Characterization of a Phage Resistance Plasmid, pLKS, of Silage-Making
Lactobacillus plantarum NGRI0101

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Phage contamination has resulted in abnormal fermentation in silage. We isolated a phage-resistant strain, Lactobacillus plantarum NGRI0101 from silage. The strain carried two plasmids, pLKL (6.8 kb) and pLKS (2.0 kb). By curing and retransformation of the plasmids, we clarified that pLKS has phage resistant activity, characterized as no adsorption inhibition. pLKS has 2,025 bp and three orfs, orf123, orf132, and orf918. The predicted amino acid sequence of the orf918 product showed high similarity to those of Rep proteins of Pediococcus halophilus plasmid pUC1287 and Lactobacillus acidophilus plasmid pLA103. The replication origin (ori) was upstream from orf918. There was no gene similar to typical phage resistant genes encoded by known plasmids. The phage resistance of L. plantarum NGRI0101 may possibly be due to a plasmid-encoded abortive infection.

Key words: phage resistance; plasmid; silage-making Lactobacillus

Silage is a storable feed which makes use of lactic fermentation.5) Phage contamination can result in abnormal fermentation in silage, and in particular, infection is at a high frequency in silage of southwestern Japan such as Kyushu and Okinawa.2,3) This is attributed to high temperatures (over 30°C) and high moisture contents of plant materials (over 80%). Such being the case, phage control is needed for processing and preservation of high quality silage.

There are reports of phage resistance in lactococci,4,5) but not in lactobacilli. Extensive research has been done on interactions between lactococcal phages and their hosts. L. lactis was found to have many plasmids coding for natural defense mechanisms against phages, and over 40 plasmids with phage defense barriers have been identified.4-8) The mechanisms of plasmid-encoded phage resistance are grouped as follows: phage adsorption interference, phage DNA injection inhibition, restriction and modification systems, and abortive phage infection (Abi).9) Constructing phage-resistant strains by introducing these natural plasmids into phage-sensitive strains is useful for silage fermentation as it is for dairy fermentation.

We isolated many silage-making lactobacilli with high lactic acid production from silage from Kyushu and Okinawa.9) In these strains, Lactobacillus plantarum NGRI0101 showed phage resistance and had two plasmids. These two plasmids are characterized in this work.

Materials and Methods

Bacterial strains, phages, and media. Silage-making lactobacilli used were isolated form Kyushu and Okinawa islands, southwestern Japan, and were grown at 37°C in MRS or GYP medium (Difco Laboratories, Detroit, MI, USA).30) Escherichia coli JM109 was grown at 37°C in LB medium.31) Erythromycin (5 μg/ml) for lactobacilli, ampicillin (50 μg/ml), and erythromycin (100 μg/ml) for E. coli were added to these media, as required.

Ten phages isolated from silage2,3) were classified into seven groups (I, II, III, IV, V1, V2, and V3)2,3) depending on host ranges, DNA homologies, DNA restriction patterns, and protein profiles: group V1 was newly classified from group V2 for this work. They were propagated on the common host Lactobacillus pentosus NGRI0524.3) The solution of each phage was kept at -20°C with 20% (vol/vol) glycerol until use.

Isolation of phage-sensitive mutants. Plasmid-curing experiments were done as described by Ruiz-Barba et al.13) L. plantarum NGRI0101 was incubated with various concentrations of SDS (0.1-1.0 mg/ml) and novobiocin (0.1-0.7 μg/ml) for 72 hr. Cultures that grew at the highest concentration of curing reagents were serially diluted with MRS broth and plat-

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ed on MRS agar plates. After 48 hr of incubation at 37°C, the colonies were replica-plated and examined for the presence of plasmids.

