspended in fresh M17 broth and incubated overnight at 37°C. The resulting culture was transferred once more in M17-Ca broth with added φA1 to a multiplicity of infection 1:10 and grown overnight at 37°C. Then, cells were plated on M17 agar and single colonies were picked up. Mutants, insensitive to all eight phages, were obtained after repeating the same procedure once again by challenging φA1-insensitive BIMs with φA7. The phage insensitivity of the potentially resistant mutants was confirmed by the lack of lysis in M17-Ca broth, absence of clear zones in the spot test and EOP values less than 10⁻⁷. Phage resistant mutants AR-s¹, insensitive to φA1 and AR-s³ ₃, insensitive to all eight phages, were selected after the acidification rate of BIMs was compared with the mother strain LBB.A. The acidification curves were prepared on the base of pH data of milk inoculated with 1% of culture and incubated at 37°C.

**Phage DNA isolation**

For phage DNA isolation, bacteriophages were propagated overnight on the host strain LBB.A in milk. To complete cell lysis chloroform was added to a final concentration of 0.5% (v/v) and when needed milk was coagulated with 50% lactic acid solution. Samples were then centrifuged at 10 000 g for 10 min and 10 ml of the resulting whey were used to prepare acid solution. Samples were then centrifuged at 10 000 g for 10 min and 10 ml of the result of which were used to prepare phage DNA with the Quiagen Lambda Mini Kit (Quiagen, Hilden, Germany) according to the manufacturer’s protocol.

**Determination of the phage genome size**

The size of the genome of the phage isolates was evaluated with Pulsed Field Gel Electrophoresis (PFGE) as described by Binetti et al. (1). Briefly, PFGE was performed on a CHEF-DRII electrophoresis unit (Bio-Rad, Hercules, CA) with 1.2% pulsed-field-certified agarose gel (Bio-Rad) in 0.5 x TBE buffer. The electrophoresis parameters were as follows: pulse ramp 1-6 s at 5 V cm⁻¹ for 17 hours and buffer temperature of 14°C. Low range molecular marker λ DNA x HindIII and BIOTECHNOL. & BIOTECHNOL. EQ. 23/2009/3

50-1000 kb – Pulse Marker (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) were used as standards. The genome size was determined with the GelCompar (Applied Maths BVBA, Koptrijk, Belgium) gel analysis software.

**Restriction fragment length polymorphism analysis**

Five hundred nanograms of phage DNA were digested with 20U of one of the two restriction enzymes HindIII or EcoRV, overnight at 37°C. Next, restricted DNA was incubated for 15 min at 65°C to denature the cohesive termini of cos-type phages. Restriction fragments were electrophoretically separated on a 1.2% agarose gel at 100V in 1 x TAE buffer. The 1 kb Plus DNA ladder (USB Corporation, Ohio, USA) was used as size standard. The obtained restriction patterns were analyzed with the GelCompar (Applied Maths BVBA, Koptrijk, Belgium) gel analysis software. Clustering of patterns was performed by the Dice-UPGMA method.

**PCR amplifications**

Phage DNA was used to amplify the gene of the small major structural protein of cos-type *S. thermophilus* phages using the primers mpSF 5'-ATGGCAATTGAGTCTTGT-3' and mpSR 5'-GCTTGAGATATTAGA-3' (15); a conservative DNA sequence of *S. thermophilus* bacteriophages with the primers BrF 5'-GCCATTCTTAAAGGAG-3' and BrR 5'-CGCTGCAAAACCCAGTC-3' (3, 15); the VR2 variable region of the phage antireceptor gene with primers HOST1 5'-GAATGATACTGCTGGCACTATTTTCGTTG-3' and HOST5 5'-AGCTCATGTAGCTATCGATGAAATTCAACG-3' (1). Amplification was performed on a 9600 GeneAmp PCR System (Perkin-Elmer, Norwalk, Connecticut) in a 25 µl reaction mixture consisting of puReTaq Ready-To-Go PCR System (Perkin-Elmer, Norwalk, Connecticut) in a 25 µl reaction mixture consisting of puReTaq Ready-To-Go PCR Beads (Amersham Biosciences AB, Uppsala, Sweden), 50 ng template DNA and 10 pmol of each primer. The PCR program was as follows: 1 cycle of 3 min at 95°C; 35 cycles of 30 s at 94°C, 30 s at 50°C, 30 s at 72°C and 1 cycle of 7 min at 72°C. With this program the majority of phages did not yield PCR products with primers HOST1/HOST5, therefore additional amplification experiments were carried out at annealing temperature of 45°C.

