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Efficient Isolation and Functional Analysis of Spontaneous *Streptococcus thermophilus* Bacteriophage-Insensitive Mutants

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Research Article

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ABSTRACT

The use of bacteriophage-resistant strains that have satisfactory technological properties is essential for preventing phage infections in industrial fermentation processes. Here, we describe an improved method for the isolation of spontaneous *Streptococcus thermophilus* bacteriophage-insensitive mutants (BIMs). The concentration of large volumes of *S. thermophilus* secondary cultures, their subculture in skim milk with high titers of bacteriophages, and their inoculation with the fast milk acidifying phenotype over multiple passages were critical to increasing the probability of obtaining mutants, improving isolation efficiency, and maintaining technological performance. We obtained large quantities of BIMs after every round of screening, with isolation efficiency in excess of 85%. We analyzed nine BIMs, and these were all similar to their parent strain *S. thermophilus* St1 with respect to syneresis, water holding capacity, and apparent viscosity. In comparison to the parent strain, seven of the nine variants possessed equivalent acidifying activities, with the remaining two variants exhibiting excellent acidification performance. One or two new spacers from corresponding phages were found in all nine BIMs, demonstrating that the CRISPR/Cas system was responsible for the phage resistance of *S. thermophilus*.

INTRODUCTION

Streptococcus thermophilus is an important starter strain that is used extensively in the dairy industry for the manufacture of fermented foods such as yogurt and a variety of cheeses [1]. Despite the advancements in production technology and the implementation of strict operating procedures, the infection of *S. thermophilus* with bacteriophages continues to result in failure or retardation of dairy fermentation processes [2,3]. Consequently, research into strategies by which bacteriophage infection of *S. thermophilus* can be minimized is ongoing. Currently, the use of bacteriophage-resistant starter strains with satisfactory technological properties is the primary method of avoiding bacteriophage infection [4-6].

The isolation of bacteriophage-insensitive mutants (BIMs) and their subsequent use in industrial processes are challenging

issues [7, 8]. The rates of spontaneous mutations in microbes are generally very low (around 10−8–10−9), with the rate of mutation in bacteriophage-resistant strains being even lower [9,10]. Because of these low mutation rates, the process of capturing and screening BIMs is laborious. The traditional methods used to isolate BIMs include double-layer agar and the secondary culture method, by which obtaining a true BIM is difficult and time-consuming.

There are four major bacteriophage defense mechanisms: inhibition of phage adsorption, inhibition of phage DNA injection, restriction-modification (R-M) systems, and abortive infection systems [2,11]. However, according to previous works, they were found in a few cases in *S. thermophilus* strains [12]. It was long believed that the acquisition of resistance to bacteriophages could be attributed to nonspecific point mutations in genes encoding cell receptor sites [13]. Recently, Barrangou et al. [14] discovered and described clustered regularly interspaced short palindromic repeats (CRISPR) and suggested that CRISPR-associated (Cas) system was linked to acquired resistance against bacteriophages.

Here, we developed a highly efficient method for the isolation of spontaneous *S. thermophilus* BIMs and found that their performance during fermentation was not adversely affected. In addition, we analyzed the technological performance and resistance mechanisms of these BIMs.

MATERIALS AND METHODS

Bacterial cultures and propagation of bacteriophages

S. thermophilus St1 was isolated from the commercial Direct Vat Set yogurt starter A (Danisco Company, Copenhagen, Denmark) and three different phages, Φ101, Φ102, and Φ108 were previously identified as its virulent bacteriophages [15]. The St1 strain of *S. thermophilus* was routinely cultured at 42°C in M17 broth (Oxoid, Basingstoke, England), supplemented with 0.5% (w/w) lactose (LM17 broth). To propagate bacteriophages, *S. thermophilus* was cultured in LM17 broth supplemented with 10 mM $CaCl_2$ (LM17-Ca) and the phages at the exponential phase of bacterial growth until the cultures became clear. The bacteriophage lysates prepared were passed through filters with a pore size of 0.45 μm (Millipore, Billerica, MA, USA), and subjected to the double-layer plaque titration method, as described previously [16].

