Prophage Origin of a Virulent Phage Appearing on Fermentations of *Lactobacillus casei* S-1

MARIKO SHIMIZU-KADOTA,* TOSHIZO SAKURAI, AND NOBUO TSUCHIDA

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

For protection from the abnormal fermentation of *Lactobacillus casei* S-1 caused by contamination of a virulent phage, φFSV, the origin of this phage was studied. Morphologies, viral structural proteins, and DNA structures of three independent isolates of φFSV were compared with those of φFSW, which is lysogenized in strain S-1. The results showed (i) that the morphology of φFSV phages is indistinguishable from that of φFSW and (ii) that all viral structural components found in φFSW are present in the particles of φFSV's. In addition, restriction endonuclease analyses of viral DNA showed that the *Hind*III-digested fragments of φFSW DNA, the sum of which covered at least 94.7% of this phage genome, were conserved in the φFSV DNA digests. Results of Southern filter hybridization of the S-1 and prophage-cured cell (C239) DNAs with φFSV DNA as a probe revealed that C239 had lost most of the φFSV DNA sequence, whereas S-1 had about one copy of the φFSV DNA sequence. These results indicate that virulent phage φFSV is derived from the lysogenized phage φFSW. Therefore, the appearance of φFSV can be eliminated by using the prophage-cured derivative of S-1.

Protection of culture fermentations from bacteriophage contamination is important in the fermentation industry. Lysogens have been used frequently as starter culture strains in cheese and yogurt production (12, 16). During fermentation with lysogenic starters, the occurrence of virulent phages has often been observed. Although temperate phages lysogenized in these strains have been suspected to be the source of virulent phages (7, 9, 14, 18), the ultimate sources of these virulent phages are unknown.

Lactobacillus casei strain S-1 has been used industrially to produce lactic acid beverages. This strain harbors a temperate phage φFSW and is thus resistant to this phage. When strain S-1 was used as a single strain starter for beverage production, a virulent phage, φFSV, which could grow on strain S-1 but could not be lysogenized with the strain (unpublished results), appeared frequently despite cultivation in a closed tank system. These φFSV's were serologically indistinguishable from φFSW (19). These data suggest that φFSV originates from strain S-1, and it is probable that φFSV is derived from φFSW. In the present study, we compared the biochemical characteristics of φFSV with those of φFSW. Moreover, φFSV-related DNA sequences were studied in both the lysogen S-1 and the φFSW-cured strains. The results showed that φFSV's were derived from φFSW.

MATERIALS AND METHODS

Bacteria and phage strains. Group B *L. casei* S-1, which carries a temperate phage φFSW, and strain C239, which is a φFSW-cured derivative of S-1, have been described previously (19). φFSW-TI is a thermoinducible mutant of φFSW (19). Virulent phages φFSV-A, φFSV-B, and φFSV-C, which can grow on φFSW lysogen, were isolated independently from abnormal fermentation products of strain S-1. Another virulent phage, J1 (8), was used to prepare endolysin.

Enzymes. Restriction endonuclease *Hind*III was purchased from Takara Shuzo Co. Ltd. (Kyoto, Japan). DNA was digested under the conditions suggested by the supplier.

Endolysin was prepared from the lysate of J1-infected C239 as follows. Cells were grown in 500 ml of MRT medium (17) at 37°C to a density of about 2×10^8 cells per ml and infected with J1 at a multiplicity of 3. After incubation for 5 h, cell debris was removed by centrifugation. Endolysin was precipitated in a 70% saturation of ammonium sulfate and collected by centrifugation. The pellet was dissolved in a minimum volume of L buffer (0.1 M potassium phosphate buffer [pH 6.0] containing 0.01 M 2-mercaptoethanol) and dialyzed against L buffer. After sonication to reduce the viscosity, the solution was loaded on a carboxymethyl cellulose column (CM52; Whatman, Kent, England; 25 by 1.5 cm). A 300-ml (total volume) linear

gradient of 0 to 0.5 M NaCl in L buffer was applied at a flow rate of 30 ml/h. The fractions containing endolysin activity, eluted at a concentration between 0 and 0.1 M NaCl, were dialyzed against L buffer and used as crude endolysin solution. One unit of lytic activity was defined as the amount of endolysin which lysed 1 μg of lyophilized cells of *L. casei* S-1 per h at 37°C. The reaction mixture consisted of L buffer containing 100 μg of the lyophilized cells per ml and an appropriate amount of endolysin solution.