**TABLE 1**

List of isolated bacteriophage cultures infecting *Streptococcus thermophilus* LBB.A

<table>
<thead>
<tr>
<th>Bacteriophage</th>
<th>Year of isolation</th>
<th>Source</th>
<th>Bacteriophage</th>
<th>Year of isolation</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>φA1</td>
<td>2002</td>
<td>yogurt</td>
<td>φA2</td>
<td>2006</td>
<td>yogurt</td>
</tr>
<tr>
<td>φA2</td>
<td>2002</td>
<td>yogurt</td>
<td>φDmA</td>
<td>2006</td>
<td>raw milk</td>
</tr>
<tr>
<td>φA5</td>
<td>2005</td>
<td>yogurt</td>
<td>φSA</td>
<td>2006</td>
<td>yogurt</td>
</tr>
<tr>
<td>φA6</td>
<td>2006</td>
<td>yogurt</td>
<td>φPtA</td>
<td>2007</td>
<td>yogurt</td>
</tr>
<tr>
<td>DmA 2006 yogurt</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Results and Discussion**

The genome size of the phage isolates in this study was as follows: φA1 – 36.6 kbp; φA2 – 37.3 kbp; φA5 and φA6 –
- 35.3 kbp; \( \phi \text{DmA} \) – 36.0 kbp; \( \phi \text{SfA} \) – 35.3 kbp; \( \phi \text{PtA} \) and \( \phi \text{A7} \) – 33.4 kbp (Fig. 1). This is in good agreement with the established genome sizes of other virulent cos-type phages of \textit{S. thermophilus}, reported to be 33.8 – 36.7 kbp for nine Subgroup III phages (22); 36.2 kbp and 35.6 kbp for \( \phi \text{A1.1} \) and \( \phi \text{B1.2} \) respectively (13); 34.8 kbp for \( \phi \text{DT1} \) (29), and 30.8 kbp – 39.7 kbp for 10 other cos-type phage species (1).

With the exception of a common 2.0 kbp \textit{HindIII} fragment, the restriction profiles of the eight phage cultures with \textit{HindIII} and \textit{EcoRV} displayed high genetic variability (Fig. 2). Only phages \( \phi \text{A5} \) and \( \phi \text{A6} \) were closely related and \( \phi \text{SfA} \), \( \phi \text{PtA} \) and \( \phi \text{A7} \) formed a group, distinguishable from the rest of the phages. These observations were confirmed by the dendrogram obtained by calculating the pattern similarities, clustering together \( \phi \text{A5} \) and \( \phi \text{A6} \), grouping \( \phi \text{SfA} \), \( \phi \text{PtA} \) and \( \phi \text{A7} \) (>90% similarity) and displaying \( \phi \text{A1} \), \( \phi \text{A2} \) and \( \phi \text{DmA} \) as unrelated isolates with less than 65% similarity (Fig. 3). The analysis of the restriction fragment length polymorphism of the phage isolates showed that at least five genetically unrelated phage cultures infected the host strain \textit{S. thermophilus} LBB.A.

![Fig. 1. Determination of the phage genome size by pulsed field gel electrophoresis. Lane number and phage isolate: 1. \( \phi \text{A1} \); 2. \( \phi \text{A2} \); 3. \( \phi \text{A5} \); 4. \( \phi \text{A6} \); 5. \( \phi \text{DmA} \); 6. \( \phi \text{SfA} \); 7. \( \phi \text{PtA} \) and 8. \( \phi \text{A7} \). Size markers: m - low range molecular marker \( \lambda \) DNA x \textit{HindIII}; M - Pulse Marker 50-1000 kb](image)

![Fig. 2. Restriction patterns of eight bacteriophages, obtained with \textit{HindIII} (lanes 1-8) and \textit{EcoRV} (lanes 9-16). Phage (lane): \( \phi \text{A1} \) (1, 9), \( \phi \text{A2} \) (2, 10), \( \phi \text{A5} \) (3, 11), \( \phi \text{A6} \) (4, 12), \( \phi \text{DmA} \) (5, 13), \( \phi \text{SfA} \) (6, 14), \( \phi \text{PtA} \) (7, 15) and \( \phi \text{A7} \) (8, 16); M- molecular size marker](image)
All phage isolates gave positive amplification of the gene for the small major structural protein of cos-type \textit{S. thermophilus} phages (Table 2). Therefore they were classified as phages of the cos-type group. This conclusion was also confirmed by observed differences in the electrophoretic pattern of pre-heated and untreated restricted phage DNA (results not shown). Lévesque et al. (16) suggest that most phage-sensitive \textit{S. thermophilus} strains are infected either by cos- or pac-type phages. Indeed, all eight phages infecting strain LBB.A proved to be only cos-type phages. Other phage sensitive \textit{S. thermophilus} strains from the LBB culture collection were also selective for these two phage types (results not shown). Nevertheless, other authors (15, 23) report that some \textit{S. thermophilus} strains are sensitive to both cos- or pac-type phages.

