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Characterization of spontaneous phage-resistant variants of *Streptococcus thermophilus* by randomly amplified polymorphic DNA analysis and identification of phage-resistance mechanisms

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Abstract

A total of 100 spontaneous phage-resistant mutants isolated from nine commercial *Streptococcus thermophilus* strains were characterized preliminarily by randomly amplified polymorphic DNA (RAPD) and the nature of their phage-resistance mechanisms was investigated. Only for mutants isolated from one strain, free phages were detected in their culture supernatants when these were titrated on the sensitive strain, suggesting that the mutants could have acquired the resistance phenotype by integrating the phage in their genomes (lysogeny). Adsorption interference was observed in the derivatives isolated from two strains. For mutants isolated from two other strains, restriction–modification (R–M) type systems were detected. In one of these cases, R–M was probably combined with another intracellular anti-phage system. In most cases, the molecular profiles (RAPD fingerprints) obtained with four arbitrary primers showed a high similarity among parent strains and their respective phage-resistant mutants. Some of these mutants were identified as potentially improved strains for industrial use.

Keywords: *Streptococcus thermophilus*; Phages; RAPD-PCR

1. Introduction

Despite the development of a variety of countermeasures (culture rotation, improved sanitation strategies and use of bacteriophage-resistant starter strains) phage infection during product manufacture continues to be the leading cause of failed or retarded dairy fermentations (Brüssow & Desière, 2001; Coffey, Coakley, Mc Garry, Fitzgerald, & Ross, 1998; Forde & Fitzgerald, 1999; Klaenhammer & Fitzgerald, 1994; Neve, 1996; Vadeboncoeur & Moineau, 2004). Several factors, such as lysogenic lactic acid bacteria (LAB) present in raw milk processed daily and the use of non-sterile fermentation media (pasteurized milk) determine the entrance and dynamics of phage populations in dairy plant environments. Thus, the success of commercial lactic starter cultures depends, primarily, on the selection of phage-unrelated strains, which are able to withstand viral infections. The isolation of phage-resistant mutants with satisfactory technological performance from sensitive-strains represents a very interesting approach for obtaining improved strains for industrial purposes (Coffey et al., 1998; Klaenhammer, 1984; Guglielmotti et al., 2006; Quiberoni, Reinheimer, & Tailliez, 1998). Although this technique has the advantage of simplicity and rapidity, it is rarely used for commercial *Lactococcus* strains since bacteriophage insensitive mutants often exhibit a variety of negative qualities that may exclude them for being used in industrial dairy fermentations (Coffey et al., 1998; Forde

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& Fitzgerald, 1999; Klaenhammer, 1984; Moineau, 1999; Sturino & Klaenhammer, 2004). However, it was successfully employed to isolate phage-resistant variants with good technological abilities from *Lactobacillus helveticus*, allowing their use in cheese making (Quiberoni, Reinheimer, & Suárez, 1999).

Streptococcus thermophilus is one of the most important thermophilic LAB because of its worldwide use in the dairy industry. In Argentina, besides its relevance as a starter in the yoghurt industry, this species is used in the production of other fermented milks and a large variety of hard and semi-hard cheeses (Reinheimer et al., 1997). From this kind of processes many specific phages have been isolated over the past years (Suárez, Quiberoni, Binetti, & Reinheimer, 2002). Low frequencies at which *S. thermophilus* spontaneous phage-resistant mutants occur were previously reported (Moineau, 1999; Viscardi, Capparelli, & Iannelli, 2003; Viscardi, Capparelli, Di Mateo et al., 2003). Notwithstanding, we have recently isolated (Binetti, Bailo, & Reinheimer, 2007) such variants from sensitive commercial strains used in Argentinean dairy plants by the secondary culture method with a frequency that was strain-dependent. Since some of these mutants showed excellent levels of phage resistance and stability, as well as acidifying and proteolytic activities, they could be used as improved strains for industrial purposes. However, the mechanisms involved in their resistance have not been elucidated yet.

The aim of this work was to investigate, by means of a primary identification, the resistance mechanisms present in phage-resistant mutants of *S. thermophilus* and characterize these derivatives based on randomly amplified polymorphic DNA (RAPD) fingerprints.