Isolation and verification of BIMs

A bacteriophage-sensitive strain St1of *S. thermophilus* was cultured in a large volume of LM17-Ca at 42°C to the exponential phase. Appropriate virulent bacteriophages were then added to cultures at a multiplicity of infection (MOI) of 2. The majority of *S. thermophilus* cells were considered lysed once the cultures appeared clear; however, they were incubated for a further 24 h at 42°C. All the secondary cultures were centrifuged (9,000 × g, 5 min, 4°C), and cell pellets were resuspended in 10 mL of sterile 12% (w/w) reconstituted skim milk (RSM) containing high titers of bacteriophages (108 PFU/mL). Samples were incubated at 42°C until the milk coagulated.

The coagulated milk samples were used at a final concentration of 5% (v/v) to inoculate 10 tubes, each containing 20 mL of RSM containing appropriate bacteriophages (108 PFU/mL). Samples were incubated for 12 h at 42°C. Of the 10 tubes of fermented milk, the sample with the lowest pH was used to further inoculate 10 tubes of 20 mL RSM containing bacteriophages (108 PFU/mL). Sub culturing was continued for 3–5 passages. After the final passage, the first coagulated milk sample, or the milk sample with the lowest pH, was serially diluted and streaked onto an LM17 agar plate. Individual colonies were picked up and were confirmed to be of *S. thermophilus* strains by primer-specific polymerase chain reaction (PCR) [17]. We verified the bacteriophage resistance of these isolates using the inhibition of acid production test in skim milk, and turbidity tests in LM17 broth [15], with high titers of bacteriophage used (108 PFU/mL).

Only one verified BIM was randomly chosen in a round of screening. The BIMs obtained over the first, second, and third rounds of screening with phage Φ101, Φ102, and Φ108 were designated BIM1-1, BIM1-2, BIM1-3, BIM2-1, BIM2-2, BIM2-3, BIM8-1, BIM8-2, and BIM8-3, respectively. For these nine BIMs, we analyzed the stability of their phage resistance, their technological performance, their cross-resistance to the phages Φ101, Φ102, and Φ108, and their mechanisms of resistance.

Stability of bacteriophage resistance

Stable bacteriophage resistance of the nine BIMs was determined by cultivating these mutants in LM17-Ca broth for 20 generations, with booster doses of the corresponding bacteriophage (10^9 PFU/mL) added at each passage. Plaque assays and turbidity tests were repeated to ensure the phage resistance phenotype was maintained.

Technological performance of the isolated BIMs

We analyzed *S. thermophilus* St1 and the nine BIMs with respect to their ability to ferment, susceptibility to syneresis (STS), water-holding capacity (WHC), apparent viscosity performance, and cross-resistance to other phages. We sterilized 12% (w/w) RSM at 110°C for 15 min. Once the RSM samples had cooled to 42°C, they were inoculated with *S. thermophilus* St1 or one of the BIMs at a concentration of 5×10^6 CFU/mL. The pH of the samples was determined with a 420A pH-meter (Thermo Orion, Beverly, MA, USA) at 12 h after inoculation. The pH of the samples was also monitored using a Cinac system (Alliance Instruments, Mery-Sur-Oise, France), which automatically recorded pH values every 5 min over 24 h while fermentation was taking place.

Fermentation was terminated once the pH of the samples was 4.5. Fermented milk samples were stirred (500 rpm, 5 min) using a mechanical stirrer (RW20; IKA, Staufenim, Germany) and then stored at 4°C for 24 h.

Apparent viscosities of fermented milk samples were measured at 20°C using an R180 viscometer (proRheo, Althengstett, Germany) with a No. 2 spindle at 64 rpm. STS and WHC were determined as previously described [18]. STS results were calculated using the formula:

$$STS\ (\%) = V1/V2 \times 100$$

Where V1 was the volume of whey collected after drainage and V2 was the volume of the fermented milk sample. WHC was calculated using the following formula:

$$WHC\ (\%)=(1 - W1/W2) \times 100$$

Where W1 was the mass of whey after centrifugation and W2 was the total fermented milk mass.