Preparation, manipulation, and transformation of plasmid DNA. Plasmid DNAs were isolated from _L. plantarum_ NGR10101 and its derivatives using the method of Anderson and Mckay,16 and from _E. coli_ as described by Sambrook et al.10 Restriction digestion of plasmid DNA was based on the instructions of suppliers. Conventional agarose gel electrophoresis and general cloning techniques were done as described by Sambrook et al.11

The _E. coli_ strain was transformed using the CaCl$_2$ method.10 Lactobacilli were transformed by electroporation with a transformation system ECM600 (BTX, San Diego, USA), following the procedures described by Fan et al.15

Southern hybridization. Total DNAs from _L. plantarum_ NGR10101 and its derivatives were separated by agarose gel electrophoresis and transferred by Southern transfer16 onto nitrocellulose filters (BA85; Schleicher & Schuell, Germany). The chemical labeling of probes, hybridization, and detection of hybridized DNA fragments were done using the DIG DNA labeling and detection kit (Roche Diagnostic, Mannheim, Germany) as specified by the supplier.

Phage sensitivity. Phage sensitivity of silage-making lactobacilli and derivatives were examined by a spot test using double layer MRS agar (top, 0.7% agarose; bottom, 2% agarose). A small drop (0.02 ml) of phage solution was spotted on the surface of top agar plates containing young cells of strains to be examined, as described elsewhere.3,17 Plates were incubated at 37°C for 24 hr.

Plaque formation was examined to confirm phage sensitivity.

Phage adsorption. The rate of adsorption of phages to _Lactobacillus_ was measured according to the description by Adams.18

DNA sequencing. DNA sequencing was done using the dye-deoxy chain termination method and a Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, UK). The identity and similarity values of DNA and amino acid sequences were analyzed using the GENETYX software program (Software Development Co. Ltd., Japan).

Results and Discussion

Isolation of phage resistant plasmids from silage-making lactobacilli

As reported,2,3 much spoiled silage from southwestern of Japan, were damaged by phage infection. To avoid this infection, phage-resistant lactobacilli were screened for with use of all the phages isolated from the spoiled silages.2,3 In more than 50 NGRI and type strains of _Lactobacillus_ tested,2,3 _L. plantarum_ NGR10101 distinctly showed resistance toward phages of groups II, V1, and V2 (Table 1); these phages were adsorbed. However, phages of groups I, III, IV, and V3 showed little adsorption.

Strain NGR10101 had two plasmids, pLKL and pLKS, as shown in Fig. 1(A). Sizes of pLKL and pLKS were approximately 6.8 kb and 2.0 kb, respectively. To observe the similarity between pLKL and pLKS, Southern hybridization using both plasmids as probes was done. Figure 1(B) shows that pLKL has no similarity to pLKS.

In plasmid-curing experiments, two strains, which had phage sensitivity, were obtained: strain NGR10101-L cured pLKS, and strain NGR10101-N cured both pLKL and pLKS (Fig. 1 and Table 1). Therefore, we considered that pLKS had the phage resistance of strain NGR10101. The efficiency of plating (EOP) of strain NGR10101-L was higher than that of strain NGR10101-N. Growth of strain NGR10101-N was slow. Useful characteristics of su-

| Table 1. Phage Sensitivity of _Lactobacillus plantarum_ NGR10101, _L. pentosus_ NGR10225 and Their Derivatives

<table>
<thead>
<tr>
<th>strain</th>
<th>plasmid</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
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<td>(A) <em>Lactobacillus plantarum</em></td>
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<tr>
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<td>pUCLSEm</td>
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<td>(B) <em>Lactobacillus pentosus</em></td>
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-, no plasmid; +, sensitive to phage; -, resistant to phage.
Phage Resistance Plasmid of Silage-making *L. plantarum*

Figure 1. Agarose Gel Electrophoresis and Hybridization of Plasmids in Silage-making *Lactobacillus plantarum* NGR10101 and Its Derivatives.

(A), Agarose gel electrophoresis; (B), Hybridization.

Lane 1 (A), λ DNA digested with HindIII; lane 1 (B), purified plasmids pLKL and pLKS, lane 2 (A, B), *L. plantarum* NGR10101 (carrying pLKL and pLKS); lane 3 (A, B), *L. plantarum* NGR10101-L (carrying pLKL); lane 4 (A, B), *L. plantarum* NGR10101-N (carrying no plasmid). Probe: B1, pLKL; B2, pLKS.

gar fermentation and consumption, and antibiotics resistance are encoded by plasmids. The poor growth of strain NGR10101-N may be due to a lack of plasmid pLKL encoding useful characteristics for growth.