2. Materials and methods

2.1. Bacterial strains, bacteriophages and culture conditions

Spontaneous phage-resistant variants were obtained from nine *S. thermophilus* strains (identified as 4-C, 5-C, YDS10-C, Jo1-C, M1-C, M8-C, M11-C, MiC1 and MiC7), isolated from commercial starters used in Argentinean dairy industries (INLAIN Culture Collection), and three Italian *S. thermophilus* strains (identified as I49, I53 and I54) belonging to the Istituto Sperimentale Lattiero Caseario (ISLC, Lodi, Italy) Culture Collection. Bacteriophages used were nine autochthonal *S. thermophilus* phages (ϕ021-4, ϕ031, ϕCYM, ϕCYS1, ϕQP2, ϕQPL2, ϕQPL1′, ϕMi2 and ϕMi1) isolated from Argentinean dairy plants (INLAIN Phage Collection) and three Italian *S. thermophilus* phages (ϕ49, ϕ53 and ϕ54) (ISLC Phage Collection) (Table 1). To isolate spontaneous phage-resistant mutants, the secondary culture method was used (Carminati, Zennaro, Neviani, & Giraffa, 1993). Strains and phage-resistant derivatives were grown in M17 broth or M17 agar (Biokar, Beaubois, France) at 42 °C and stored (−80 °C) in M17 broth supplemented with 15% (v/v) glycerol and in non-fat dry skim milk (Merck, Darmstadt, Germany). Phage enumerations were carried out by double-layer plaque titration method from IDF Standards (1991) using M17 soft agar on M17 agar supplemented with 10 mM $CaCl_2$ (M17-Ca) and 100 mM glycine (Lillehaug, 1997).

Table 1
Phage-resistance mechanisms present in phage-resistant mutants isolated from commercial *S. thermophilus* strains

Sensitive strain	Phage	n_R[a]	Lysogeny[b]	Adsorption rate[c]
4-C	ϕ021-4	3	−	85.5
5-C	ϕ031	3	−	93.3
YSD10	ϕCYM	2	−	89.9
M1-C	ϕQP2	6	−	82.1 ± 18.1
M8-C	ϕQLP2	10	−	80.6 ± 3.4
M11-C	ϕQLP1′	1	−	99.9
Jo1-C	ϕCYS1	1	−	95.7
MiC1	ϕMi2	4	−	96.1 ± 3.6
MiC7	ϕMi1	8	−	48.5 ± 3.2
I49	ϕ49	26	+	96.2 ± 6.6
I53	ϕ53	17	−	83.1 ± 7.1
I54	ϕ54	9	−	49.2 ± 9.0

[a] n_R: Number of phage-resistant mutants isolated; −: absence; +: presence.
[b] Spontaneous induction of free phages, detected in the supernatants of mutants cultures.
[c] % (mean value of each group) of adsorbed phages in M17-Ca broth after 30 min at 45 °C. Standard deviation was calculated when $n_R > 3$.

2.2. Characterization of strains

For *S. thermophilus* strains and their phage-resistant variants, cell (phase contrast, 1000 ×, Microscope Jenamed 2 Carl Zeiss, Jena, Germany) and colony (on M17 agar) morphologies were observed. To evaluate sugar fermentation patterns, API 50 CHS (Bio Merieux, Marcy l'Etoile, France) galleries were used, according to the manufacturer's instructions.

2.3. Phage-resistance mechanisms

Lysogeny and adsorption rates were determined for all phage-resistant mutants as previously described (Quiberoni et al., 1998). All assays were performed in triplicate.

In the case of mutants that exhibited a relatively high adsorption rate of phage particles and a late lysis in broth, the presence of restriction–modification (R–M) type resistance mechanisms was investigated according to de los Reyes-Gavilán, Limsowtin, Tailliez, Séchaud, and Accolas (1992) modified as follows: a phage suspension was titrated on the sensitive strain and on the phage-resistant mutant, and Efficiency of Plaquing (EOP) (first value) was calculated. One or two lysis plaques obtained from the titration on the phage-resistant variant were picked up and suspended in 5 mL of M17-Ca broth. Phage suspensions were kept 24 h at 4 °C and then inoculated with