The presence of a conserved DNA sequence of \textit{S. thermophilus} bacteriophages (3) was confirmed for only 6 of the analysed phage isolates and it was absent in \textit{f}A1 and \textit{f}DmA (Table 2). Brüssow et al. (2) identified this sequence and used it to develop the first method for direct PCR detection of \textit{S. thermophilus} phages in dairy products. However phages which do not contain this sequence were also described (3). This variation was also observed by Le Marrec et al. (15), who confirmed the presence of this sequence for 9 phage cultures out of 19 cos-type \textit{S. thermophilus} phages analysed. In our study the absence of this conserved DNA target sequence in phage isolates \textit{f}A1 and \textit{f}DmA gave additional evidence that \textit{S. thermophilus} LBB.A was infected by genetically unrelated phages.

At annealing temperature of 50°C PCR amplification of the variable VR2 region of the antireceptor gene of the phage isolates yielded no product for \textit{f}A1, \textit{f}DmA, \textit{f}SfA, \textit{f}PtA and \textit{f}A7, whereas a 700 bp product for \textit{f}A2 and an 800 bp product for \textit{f}A5 and \textit{f}A6 were obtained (Table 2). Lowering the annealing temperature to 45°C resulted in the amplification of 800 bp product for \textit{f}A1, \textit{f}DmA, \textit{f}SfA, \textit{f}PtA and \textit{f}A7, but a second smaller unspecific band was also observed, indicating further difference in the target sequence in the region of the applied primers. The size of the obtained products was within the range of 700-800 bp determined for the VR2 sequence in other \textit{S. thermophilus} phages by Binetti et al. (1). The same authors demonstrate that clustering of \textit{S. thermophilus} phages according to the sequence variation of the VR2 region correlates with their host range. In our study all eight phages were highly specific for \textit{S. thermophilus} LBB.A. Therefore the different results from the amplification of the VR2 region of the antireceptor gene of the eight bacteriophages suggest potential differences in the recognition of the host \textit{S. thermophilus} LBB.A during adsorption by a particular phage isolate. These results may also suggest that the eight phage cultures adsorb to different receptors on the surface of strain LBB.A or to different structures within the same receptor. Although the phage receptors in \textit{S. thermophilus} are not yet identified, in other Gram-positive bacteria, such as \textit{Lactococcus lactis} and \textit{Bacillus subtilis}, phage adsorption is attributed to interaction of the phage antireceptor with cell wall carbohydrates and/or specific membrane proteins of the host (7, 9, 24).

**Table 2**

<table>
<thead>
<tr>
<th>Bacteriophage</th>
<th>Primer pair mpSF/mpSR</th>
<th>Primer pair BrF/BrR</th>
<th>Primer pair HOST1/HOST5</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{f}A1</td>
<td>+</td>
<td>-</td>
<td>800 bp*</td>
</tr>
<tr>
<td>\textit{f}A2</td>
<td>+</td>
<td>+</td>
<td>700 bp</td>
</tr>
<tr>
<td>\textit{f}A5</td>
<td>+</td>
<td>+</td>
<td>800 bp</td>
</tr>
<tr>
<td>\textit{f}A6</td>
<td>+</td>
<td>+</td>
<td>800 bp</td>
</tr>
<tr>
<td>\textit{f}DmA</td>
<td>+</td>
<td>-</td>
<td>800 bp*</td>
</tr>
<tr>
<td>\textit{f}SfA</td>
<td>+</td>
<td>+</td>
<td>800 bp*</td>
</tr>
<tr>
<td>\textit{f}PtA</td>
<td>+</td>
<td>+</td>
<td>800 bp*</td>
</tr>
<tr>
<td>\textit{f}A7</td>
<td>+</td>
<td>+</td>
<td>800 bp*</td>
</tr>
</tbody>
</table>

* PCR amplification at lower annealing temperature (see text)

