Preparation of Phage-insensitive Strains of *Tetragenococcus halophila* and Its Application for Soy Sauce Fermentation

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We attempted to breed phage-insensitive strains of *Tetragenococcus halophila* D10. Phage contact during selection initially caused the occurrence of lysogeny. Subsequently, we screened phage-insensitive mutants by replica plating so that mutant cells did not touch the phage during selection. Two strains were selected from about 150,000 strains. They grew normally in soy sauce mash (moromi) in the presence of phage φD-10, although they had a similar extent of adsorption of φD-10 as did the parent strain.

Key words: bacteriophage; phage-insensitive strain; soy sauce; *Tetragenococcus halophila*; lactic acid bacteria

In the process of soy sauce fermentation, lactic acid fermentation is done by *Tetragenococcus halophila* (formerly known as *Pediococcus halophilus*) before alcohol fermentation by yeast. Lactic acid fermentation decreases the pH of the soy sauce mash (moromi) and makes the sauce slightly sour. Recently, the use of starter cultures of *T. halophila* instead of traditionally-used natural floras has become popular for control of lactic acid fermentation. However, repetitive use of the same strain often results in insufficient lactic acid fermentation, caused by bacteriophage contamination. Two typical virulent phages, named φ7116 and φD-86, have been isolated from *T. halophila*, and both were found to have isometric heads. Fermentation tanks of soy sauce are not closed microbiologically; thus, it is difficult to prevent phage contamination absolutely.

In the dairy industry, phage contamination has been a major problem, and a variety of phage-insensitive strains have been bred to overcome this problem. Therefore, we attempted to isolate phage-insensitive strains of *T. halophila* to stabilize lactic acid fermentation of soy sauce moromi.

*T. halophila* D10 is a typical industrial strain of soy sauce lactic acid bacteria. This strain is resistant to most tetragenococcal phages but susceptible to a phage designated φD-10. This phage belongs to siphoviridae; it has a hexagonal head (length, 55 nm; width, 55 nm) and noncontractile tail (length, 200 nm; width, 9 nm). Although most φD-10 infecting D10 as a host develop lytically, the infected host cells are lysogenized at a rate of 10^{-3} to 10^{-6}. Phage-insensitive strains of D10 obtained as surviving colonies in the presence of φD-10 have been mainly φD10 lysogens unsuitable for industrial use because of the potential risk of phage induction. Thus, we selected phage-insensitive mutants by replica plating so that mutant cells did not touch the phage during selection.

The microorganisms and phages used are listed in the Table. All the strains were from stock cultures in our laboratory. Bacteria were grown in MRS-15, in which lactobacilli MRS broth (Difco Laboratories, Detroit, MI, USA) was supplemented with 15% NaCl. For plate culture, BYPS-15 plates were used. All plate cultures were incubated at 30°C anaerobically in a GasPack jar (B. L. Microbiology Systems, Cockeysville, MD, USA). Adsorption broth is a modified lactobacilli MRS broth with 15% NaCl, 0.015% CaCl₂·2H₂O, 0.02% MgSO₄·7H₂O, 0.005% MnSO₄·5H₂O, and 0.01% gelatin. Before sterilization, the pH of these media was adjusted to 7.0 with 1 N NaOH. Moromi was prepared as described previously. Propagation of the phage was done as previously described except for the use of MRS-15 as a medium. Plaques of tetragenococcal phages are difficult to detect by the standard agar layer method. However, their plaques are detectable by directly spreading a host-phage mixture on the surface of agar plates as described previously, and this method was used in this study.

Before selection of phage-insensitive strains, D10 cells were mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine (NTG). D10 cells in the early stationary phase were harvested by centrifugation at 2,000 x g for 10 min and suspended in NTG solution at the final concentration of 100 µg/ml. The cell suspension was incubated at 30°C for 1 h and washed twice with 15% NaCl solution.

