APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1982, p. 1284–1287
0099-2240/82/061284-04$02.00/0

Vol. 43, No.6

Prophage Curing in *Lactobacillus casei* by Isolation of a Thermoinducible Mutant

MARIKO SHIMIZU-KADOTA* AND TOSHIZO SAKURAI

Yakult Central Institute for Microbiological Research, Tokyo, 186, Japan

Received 8 July 1981/Accepted 22 February 1982

To eliminate the occurrence of virulent phage in industrial fermentation, attempts were made to obtain prophage-cured derivatives from *Lactobacillus casei* lysogenic strain S-1. A thermoinducible mutant lysogen was isolated from mutagenized strain S-1, since S-1 cannot be induced under laboratory conditions. The mutation responsible for thermoinducibility was located on the prophage. Prophage-cured strains were selected after heat induction of the mutant. These cured strains did not produce the virulent phage and should be valuable for industrial fermentation.

Group B *Lactobacillus casei* strain S-1 has been used industrially to produce lactic acid beverages (8, 11). The manufacturing process involves fermentation of skim milk with strain S-1 in a closed tank system. During fermentation, the product is occasionally contaminated with virulent phages, causing abnormal fermentation. Although many virulent phages have been isolated independently at several factories, the phages have been serologically indistinguishable. These phages were thought to have a common origin and thus were designated φFSV. It has been proposed that virulent phage φFSV did not originate from the environment, but rather appeared from a temperate phage harbored in strain S-1. Lysogeny is known to be widespread in lactobacilli (1, 10, 12, 13). Actually, strain S-1 was found to be a lysogen because the supernatant of a culture contained a small amount of free phage and strain S-1 was resistant to this phage (unpublished data). This temperate phage was classified in the same serological group as virulent phage φFSV and was designated φFSW, since it was supposed to be the wild type of virulent phage φFSV. If this is the case, it should be possible to obtain a prophage-cured strain which no longer produces virulent phage φFSV. In this paper we describe the process of obtaining such a strain.

TABLE 1. *L. casei* group B strains used

Strain	Prophage harbored	Source
S-1	φFSW	ATCC 27139; from our laboratory collection[a]
TS276	φFSW-TI	Mutant thermoinducible for φFSW-TI from mutagenized S-1
TR1	φFSW-TR	From thermoresistant spontaneous revertant of TS276
C239	Cured	From survivors of TS276 after incubation at 42°C
C239(φFSW)	φFSW	C239 lysogenized by φFSW
C239(φFSW-TI)	φFSW-TI	C239 lysogenized by φFSW-TI
C239(φFSW-TR)	φFSW-TR	C239 lysogenized by φFSW-TR
C9		ATCC 29599; from M. E. Sharpe; indicator strain for φFSW

[a] See reference 6.

MATERIALS AND METHODS

Bacterial and phage strains. The *L. casei* group B strains are listed in Table 1. Temperate phage φFSW was isolated from strain S-1. A thermoinducible mutant of φFSW, designated φFSW-TI, was isolated from strain TS276. A revertant of φFSW-TI, designated φFSW-TR, was isolated from strain TR1. Virulent phage φFSV was isolated from abnormal fermentation products of strain S-1.

Media and drugs. MRT medium (7) consists of the following (per liter): polypeptone (Daigo Eiyo Inc., Tokyo, Japan), 10 g; glucose, 10 g; sodium acetate, 10 g; yeast extract (Oriental Kobo Inc., Tokyo, Japan), 3 g; beef extract (Kyokuto Inc., Tokyo, Japan), 3 g; $CaCl_2$, 1.5 g; NaCl, 1 g; $MgSO_4 \cdot H_2O$, 0.2 g; $MnSO_4 \cdot H_2O$, 10 mg; and $FeSO_4 \cdot 7H_2O$, 1 mg. Rogosa medium (2) contains the following (per liter): glucose, 20 g; Trypticase peptone (BBL Microbiology Systems, Cockeysville, Md.), 10 g; yeast extract, 5 g; tryptose (Difco Laboratories, Detroit, Mich.), 3 g; K_2HPO_4, 3

g; KH_2PO_4, 3 g; ammonium citrate, 2 g; sodium acetate, 1 g; Tween 80, 1 g; $MgSO_4 \cdot H_2O$, 0.575 g; L-cysteine hydrochloride, 0.5 g; $MnSO_4 \cdot 2H_2O$, 0.12 g; and $FeSO_4 \cdot 7H_2O$, 34 mg. The pH was adjusted to 6.8 by adding 4 N NaOH. Plates were made with 1.4% agar, and the top agar for assays of phage titers contained 0.6% agar. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine was purchased from Wako Chemicals, Tokyo, Japan.

Assay for bacteria and phage. The numbers of viable cells were measured on Rogosa agar plates at 30°C. The phage titers were assayed on MRT agar plates by using a standard phage overlay technique at 30°C.