The eight phage isolates, infecting \textit{S. thermophilus} strain LBB.A displayed extremely narrow host range. Sixty other \textit{S. thermophilus} strains were found to be completely insensitive to the studied phage cultures as no clear zones with the spot test and no lysis in M17-Ca broth was observed. Beside strain LBB.A, the spot test was positive for strain LBB.T12/14 with \textit{f}DmA and strain LBB.K9/2 with \textit{f}A1. Lysis in M17-Ca broth was observed only for LBB.T12/14 treated with \textit{f}DmA. Plaques of \textit{f}DmA and \textit{f}PtA were observed on \textit{S. thermophilus} strain LBB.T12/14 and of \textit{f}A1 on \textit{S. thermophilus} strain LBB.K9/2 with effectiveness of plaquing (EOP) of $10^3-10^4$ (Table 3). Practically phages \textit{f}A2, \textit{f}A5, \textit{f}A6, \textit{f}SfA and \textit{f}A7 were specific only to \textit{S. thermophilus} strain LBB.A. With so narrow host specificity it was not possible to differentiate the eight phage cultures by their host range with the exception of \textit{f}DmA, suggesting that this phage, unlike the other seven phage cultures, can recognize a common receptor on \textit{S. thermophilus} strains LBB.A and LBB.T12/14.
Plaque formation (+ or -) and effectiveness of plaquing (EOP) of phage isolates infecting alternative *Streptococcus thermophilus* strains and BIMs AR₂<sup>A₁</sup>, insensitive to φA₁ and AR₃₃<sup>₃</sup><sup>₁₃</sup><sup>A₁₇</sup> sensitive to all eight phages. EOP for each phage on the original host strain LBB.A is considered 1; the lack of any plaque formation in the experiment corresponds to EOP<sub>≤</sub>10<sup>⁻⁷</sup>.

![Fig. 4](image_url)

**TABLE 3**

<table>
<thead>
<tr>
<th>Streptococcus thermophilus strain</th>
<th>Bacteriophage</th>
<th>LBB.A</th>
<th>LBB.T12/14</th>
<th>LBB.K9/2</th>
<th>AR₂&lt;sup&gt;A₁&lt;/sup&gt;</th>
<th>AR₃₃₃&lt;sup&gt;₃&lt;/sup&gt;&lt;sup&gt;₁₃&lt;/sup&gt;&lt;sup&gt;A₁₇&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>φA₁</td>
<td>+</td>
<td>-</td>
<td>+ (10&lt;sup&gt;⁻⁵&lt;/sup&gt;)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>φA₂</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>φA₅</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>φA₆</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>φDmA</td>
<td>+</td>
<td>-</td>
<td>+ (10&lt;sup&gt;⁻⁵&lt;/sup&gt;)</td>
<td>-</td>
<td>+ (10&lt;sup&gt;⁻⁵&lt;/sup&gt;)</td>
<td>-</td>
</tr>
<tr>
<td>φSfA</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+ (10&lt;sup&gt;⁻⁵&lt;/sup&gt;)</td>
<td>-</td>
</tr>
<tr>
<td>φPtA</td>
<td>+</td>
<td>-</td>
<td>+ (10&lt;sup&gt;⁻⁵&lt;/sup&gt;)</td>
<td>-</td>
<td>+ (1)</td>
<td>-</td>
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<tr>
<td>φA₇</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+ (10&lt;sup&gt;⁻¹&lt;/sup&gt;)</td>
<td>-</td>
</tr>
</tbody>
</table>

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) present in the genome of *S. thermophilus* (5, 11). This integration event correlates with the acquisition of a phage resistant trait in the bacterial population.

Phage resistant mutants AR₂<sup>A₁</sup> and AR₃₃₃<sup>₃</sup><sup>₁₃</sup><sup>A₁₇</sup> acidified milk at a rate comparable to the mother strain LBB.A (Fig. 4). Consequently AR₃₃₃<sup>₃</sup><sup>₁₃</sup><sup>A₁₇</sup> was recommended as a potential replacement of the sensitive mother strain in yogurt production.

**Conclusions**

*Streptococcus thermophilus* strain LBB.A was infected by at least five genetically unrelated cos-type phages, which, at the same time, were highly specific for their host. The results from our study provide further evidence that the specialization of bacteriophages to a particular *S. thermophilus* strain and the overall evolution of the phage genome are two independent processes (6, 17). The diversity of phages infecting strain LBB.A questioned the success of selection procedures for obtaining bacteriophage insensitive derivatives of this culture. Nevertheless, by successive challenge of the mother culture with φA₁ and φA₇ in a two-step selection procedure BIMs of technological interest, resistant to all eight phages were obtained. Consequently, we consider strain LBB.A an excellent model for studies of phage-host interaction of *S. thermophilus* phages.

**REFERENCES**