Mechanisms of bacteriophage resistance

Lysogeny and adsorption rates were determined for the nine BIMs as previously described [19]. The presence of R-M mechanisms was investigated according to the methods of Binetti et al. [20]. Genomic sequences corresponding to the CRISPR array were investigated for all the nine BIMs and the parental *S. thermophilus* St1 strain. Total DNA was obtained using a phenol-chloroform extraction procedure [21]. The DNA obtained was quantified by electrophoresis on 0.8% (w/v) agarose gels (Biowest, Barcelona, Spain). Genomic sequences corresponding to three CRISPR loci were amplified by PCR using the primer pairs CR1-Forward (5′-TGC TGA GAC AAC CTA GTC TCT C-3′) and CR1-Reverse (5′-TAA ACA GAG CCT CCC TAT CC-3′), CR2-Forward (5′-TTA GCC CCT ACC ATA GTG CTG-3′) and CR2-Reverse (5′- TTA GTC TAA CAC TTT CTG GAA GC-3′), or CR3-Forward (5′- CTG AGA TTA ATA GTG CGA TTA CG-3′) and CR3-Reverse (5′-GCT GGA TAT TCG TAT AAC ATG TC-3′), as outlined by Horvath et al. [22]. Reactions were performed in a total volume of 50 µL using pfu Polymerase (Transgen Co. Ltd., Beijing, China). Amplicons were purified with an Omega Cycle-Pure Kit (Omega bio-tek, Norcross, GA, USA), and Sanger DNA sequencing was performed.

Southern blotting analysis

We obtained biotin-labeled single-stranded oligonucleotide probes synthesized according to new spacer sequences (Life Technologies Co. Ltd., Shanghai, China). We used Southern blotting to analyze the genomic DNA of bacteriophages according to standard protocols [23]. Bacteriophage genomic DNA was extracted according to the procedure of Zinno et al. [3]. Bound probes were detected using a Chemiluminescent Biotin-labeled Nucleic Acid Detection Kit (Beyotime, Shanghai, China).

STATISTICAL ANALYSIS

All experiments were conducted in triplicate, and results have been expressed as the mean ± standard deviation (SD). Data were subjected to analysis of variance using Statistica 9.2 (Stat Soft, Inc., Tulsa, OK, USA). Comparison of means was conducted using the Tukey's significant difference test, where a p-value less than 0.05 were considered statistically significant.

RESULTS

Isolation of BIMs

We cultured *S. thermophilus* St1 in a 1 L volume with the Φ101, Φ102, or Φ108 bacteriophage. Secondary cultures were centrifuged to concentrate any possibly present BIMs and further enrichment was performed. We obtained a large number of BIMs after every round of screening and confirmed that the isolation efficiency was greater than 85%. As the passage number increased, the isolation efficiency increased accordingly **(Table 1).** After the five passages of screening and enriching in the presence of the appropriate phage, the 100 individual colonies randomly selected from LM17 agar plates were almost BIMs.

Table 1. BIMs obtained from *S. thermophilus* St1 strains with its specific bacteriophage n_P, number of presumptive BIMs isolated; n_R, number of confirmed BIMs isolated; Isolation efficiency was $n_R/n_P \times 100\%$.

Mutant strains	Phage	Passage times	n_P	n_R	Isolation efficiency
BIM 1-1	Φ101	3	100	85	85%
BIM 1-2	Φ101	4	100	89	89%
BIM 1-3	Φ101	5	100	98	98%
BIM 2-1	Φ102	3	100	87	87%
BIM 2-2	Φ102	4	100	91	91%
BIM 2-3	Φ102	5	100	99	99%
BIM 8-1	Φ108	3	100	86	86%
BIM 8-2	Φ108	4	100	92	92%
BIM 8-3	Φ108	5	100	100	100%

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Bacteriophage resistance stability and cross-resistance

Resistance to bacteriophages was highly stable for the nine BIMs investigated. All derivatives of the BIMs maintained their bacteriophage-resistant phenotype over 20 generations (data not shown).