Cultivation and purification of phages. For preparation of phage ϕFSW-TI, C239 (ϕFSW-TI) was grown at 30°C in MRT medium to a density of about 5×10^8 cells per ml. An equal volume of MRT medium, prewarmed at 55°C, was added. The culture was incubated at 42°C for 30 min and then at 37°C until complete lysis occurred. Each of the ϕFSV or wild-type ϕFSW lysates was prepared with C239 as a host by the plating method (1) in MRT medium. Phages in the lysate were precipitated with 10% (wt/vol) polyethylene glycol 6000 and 0.5 M NaCl at 0°C and collected by centrifugation at 8,000 $\times$ *g* for 20 min (23); the precipitated phages were resuspended in phage buffer (0.05 M Tris-hydrochloride [pH 7.3], 0.15 M NaCl, 0.02 M NH$_4$Cl, 0.01 M MgCl$_2$, 0.001 M CaCl$_2$, and 0.2% gelatin). After cell debris was pelleted, the phage suspension was treated with 100 μg of RNase A (Sigma Chemical Co., St. Louis, Mo.) per ml and 10 μg of DNase I (Miles Laboratories, Inc., Elkhart, Ind.) per ml. Phage particles were purified by banding in two successive CsCl step gradients (23).

SDS-polyacrylamide gel electrophoresis. Approximately 2×10^{10} PFU of purified phage particles suspended in 40 μl of a solution containing 0.05 M Tris-hydrochloride (pH 6.8), 0.02 M sodium EDTA, 10% 2-mercaptoethanol, and 1% sodium dodecyl sulfate (SDS) were disrupted by boiling at 100°C for 10 min, electrophoresed in a gel slab (15% acrylamide; 0.2 by 14 by 10 cm) by using the Laemmli discontinuous buffer system (11), and stained with Coomassie brilliant blue R250 as described previously (21). Standard proteins for molecular weight estimation (Pharmacia, Uppsala, Sweden) used were: lactate dehydrogenase from beef heart (140,000), phosphorylase *b* from rabbit muscle (94,000), albumin from bovine serum (67,000), ovalbumin from egg white (43,000), carbonic anhydrase from bovine erythrocytes (30,000), trypsin inhibitor from soybeans (20,100), and α-lactalbumin from bovine milk (14,400).

Preparation of DNA. For phage DNA preparation, 0.3 ml of a suspension containing 10^{12} PFU of purified phage was heated at 65°C for 10 min in the presence of 1% SDS and 0.02 M sodium EDTA, and treated with 100 μg of fungal proteinase K (Merck, Darmstadt, West Germany) per ml at 37°C for 20 min. The sample was extracted twice with 2 volumes of a phenol-chloroform mixture (1:1 [vol/vol] mixture of water-saturated phenol and chloroform-isoamyl alcohol [24:1 (vol/vol)]) and dialyzed against DNA buffer (0.01 M Tris-hydrochloride [pH 7.6] and 0.1 mM sodium EDTA).