Anti-phage sera. A phage lysate (5×10^{10} PFU/ml) was injected intravenously into a 3-kg rabbit six times at about 4-day intervals, starting with 0.5-ml injections and gradually increasing to 5 ml/dose. The rabbit was bled 7 days after the last injection by using standard procedures. Antiserum was heated at 50°C for 30 min and stored at −20°C without preservatives.

Isolation of mutant lysogens thermoinducible for φFSW. Cells of strain S-1 grown to a density of 2×10^8 cells per ml at 37°C in MRT liquid medium were washed and suspended in 0.1 M sodium citrate buffer (pH 5.5) containing 100 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml at a density of about 4×10^8 cells per ml, and these cells were then incubated at 37°C for 30 min. After washing with 0.1 M potassium phosphate buffer (pH 7.0), the mutagenized cells were suspended in MRT medium to a density of 4×10^7 cells per ml, grown at 30°C for 18 h, and plated onto Rogosa agar plates. About 60,000 colony-forming bacteria were tested for the ability to grow at 42°C by a replica-plating method, and 570 thermosensitive mutants were isolated. Each of the thermosensitive mutants was grown exponentially at 30°C in MRT medium and was incubated at 42°C for 30 min (heat treatment) and then at 30°C for additional 18 h. The supernatants of these cultures were spotted onto a lawn of strain C9 as an indicator for φFSW, and the mutant lysogens whose productivities of φFSW were increased by heat treatment were selected.

Isolation of strains cured of prophage φFSW. Cells of strain TS276 were grown at 30°C for about 24 h in MRT medium containing anti-φFSV serum (K [velocity constant of inactivation] = 1) to inactivate free φFSW. The preparations were subcultured twice to concentrate the prophage-cured progeny, and the third culture, which contained 3×10^8 cells per ml, was exposed to 42°C for 40 min. Then the surviving cells were selected as colonies which could grow at 37°C. These colony-forming bacteria were tested for the ability to act as a host for φFSW, and φFSW-sensitive strains were selected.

RESULTS

Mutant lysogen TS276 thermoinducible for φFSW. The curing of lysogens is usually carried out by selection of phage-sensitive strains from surviving cells after induction of the lysogens. However, phage φFSW in strain S-1 cannot be induced by either UV irradiation or mitomycin C treatment (data not shown). Therefore, we attempted to isolate the mutant lysogens which are inducible for φFSW by heat treatment. These mutant lysogens were expected to have a thermosensitive phenotype.

One thermosensitive mutant that produced φFSW in higher yields after heat treatment was isolated from strain S-1 by mutagenesis (see above); this mutant was designated strain TS276. Table 2 shows that the phage productivity of strain TS276 was increased by heat treatment. Cells of strain TS276 were lysed after heat treatment, and the phage produced was designated φFSW-TI. A neutralization test with anti-φFSW-TI serum showed that φFSW-TI was serologically indistinguishable from φFSW. Thus, these results suggested that φFSW-TI is a derivative of φFSW and that strain TS276 is thermoinducible for φFSW-TI.

A culture of strain TS276 contained thermoresistant spontaneous revertants which were shown to be lysogenic at a frequency of about 3×10^{-6}. The φFSW productivity of a representative revertant (TR1) was similar to that of parental strain S-1 (data not shown). Therefore, the thermoinducibility of strain TS276 seems to be caused by a single mutation.

φFSW prophage-cured strains. Attempts were made to obtain cured derivatives, as described above. Of 400 survivors of strain TS276 after heat induction, 80 were φFSW sensitive and did not release φFSW-TI at any temperature tested. These φFSW-sensitive cells were cured of φFSW prophage. Among these survivors, a clone designated C239 was found to be a suitable host for φFSW, since its efficiency of plating was the highest. The phage titers of both S-1 and TS276 supernatants (Table 2) seemed to be underestimated because of the low efficiency of plating on indicator strain C9. When strain C239 was used as the indicator, the plaque-forming ability increased 10^5 times compared with that on strain C9. Then 20 φFSW-resistant strains were isolated from the centers of turbid plaques of φFSW on a lawn of strain C239. All of these resistant strains were shown to be lysogens which released a small amount of φFSW sponta-

TABLE 2. Effect of heat treatment on φFSW productivity of strain TS276

Strain	Supernatant of culture titrated on C9 (PFU/ml)[a]	
	+Heat	−Heat
S-1	2.0×10^2	2.1×10^2
TS276	1.1×10^5	5.3×10^3

[a] Cells were grown at 30°C to a density of about 3×10^8 cells per ml in MRT medium, and the culture was divided into two portions. One-half of the culture was heated at 42°C for 30 min (+Heat), and the other half was not heated (−Heat). These preparations were incubated at 30°C for an additional 18 h and centrifuged, and the supernatant was titrated.

neously [designated C239(ϕFSW)]. Strain C239 could also be lysogenized by ϕFSW-TI or ϕFSW-TR1. C239, C239(ϕFSW), and C239(ϕFSW-TR1) could grow at 42°C, but C239(ϕFSW-TI) could not. Moreover, only C239(ϕFSW-TI) could be induced for the phage by heat treatment, as described below. These results indicate that the mutation which is responsible for the thermoinducibility and thermosensitivity of host cells is located on ϕFSW-TI prophage. Therefore, ϕFSW-TI prophage-cured strains, such as C239, are resistant to heat induction.