Inhibition of acid production tests and turbidity tests revealed that all BIMs were cross-resistant to the various other phages used in this study. Namely, BIM1-1, BIM1-2, and BIM1-3 were insensitive to phages Φ102 and phage Φ108, while BIM2-1, BIM2-2, and BIM2-3 were insensitive to phages Φ101 and Φ108. In addition, BIM8-1, BIM8-2, and BIM8-3 were insensitive to phages Φ101 and Φ102.

Technological performance of BIMs

Representative acidification curves for *S. thermophilus* St1 and the three groups of BIMs were determined **(Figure 1).** Of the nine BIMs we identified, seven exhibited acidifying activity similar to that of the parent strain, with the majority of the BIMs possessing some acidifying activity. The acidification curves for BIM3-3 and BIM8-3 for the initial 4 h of fermentation were similar to that seen for *S. thermophilus* St1 over the same period. However, the rates of acidification for these two BIMs from 4 to 24 h of fermentation were more rapid than that for *S. thermophilus* St1 **(Figure 1).** The mutants BIM3-3 and BIM8-3 also exhibited significantly (P<0.05) lower pH values than *S. thermophilus* St1 at 12 h **(Table 2).**

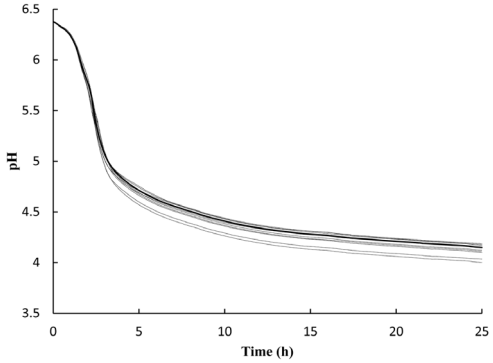

Figure 1. Kinetics of acidification for *Streptococcus thermophilus* St1 and its bacteriophage-insensitive mutants (BIMs). The solid bold line represents *S. thermophilus* St1, while the various dashed lines represent one of the nine BIMs that were isolated.

Table 2. Technological performance of *S. thermophilus* St1and its BIMs STS, Susceptibility to syneresis; WHC, water holding capacity. The pH values were measured at 12 h. All values are mean of three replications ± SD. Different superscript letters in the same column indicate significant differences at P<0.05.

Strains	pH values	STS (%)	WHC (%)	Apparent viscosity (Pa·s)
St1	4.34 ± 0.05[a]	48.4 ± 2.1[a]	16.3 ± 0.6[a]	0.240 ± 0.032[a]
BIM 1-1	4.30 ± 0.07[a]	49.1 ± 1.1[a]	15.6 ± 0.7[a]	0.243 ± 0.027[a]
BIM 1-2	4.31 ± 0.06[a]	50.3 ± 1.3[a]	16.1 ± 0.9[a]	0.246 ± 0.019[a]
BIM 1-3	4.17 ± 0.05[b]	52.0 ± 1.6[a]	15.2 ± 0.8[a]	0.245 ± 0.022[a]
BIM 2-1	4.32 ± 0.06[a]	52.2 ± 2.0[a]	16.5 ± 1.1[a]	0.239 ± 0.028[a]
BIM 2-2	4.36 ± 0.05[a]	47.1 ± 1.5[a]	16.1 ± 0.9[a]	0.237 ± 0.026[a]
BIM 2-3	4.37 ± 0.08[a]	48.9 ± 1.7[a]	17.2 ± 1.0[a]	0.245 ± 0.034[a]
BIM 8-1	4.36 ± 0.05[a]	50.3 ± 1.9[a]	16.1 ± 1.2[a]	0.241 ± 0.027[a]
BIM 8-2	4.33 ± 0.04[a]	47.8 ± 2.4[a]	15.9 ± 0.9[a]	0.248 ± 0.035[a]
BIM 8-3	4.19 ± 0.06[b]	50.6 ± 2.3[a]	15.6 ± 0.7[a]	0.238 ± 0.026[a]

All nine BIMs and *S. thermophilus* St1 exhibited STS values of 50% and WHC values of approximately 16%. Syneresis and WHC did not significantly differ (P > 0.05) between *S. thermophilus* St1 and the nine BIMs **(Table 2).** The nine BIMs were also similar in viscosity to *S. thermophilus* St1. The apparent viscosities of ten mature fermented milk samples ranged from 0.237 to 0.248 **(Table 2)**; the difference between viscosities of BIM strains and parent strains were not statistically significant (P>0.05).