High-molecular-weight DNAs of various strains of *Lactobacillus* were prepared by the following procedure. Cells were grown in 500 ml of Rogosa medium (5) containing 0.5 U of potassium penicillin G (Meiji, Tokyo, Japan) per ml at 37°C to a density of about 5×10^7 cells per ml. Cells were harvested and washed first with 0.05 M Tris-hydrochloride (pH 8.0)–0.02 M sodium EDTA and then with 75% ethanol–2% phenol–0.05 M Tris-hydrochloride (pH8.0)–0.01 M sodium EDTA. Then cells were suspended in 2.5 ml of H buffer (0.05 M sodium phosphate buffer [pH 6.0] containing 25% sucrose, 0.02 M sodium EDTA, and 0.01% diethylpyrocarbonate) and heated at 70°C for 15 min to inactivate the nucleases. A 0.4-ml amount of lysozyme (Sigma) (7 mg/ml) and 0.4 ml of J1-induced endolysin (175 U/ml) were added, and the cell suspension was incubated at 37°C for 1 h. Cells were collected, suspended gently in 0.2 ml of H buffer, and frozen in an ethanol-dry ice bath. Then 9 volumes of a solution containing 0.05 M Tris-hydrochloride (pH 8.0), 2.5% SDS, 0.02 M sodium EDTA, 0.01% diethylpyrocarbonate, and 50 μg of proteinase K per ml were preheated at 65°C and added to the cell suspension. After incubation at 65°C for 5 min, the lysate was treated with 500 μg of proteinase K per ml at 37°C for 3 h. CsCl was added to a final density of 1.7 g/ml, and then the resulting solution was centrifuged at 36,000 rpm at 15°C for 20 h in a Hitachi RPS65 rotor. After centrifugation, the viscous DNA fraction was collected from the bottom of the tube and dialyzed against DNA buffer.

Agarose gel electrophoresis and Southern filter hybridization. DNA digested with restriction endonuclease was electrophoresed in an agarose (SeaKem, Rockland, Me.) gel slab (0.3 by 14 by 12 cm) with Loening buffer (36 mM Tris, 32 mM NaH$_2$PO$_4$ [pH 7.8], 1 mM sodium EDTA) (13). *Hin*dIII-digested λ DNA (BRL, Rockville, Md.) was used as a size marker (2). For Southern filter hybridization, the gel was first treated with 1 N acetic acid for 30 min (22) and then transferred to a nitrocellulose filter as described previously (20). The filter was baked at 80°C for 2 h and treated for 1 h at 42°C with prehybridization mixture containing 5$\times$ SSC (1$\times$ SSC, 0.15 M NaCl plus 0.015 M sodium citrate), 5$\times$ Denhardt reagent (3), 0.05 M piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES) (pH 6.8), 50% (vol/vol) formamide, and 250 μg of denatured salmon sperm DNA per ml. Hybridization with ^{32}P-labeled ϕFSV DNA was performed at 42°C for 16 h by saturating the filter with the hybridization mixture containing 10^6 cpm of denatured [^{32}P]DNA probe per ml, 5$\times$ SSC, Denhardt reagent, 0.01 M PIPES (pH 6.8), 50% (vol/vol) formamide, 100 μg of denatured salmon sperm DNA per ml, and 10% (wt/vol) dextran sulfate. The [^{32}P]DNA probe was prepared by a nick translation procedure (15). A 1-μg amount of DNA was incubated for 3 h at 15°C in 40 μl of a solution containing 0.05 M Tris-hydrochloride (pH 7.8), 5 mM MgCl$_2$, 0.01 M 2-mercaptoethanol, 50 μg of bovine serum albumin per ml, 2 μM [α-^{32}P]dCTP (2,000 to 3,000 Ci/mmol; Amersham, Buckinghamshire, England), and 2 μM each dATP, dGTP, and dTTP in the presence of 0.1 ng of DNase I (BRL) and 1 U of *Escherichia coli* DNA polymerase I (BRL). The reaction product was separated from the unincorporated nucleotides by gel filtration with Sephadex G-50. The specific activity of the probe was more than 5×10^7 cpm/μg of DNA. After hybridization, the filter was washed once with 150 ml of 50% (vol/vol) formamide containing 5$\times$ SSC at room temperature for 5 min, followed by two washes of 5 min each at room temperature with 250 ml of 2$\times$ SSC containing 0.1% SDS, and finally two washes at 45°C with 250 ml of prewarmed 0.1$\times$ SSC containing 0.1% SDS for 30 min

each. The filter was exposed to a Kodak XAR film with Dupont Cronex Lightning Plus intensifying screens.