Kinetics of heat induction. Heat induction of ϕFSW-TI was examined (Fig. 1). By heat treatment at 42°C for 30 min, colony-forming progenies of C239(ϕFSW-TI) were reduced at a frequency of about 2×10^{-4}, and 85% of the heat-treated cells seemed to form plaques when they were plated with indicator cells of strain C239. The turbidity of a C239(ϕFSW-TI) culture began to decrease at 2 h after heat treatment, and by 3 h complete lysis had occurred. The number of free phage in the supernatant increased rapidly between 2 and 3 h and remained constant thereafter. The average burst size of ϕFSW-TI after heat induction (mean yield of phage particles per bacterium) was 70. Without heat treatment, lysis did not occur, and the number of free phage in the supernatant increased proportionally with cell growth, which seemed to be due to spontaneous induction. The frequency of spontaneous induction of C239(ϕFSW-TI) was likely to be higher than that of C239(ϕFSW), because the supernatant of C239(ϕFSW-TI) contained about 30 times as many phage as the supernatant of C239(ϕFSW).

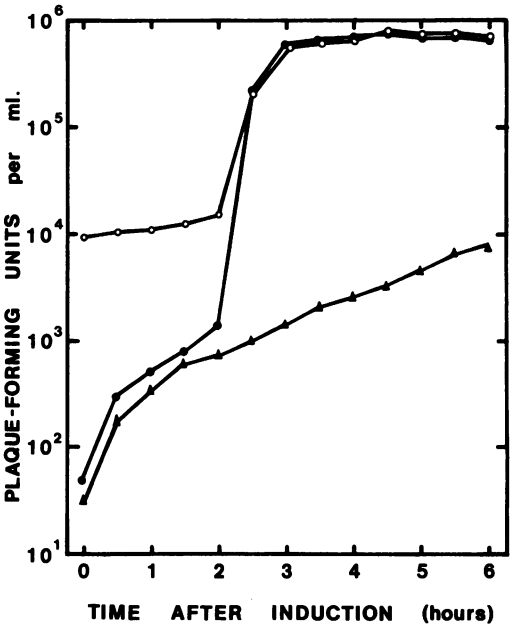

FIG. 1. Heat induction of C239(ϕFSW-TI). C239(ϕFSW-TI) was grown in MRT medium containing anti-ϕFSV serum (K = 1) at 30°C to a density of about 10^8 cells per ml and then diluted 10^4-fold. The diluted culture was heated at 42°C for 30 min, shifted at zero time to 30°C for further incubation, and titrated on a lawn of strain C239 cells (○). Free phage was titrated after centrifugation at 10,000 × *g* for 1 min (●). An unheated culture was titrated similarly after centrifugation (▲).

DISCUSSION

We isolated thermoinducible mutant phage ϕFSW-TI by direct mutagenesis of lysogen S-1, from which wild-type ϕFSW cannot be induced under laboratory conditions. ϕFSW-cured strains could be obtained easily from the lysogen of ϕFSW-TI after heat induction. For prophage curing, it is useful to obtain thermoinducible mutants.

It has been reported that the lysogenic streptococci used for dairy production may be one source of bacteriophage contamination on an industrial scale and that prophage curing may result in improvement of the strains (3–5). In acetone-butanol-producing clostridia, a virulent phage causing abnormal fermentation has been suggested to be a virulent mutant of the temperate phage lysogenized with fermenting bacteria (9). In lactobacilli, ϕFSW-cured strains (see above) were shown to be excellent in producing lactic acid beverages for the following reasons. First, there has been no occurrence of virulent phage ϕFSV during fermentation with these cured strains. This observation is consistent with the suggestion that virulent phage ϕFSV is derived from ϕFSW, although the ultimate source of ϕFSV is unknown. Second, the mutation causing thermoinducibility is located on the prophage, and the cured strains seem to retain their original properties relevant to beverage production despite mutagenesis.

These prophage-cured strains are suitable hosts not only for ϕFSW but also for ϕFSV. Studies of differences between ϕFSW and ϕFSV using these cured strains may help reveal the origin and the mechanisms of occurrence of ϕFSV.

ACKNOWLEDGMENTS

We are deeply indebted to H. Saito of the Institute of Applied Microbiology, Tokyo University, for his invaluable discussions and advice. We thank T. Khosaka of the Yakult Central Institute for Microbiological Research for his critical reading of the manuscript. We also thank S. Kodaira, Y. Kimura, and K. Sakamoto for testing the strains in practical use and A. Kato for technical assistance.

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