Analysis of phage resistance mechanisms

The nine BIMs isolated from cultures of *S. thermophilus* St1 did not contain bacteriophages were able to infect the parent strain in their broth culture supernatants **(Table 3).** This finding suggested that the resistance phenotypes were not linked to lysogeny. Obvious inhibition of bacteriophage adsorption was not detected for these variants, as their mean adsorption rates were greater than 94.5%. There were no significant differences (P>0.05) between adsorption rates for BIMs and the parent strain (Table 3). Therefore, it was apparent that inhibition of phage adsorption was not the mechanism of phage resistance employed by our isolated BIMs. We tested all nine BIMs for R-M systems and found that all the variants did not exhibit the ability to form visible plaques when they were infected with specific bacteriophages at an MOI of 2 **(Table 3).** Therefore, silent R-M mechanisms were most likely absent in *S. thermophilus* St1.

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Table 3. Bacteriophage-resistance mechanism analysis in S. thermophilus BIMs "–" represent absence or not detected. All values are mean of three replications ± SD. Different superscript letters in the same column indicate significant differences at P<0.05.

Strains	Lysogeny	Adsorption rate(%)	R-M system
St1	–	95.1 ± 1.7[a]	–
BIM 1-1	–	96.3 ± 2.1[a]	–
BIM 1-2	–	95.6 ± 1.8[a]	–
BIM 1-3	–	94.5 ± 2.3[a]	–
BIM 2-1	–	95.2 ± 1.9[a]	–
BIM 2-2	–	96.1 ± 1.7[a]	–
BIM 2-3	–	95.4 ± 2.4[a]	–
BIM 8-1	–	95.8 ± 1.8[a]	–
BIM 8-2	–	96.5 ± 2.2[a]	–
BIM 8-3	–	95.6 ± 3.1[a]	–

We detected 10 new CRISPR spacer sequences in the nine BIMs (Table 4), all of which belonged to the CRISPR1 locus. Results from other studies have indicated that CRISPR1 is the most active locus with respect to integrating new spacers [22, 24]. The 10 new spacers showed homology to reported *S. thermophilus* bacteriophage genomes **(Table 4).** Our Southern blotting results indicated that the extra spacer sequences were derived from specific bacteriophages **(Figure 2).** These results illustrate that the new spacers were acquired by the CRISPR/Cas system and subsequently led to the bacteriophage-resistant phenotypes of these strains. These results correspond with those from previous studies involving *S. thermophilus* [14, 24, 25].