RESULTS

Morphology and protein composition of phage particles. Phage particles of φFSW and three φFSV's, φFSV-A, φFSV-B, and φFSV-C, which were isolated independently from abnormal fermentation products of *L. casei* S-1, were examined under an electron microscope. Figures 1 to 4 show that their morphologies were indistinguishable from that of φFSW. Each of the phages had a hexagonal head 57 nm in diameter, a noncontractile tail 156 nm in length and 8 nm in width, and a baseplate 14 nm in diameter.

Structural proteins of φFSW and φFSV's were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 5). Disrupted purified φFSW particles gave four major bands with estimated molecular weights of 48,000, 44,000, 30,000, and 13,500, respectively, and at least eight minor bands with estimated molecular weights of 130,000, 81,000, 64,000, 43,000, 38,000, 32,000, 21,000, and 15,000, respectively. All of these bands were detected in all three φFSV's, and some additional bands were observed in φFSV-B and φFSV-C.

Comparison of DNA structure between φFSW and φFSV's. The genome DNAs of φFSW and three φFSV's were compared by restriction endonuclease analyses. *Hin*dIII cleaved φFSW DNA into at least 14 fragments, and the molecular sizes of the digested DNA fragments were estimated by their electrophoretic mobilities relative to those of lambda phage DNA digested with *Hin*dIII (Fig. 6, lane 1). The genome size of φFSW was given by the sum of the molecular sizes of these 14 fragments, which was calculated to be 41.3 kilobase pairs (kbp). The value is a minimum estimate, since small fragments could not be detected.

The pattern of *Hin*dIII digests of φFSV-A was indistinguishable from that of φFSW (Fig. 6, lane 2). φFSV-A DNA was further digested with other restriction endonucleases, and no difference was found from the digests of φFSW DNA (data not shown). All *Hin*dIII-digested fragments of φFSW DNA, except for the 2.2-kbp E fragment, were detected in φFSV-B and φFSV-C DNAs (Fig. 6, lanes 3 and 4), and the sum of their molecular sizes represents about 94.7% of the genome size of φFSW. The E fragment of φFSW was missing in φFSV-B and φFSV-C digests, but the 3.0-kbp fragment in φFSV-B digests and the 1.9- and 0.8-kbp fragments in φFSV-C digests were detected. Since small fragments of *Hin*dIII digests could not be detected in the experiment shown in Fig. 6, the phage DNAs were subjected to digestion with *Pst*I, *Bgl*II, or *Bst*EII. In each digestion, a single fragment of φFSW DNA digests was missing in each of the φFSV-B and φFSV-C DNA digests, and a new fragment which was 1.3 kbp larger than the corresponding fragment of φFSW digests was detected in each of the φFSV-B and φFSV-C DNA digests (data not shown). These observations suggest (i) that φFSV-B and φFSV-C genomes are homologous in DNA sequence to the φFSW genome, except for the sequence represented in the *Hin*dIII-E fragment of φFSW, and (ii) that the respective sequences of φFSV-B and φFSV-C DNA corresponding to that of the φFSW-E fragment are larger by 1.3 kbp. Thus,
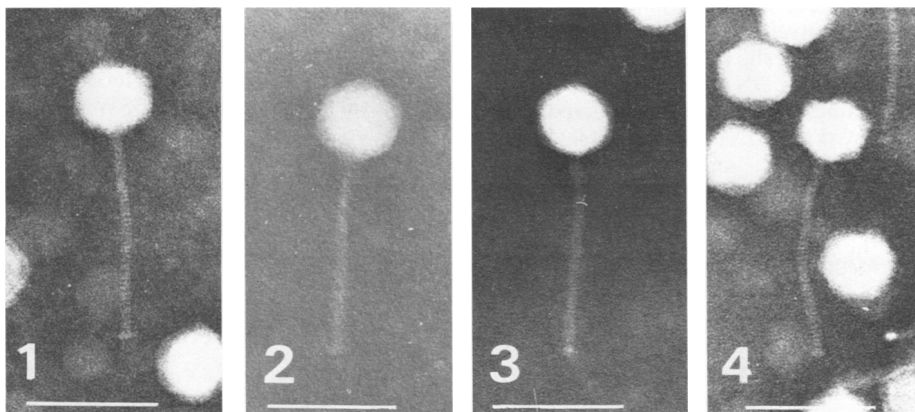