The NTG-treated cells were diluted to obtain 50 to 500 CFU/plate, then spread on MRS-15 plates. The plates were incubated at 30°C for one week, when the colony diameters reached 0.3 to 0.5 mm (phage-free original plates). These colonies were replica plated with RepliPlate Colony Transfer Pads (FMC Bio Products, Shizuoka, Japan) and then incubated at 30°C to obtain plaques.

Abbreviations: PFU, plaque-forming unit; CFU, colony-forming unit; mol, multiplicity of infection; NTG, N-methyl-N'-nitro-N-nitrosoguanidine

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Table. Strains of Microorganisms and Phages

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<td>βD-10</td>
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Fig. 1  Growth Curves of Phage-insensitive Strains in MRS-15 Broth with 10⁶ PFU/ml of βD-10.

Growth was measured by OD at 600 nm. The initial titer of βD-10 was 10⁹ PFU/ml. Symbols: ○, D10; △, strain 1; ■, strain 4; ●, D10 without phage.

Fig. 2. Growth of Tetragnococcal Cells and pH Changes in Moromi Fermented by Phage-insensitive Strains in the Presence of Phage βD-10.

The initial titer of βD-10 was 10⁶ PFU/ml. ○, D10; △, strain 1; ■, strain 4; ●, D10 without phage; ———, viable cell concentration (log CFU/ml).

ME, USA) onto MRS-15 plates on which 0.1 ml of βD-10 phage lysate containing 5 × 10⁸ PFU/ml had been spread previously (replica plates). After anaerobic incubation at 30°C for one week, only phage-insensitive mutants were expected to form colonies on the replica plates. Among about 150,000 D10 derivatives, we obtained approximately 1,500 phage-insensitive candidates from the corresponding colonies on the original plates. These candidates were cultured statically with 10⁶ PFU/ml of βD-10 in MRS-15 broth at 30°C and their growth profiles were measured with an automatic growth analyzer, Bioscreen C (Labsystems, Helsinki, Finland), to confirm their insensitivity to βD-10. We selected two strains, 1 and 4, whose growth curves were almost identical with that of D10 in the absence of the phage (Fig. 1).

We further examined the responses of these strains to βD-10 in soy sauce moromi by inoculating them with βD-10 into 360 ml of freshly prepared moromi. The initial bacterial and phage numbers were approximately 10⁸ CFU/ml and 10⁶ PFU/ml, respectively (moi was approximately 10). The moromi was incubated at 15°C for 14 days, 20°C for the next 7 days, and 25°C for the following 9 days. Unlike D10, both strains grew normally in moromi and lowered its pH as efficiently as did D10 without βD-10 (Fig. 2). As expected, the phage titer in the moromi fermented by strains 1 and 4 in the presence of βD-10 did not increase during the growth period of the bacterial cells (data not shown).

After lactic acid fermentation for 30 days, the moromi was inoculated with the yeast Zygosaccharomyces rouxii NISL3355.6) incubated at 30°C for one week with occasional agitation, and then matured at 20°C for 5 months. The final pH of the moromi fermented in strains 1 and 4 with βD-10 and fermented in D10 without βD-10 was 4.9 to 5.0.

At various stages of moromi production, we compared the taste and flavor of moromi fermented by strains 1 and 4 with those of moromi fermented by D10 without βD-10. There were no detectable differences just after lactic acid fermentation, after yeast fermentation, or even after 5 months of maturation (data not shown). These results suggest that strains 1 and 4 are non-lysogenic phage-insensitive mutants suitable for large-scale use.

It is known that most phage-insensitive mutants of lactococci lose the ability to adsorb phage.9) We assessed the phage adsorption of strains 1 and 4 as follows: Phage βD-10 lysate and washed D10 cell suspension were added to adsorption broth to final concentrations of 10⁶ PFU/ml and 10⁶ CFU/ml, respectively (moi was approximately 0.01). After incubation at 25°C for 30
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References


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