Table 4. Analysis of new CRISPR spacers in *S. thermophilus* BIMs

Mutant strains	New spacers	Extra spacer sequence	Homology
BIM 1-1	+2	S1: GACAAGCCTACAAGCTCTTGCACTTGTTT (29 bp) S2: TCCAAGTTATTTGAGGAGTTATTAAGACAT (30 bp)	φ858, φ2972 (100%) (29/29) φ858, φ2972, φSFi18, φSfi19 (100%) (30/30)
BIM 1-2	+1	S2: TCCAAGTTATTTGAGGAGTTATTAAGACAT (30 bp)	φ858, φ2972, φSFi18, φSfi19 (100%) (30/30)
BIM 1-3	+1	S3: TAATATTGCTGGTTACTTCAACGCGTTACA (30 bp)	φ858 (100%) (30/30), φSfi11 (96.7%) (29/30)
BIM 2-1	+2	S2: TCCAAGTTATTTGAGGAGTTATTAAGACAT (30bp) S4: TGGAATTATCCAAGGCTGGCTACATGGTAT (30bp)	φ858, φ2972, φSFi18, φSfi19 (100%) (30/30) φ858, φ2972, temperate φ01205 (100%) (30/30)
BIM 2-2	+2	S5: ATCCGTTTCCGTATTCGCAAGGACTTCCAA (30bp) S6: TTCCCTTCGATAATGGCAAGACCGAAACGC (30bp)	φALQ13.2, φ2972, φSfi11, φSfi18, φSfi19, temperate φ01205 (100%) (30/30) φALQ13.2, φ2972 (100%) (29/29)
BIM 2-3	+1	S7: CTCAGTCGTTACTGGTGAACCAGTTTCAAG (30bp)	φALQ13.2, φ2972, φkappa3, φDT1 (100%) (29/29)
BIM 8-1	+1	S8: GAAGTTGAAATAATTCGAGAAATAGAACTC (30bp)	φALQ13.2, φ2972, φSfi11, φSfi18, φSfi19, temperate φ01205, φSfi21 (100%) (30/30)
BIM 8-2	+1	S5: ATCCGTTTCCGTATTCGCAAGGACTTCCAA (30bp)	φALQ13.2, φ2972, φSfi11, φSfi18, φSfi19, temperate φ01205 (100%) (30/30)
BIM 8-3	+2	S9: TCGTTTTCAGTCATTGGTGGTTTGTCAGCG (30bp) S10: GTTAGGGATAAGAGTCAAGTGGCCGTCAGG (30bp)	φ2972, φSfi11, φDT1, φSfi18, φSfi19, φSfi21, temperate φ01205 (100%) (30/30) φALQ13.2, φ858, φ2972 (100%) (30/30)

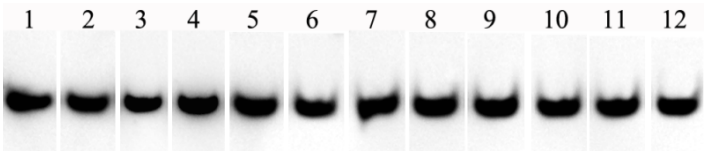

Figure 2. Southern blotting analysis of the complementary relationship between new spacer sequences and bacteriophage genomes.Lanes 1, 2 and 3 show the genomic DNA from bacteriophage Φ101 hybridized with spacers S1, S2, and S3, respectively. Lanes 4, 5, 6, 7, and 8 shows the genomic DNA from bacteriophage Φ102 hybridized with spacers S2, S4, S5, S6, and S7, respectively. Lanes 9, 10, 11, and 12 shows the genomic DNA from bacteriophage Φ108 hybridized with spacers S8, S5, S9, and S10, respectively.

DISCUSSION

The development of food-grade genetically engineered microorganisms that are bacteriophage-resistant remains in industry production controversial in certain regions such as China and Europe [26]. However, BIMs developed by spontaneous mutations could address the concerns of various governments and be employed by the food production industry.

There are some disadvantages to the traditional techniques used for the isolation of BIMs. These include the low yield of BIMs, low isolation efficiencies, and reduced technological performance of the BIMs that are eventually isolated. As an example, Binetti et al. [7] reported a low nR (0–26) and low isolation efficiency (22.6%) for true BIMs obtained from sensitive strains of *S. thermophilus*.

We developed an efficient method for generating BIMs of *S. thermophilus* as industrial starters. Our technique offers many

advantages compared with those currently used for BIM isolation. In our method, large volumes of *S. thermophilus* cultures were collected and concentrated by centrifugation. In general, the concentration of *S. thermophilus* during the exponential phase of growth is around 10^8 CFU/mL. The spontaneous mutation rate for bacteriophage-resistant mutants is low under 10-8, then, theoretically, 1 BIM should be generated from 1 mL of *S. thermophilus* secondary cultures. However, if culture volumes of 0.1–1.0 mL are used in traditional double-layer agar and secondary culture method, it would be unlikely that a single BIM could be obtained from 10 repetitions. Therefore, it is necessary that a large number of cells are used for the spontaneous generation of mutants as this greatly increases the probability of successfully obtaining mutants.

In addition, BIMs were screened for and enriched during fermentation, using high titers of bacteriophages and multiple passages. Successive sub culturing in the presence of a high titer of bacteriophages eliminated false positive variants, while true BIMs gradually became the dominant strains in cultures, significantly improving the isolation efficiency of our method.