FIG. 1–4. Electron micrographs of φFSW (1), φFSV-A (2), φFSV-B (3), and φFSV-C (4). Purified phages were diluted to about 10^{11} PFU/ml in 0.1 M ammonium acetate (pH 7.2), negatively stained with 2% (wt/vol) phosphotungstic acid (pH 6.8), and examined in a JEM-7A electron microscope (Japan Electron Optics Laboratory, Tokyo, Japan). Bars, 100 nm.
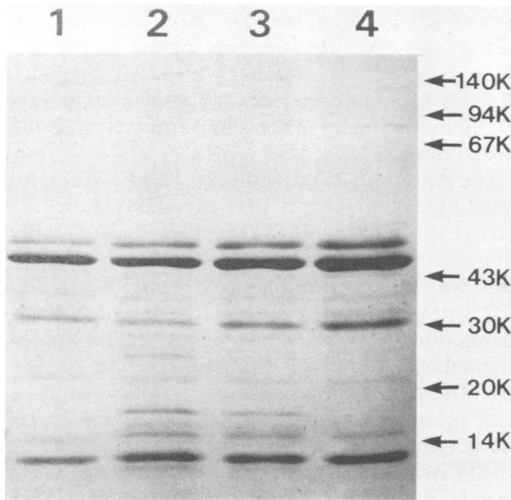
FIG. 5. Patterns of structural proteins of φFSW and φFSV's by SDS-polyacrylamide gel electrophoresis. Lane 1, φFSV-A; lane 2, φFSV-B; lane 3, φFSV-C; lane 4, φFSW-TI.

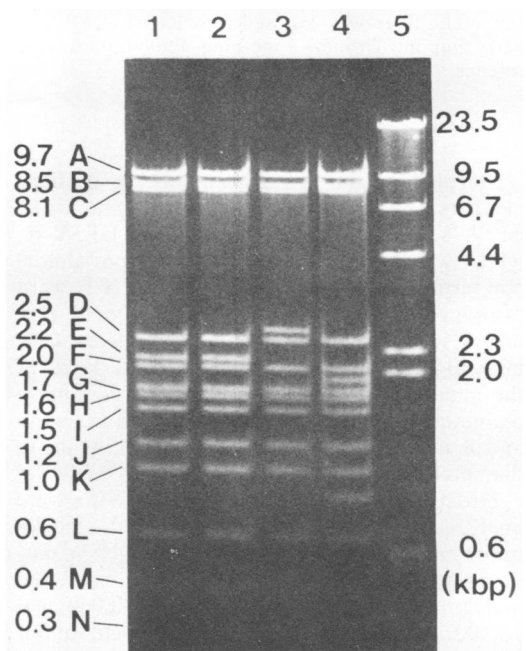FIG. 6. Patterns of HindIII-digested DNA fragments by agarose gel electrophoresis. φFSW and three φFSV DNAs were digested with HindIII and electrophoresed in a 1% agarose slab gel for 2 h at 5 V/cm. The slab gel was stained with 0.5 μg of ethidium bromide per ml for 30 min and photographed with a red filter under UV light (254 nm). Lane 1, φFSW-TI; lane 2, φFSV-A; lane 3, φFSV-B; lane 4, φFSV-C; lane 5, HindIII-digested λ DNA.