To find if pLKS is indeed concerned with the phage resistance, a recombinant plasmid, pUCLSEm, was constructed, as shown in Fig. 2. The plasmid pUCLSEm was used to transform strains NGR10101-L and NGR10101-N and other silage-making lactobacilli. Transformants were obtained from strain NGR10101-L and *Lactobacillus pentosus* NGR10225 (old name *L. plantarum* NGR10225). Phage sensitivity of each strain was detected in spot tests. As shown in Table 1, transformant NGR10101-LR showed resistance to phages φPY3 (Group II), φPY6 (Group V₁), and φPY8 (Group V₂). Although *L. pentosus* NGR10225 was sensitive to four phages (Table 1), its transformant NGR10225R was resistant to phages φPY8 (Group V₁) and φPY7 (Group V₂). The transformants adsorbed the phages as did their parents. This means that plasmid pLKS has phage-resistant activity. However we have no explanations for why the transformant NGR10225R do not express resistance against phage φPY6.

Gene structure and function of pLKS

Figure 3 shows the restriction analysis of pLKS, which has a circular DNA molecule and four unique sites (BglII, EcoRI, HincII, and XbaI). BglII-digested pLKS was cloned into the BamHI site of pUC119 and the complete nucleotide sequence proved to be

Fig. 2. Strategy for Construction of Recombinant Plasmid pUCLSEm.

pUC19 plasmid; MCS, multi-cloning site; ori, origin of replication; rep, gene encoding the replication protein.

pUC19 (2.1 kb)

BamH I

Em’

Smal

pUCLSEm

(6.2 kb)

BamH I/BglII

Em’

pUC19 (1.8 kb)

BglII

BamH I

BglII

Smal

pUC19 (2.1 kb)

BglII

BamH I

BamH I/BglII

Em’

pUC19 (1.8 kb)
Fig. 3 Physical and Genetic Map of Plasmid pLKS.
The genetic map was constructed based on the results shown in Figs. 4 and 5.

The amino acid sequence of orf918 (305 amino acids), was compared with known replication proteins (Rep). It showed high similarity with Reps in lactic acid bacteria, particularly those of the RepA of plasmid pUCL287 in *Pediococcus halophilus* (identity, 66.2%; similarity, 93.7%) and RepA of plasmid pLA103 in *Lactobacillus acidophilus* (identity, 43.0%; similarity, 82.2%) (Fig. 4). Thus, orf918 may encode a Rep of plasmid pLKS.

A putative replication origin (ori) was also identified upstream of orf918 (Fig. 5). This region showed a close similarity with the ori region of pUCL287. Three tandem direct repeats (ACCTCTTTTT) were conserved in both sequences.

We found no other similarity among genes of plasmid pLKS and other plasmids, especially the genes with plasmid-encoded resistance reported earlier. Based on four mechanisms recognized for naturally occurring phage resistance mechanisms, we can rule out mechanisms of adsorption interference and restriction and modification systems for that of plasmid pLKS. Only one report has discussed the inhibition of phage DNA injection by *L. lactis* plasmid pNP40, which encodes conjugal transfer and injection blocking. Blocking phage DNA injection would be related to tra gene function (DNA translocation) of conjugative plasmids, suggesting that phage and plasmid share similarities in factors required to pass through membrane. A similar competition affected by conjugative plasmids has been reported in case of translocation of DNA across bacterial membranes. Plasmid pLKS is very small and has no tra gene.

We consider that the phage resistant mechanisms of pLKS belong to Abi systems, which are further divided into several inhibition mechanisms. As a first approximation, we think that plasmid pLKS and phages would share similarities in factors required for replication of their DNA. Further investiga-
tion are in progress to search for the similarities in their rep and ori.

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