Results from many previous studies have shown that only a few variants exhibiting good technological performance could be used as improved starter strains for industrial processes [6, 7]. The major challenge lies in maintaining or improving the technological performance of spontaneous BIMs. In the present study, the milk sample with the lowest pH was selected for continuous subculture to screen for the variant with the greatest acidification ability and tolerance to acidity. Exopolysaccharides are responsible for the filamentous ties of the protein matrix in the yogurt microstructure [27]. The technological performances, such as Syneresis, WHC, and apparent viscosity, are associated with exopolysaccharide production. The screening method we used to determine phage resistance focused on natural selection; therefore, it was not surprising that the BIMs exhibited minimal differences in STS, WHC, and apparent viscosity in comparison to those for the parental strain. We observed that the nine BIMs that we isolated retained the essential properties of the parental strain, indicating that they were suitable for use in industrial applications.

There are few reports of bacteriophage resistance mechanisms in *S. thermophilus* starters, as this species of bacteria was thought to have limited natural phage defense mechanisms [28]. Some researchers have indicated that resistance to lysogeny is common in lactococci and lactobacilli, but is uncommon for *S. thermophilus* [6, 28]. We assessed possible mechanisms responsible for the bacteriophage-resistant phenotype of BIMs. Our findings confirm these previously published results [6, 28]. We observed no significant association between lysogeny and the bacteriophage-resistant phenotype. For the adsorption assays, we observed that the adsorption rate of phage particles was normal for all BIMs, suggesting that bacteriophage adsorption to the cell was unaffected in bacteriophage-resistant strains. The R-M systems are powerful defense mechanisms that are primarily found in the plasmids of some lactococci [13]. Few phage resistance mechanisms have been reported for *S. thermophilus* [20]. It is thought that *S. thermophilus* likely lacks R-M systems.

It has been demonstrated that *S. thermophilus* can integrate novel spacers into its CRISPR loci in response to a phage attack [14, 25]. We identified 1–2 new spacers in each of the BIMs we isolated; these spacers were from the corresponding phages used to induce these BIMs. Our results suggest that the CRISPR/Cas system of *S. thermophilus* might be the mechanism responsible for bacteriophage resistance. Restriction digestion patterns and structural protein analysis showed that the phages Φ101, Φ102, and Φ108 differed significantly from each other [15]. However, the assays we conducted showed that all nine BIMs were cross-resistant to the three phages. It is possible that these phages were homologous and that some spacer sequences were simultaneously present in the three phages. As an example, the S2 proto-spacer was identified as an extra new spacer in BIM1-1, BIM1-2, and BIM2-1 **(Table 4).** This proto-spacer was also present in both phages Φ101 and Φ102 **(Figure 2).**

S. thermophilus is susceptible to infection by bacteriophages during fermentation, which can seriously affect the production and quality of fermented dairy products. The highly efficient isolation of spontaneous *S. thermophilus* phage-insensitive mutants with appropriate technological performance would be an effective approach for overcoming the susceptibility of this species to bacteriophage infections. However, phages possess the ability to evolve through single nucleotide polymorphisms, resulting in the circumvention of bacterial phage defense mechanisms [11]. Bondy-Denomy et al. [29] found that phage-encoded anti-CRISPR genes possibly evade CRISPR systems, allowing for the phage to invade the host. The efficient screening of BIMs is crucial in improving our understanding of the co-evolution of phages and their hosts.

CONCLUSION

The rate of spontaneous mutations that confer resistance to bacteriophages is very low in microbes. Despite this hurdle, we developed an improved method that increased the probability of generating true spontaneous BIMs. We successfully obtained true BIMs after every round of screening and demonstrated isolation efficiency in excess of 85%. In general, the BIMs we isolated functioned in a similar way as the parental *S. thermophilus* strain with regard to technological performance, as estimated by parameters such as STS, WHC, and apparent viscosity. Taken together, our results indicate that the modified secondary culture method we devised was effective for BIM generation and screening. Our analysis of mechanisms that confer phage resistance showed that the CRISPR/Cas system and not lysogeny, adsorption interference, or the R-M type systems were most likely responsible for conferring phage resistance to *S. thermophilus*.

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