φFSV's were closely related to φFSW in DNA structure, which suggested that they were derivatives of φFSW.

φFSV-related sequence in DNAs of the lysogen and the cured strain. To prove that φFSV's originate from temperate phage φFSW lysogenized in *L. casei* strain S-1, φFSV-related DNA sequences were examined in cellular DNAs of the lysogen S-1 and the φFSW-cured strain C239 by Southern filter hybridization (Fig. 7). When φFSV-C DNA was used as a probe and hybridized to the HindIII-digested φFSW DNA, essentially the same fragments which were visualized by the ethidium bromide staining method (Fig. 6) were detected (Fig. 7, lanes 3 to 7) with the intensity correlating to their molecular weights. The result shows that φFSV-C DNA contains most of the DNA sequences of φFSW. When the same probe was hybridized with HindIII-digested S-1 DNA (Fig. 7, lane 2), the band pattern was the same, except that two additional bands with molecular sizes of 4.6 and 1.1 kbp were observed. These results show that φFSV-related sequences were present in S-1 DNA, and furthermore, that most of the φFSV-related DNA sequences in S-1 were structurally similar to that of φFSW. When the same amount of C239 DNA (Fig. 7, lane 1) as S-1 DNA was hybridized, the probe detected at least two faint bands, corresponding to 8.3 and 1.1 kbp, but did not detect most of the bands seen in S-1 DNA. Similar results were obtained when φFSV-A or φFSV-B DNA was used as a probe (data not shown).

For estimation of the copy number of φFSV-related sequences in S-1 DNA, the intensity of the hybridization bands of 0.5 μg of HindIII-digested S-1 DNA (Fig. 7, lane 2) was compared with that obtained with serial dilutions of HindIII-digested φFSW DNA (Fig. 7, lanes 3 to 7). S-1 bands were intermediate in intensity between the φFSW bands in lane 5 (1.0 ng) and lane 6 (2.0 ng). As the genome size of φFSW is about 2.7×10^7 daltons (1 base pair was calculated as 654 daltons) and those of bacteria were reported to range from 10^9 to 10^{10} daltons (10), S-1 DNA was estimated to have one copy of the φFSV-related sequence structurally similar to that of φFSW, and C239 was estimated to have none of the copy.

DISCUSSION

Virulent phage φFSV, which was isolated from abnormal fermentation products in single cultures of strain S-1, was shown to be closely related to the temperate phage φFSW harbored in S-1 by morphology, protein composition of the particle, and the pattern of genome DNA
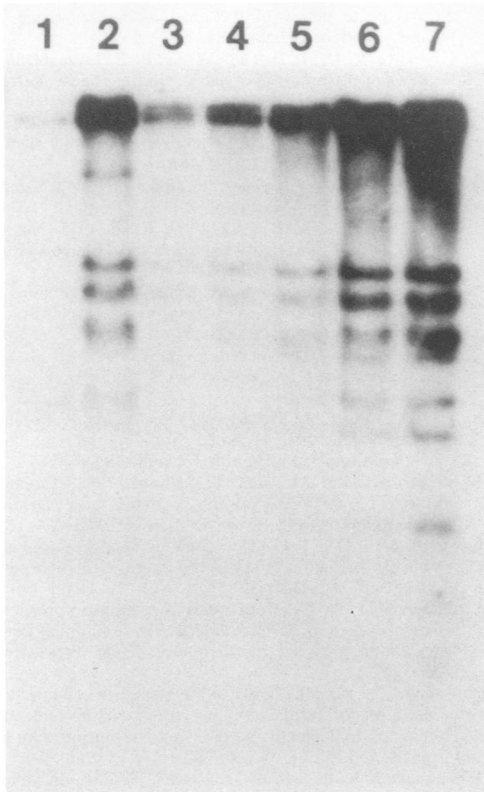
FIG. 7. Southern filter hybridization of *Hin*dIII-digested cellular DNAs. A 0.5-μg amount of *Hin*dIII-digested C239 DNA (lane 1), 0.5 μg of *Hin*dIII-digested S-1 DNA (lane 2), and *Hin*dIII-digested φFSW DNA (0.25 ng, lane 3; 0.5 ng, lane 4; 1.0 ng, lane 5; 2.0 ng, lane 6; and 4.0 ng, lane 7) were electrophoresed in a 1.5% agarose gel for 14 h at 2.6 V/cm, transferred to a nitrocellulose filter, and hybridized with ^{32}P-labeled φFSV-C DNA. The filter was exposed to an X-ray film as described in the text.

cleaved with restriction endonuclease. Furthermore, we have developed a new method to isolate high-molecular-weight DNA from *Lactobacillus* spp., which enabled us to analyze phage-related cellular DNA sequences by the Southern filter hybridization method. The results showed (i) that φFSV DNA contained most of the DNA sequences of φFSW and (ii) that S-1 DNA had a single copy of the φFSV-related sequence structurally similar to that of φFSW, but C239 did not have such sequence. These results suggest that the φFSV-related sequence present in S-1 but not in C239 is that of the φFSW prophage. Therefore, we concluded that φFSV is derived from the φFSW prophage in S-1, and thus the prophage-cured derivative is not able to produce φFSV. It is possible that the prophage is integrated into the host chromo-

some, since the 4.6-kbp *Hin*dIII-fragment observed in S-1 was detected neither in φFSW nor in C239. Although we could detect only the 4.6-kbp lysogen-specific *Hin*dIII fragment in S-1 DNA, preliminary results showed that a φFSW fragment disappeared, but two lysogen-specific fragments were detected when S-1 DNA was digested with other restriction endonucleases.

We isolated φFSW prophage-cured derivatives of S-1 by introducing a thermoinducible mutation into the prophage and selecting surviving cells after heat induction, as reported previously (19). By using these cured strains, the appearance of φFSV was eliminated during the fermentation. Thus, curing of prophage φFSW in S-1 is the most effective method of protection from contamination by virulent phage φFSV, which causes abnormal fermentation.

Two possible mechanisms of the derivation of φFSV from φFSW were proposed on the basis of the results of restriction endonuclease analysis shown in Fig. 6: (i) multiple point mutations and (ii) rearrangement of φFSW DNA or insertion of exogenous DNA sequence or both. The first mechanism was suggested from the result that the structure of φFSV-A DNA was indistinguishable from that of φFSW DNA. When φFSW particles were mutagenized in vitro by ethylmethanesulfonate under conditions of 60% survival, virulent mutants were obtained with a frequency of less than 10^{-9} (unpublished results). As this value was much lower than the estimated frequency of a single point mutation induced under the same conditions (more frequent than 10^{-5}, calculated from the genome size of φFSW and the ratio of the survivors [4]), these virulent mutants obtained by the mutagenesis of φFSW were supposed to be induced by multiple point mutations rather than by a single point mutation. Therefore, it is possible that the derivation of φFSV from φFSW on fermentations of strain S-1 occurs by multiple point mutations. Actually, in the case of a temperate coliphage λ, it was reported that more than two mutations at the two operators O_L and O_R can make the phage virulent (6). The second mechanism was suggested from the result that structures of φFSV-B and φFSV-C DNA were different from that of φFSW in the region of the *Hin*dIII-E fragment of φFSW DNA. Although the functions of the genes within this segment have not been elucidated, one of the possibilities is that by DNA rearrangement or insertion or both, φFSV-B and φFSV-C have lost genes of the parental phage which respond to growth control by the lysogen.

preparation of high-molecular-weight cellular DNA from *Lactobacillus* species. We thank S. Iwata for the preparation of the electron micrographs and N. Mizobuchi and Y. Toki for critical reading of the manuscript. We also thank M. Mutai for his interest and encouragement.

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