Cloning in *Streptococcus lactis* of Plasmid-Mediated UV Resistance and Effect on Prophage Stability

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Plasmid pIL7 (33 kilobases) from *Streptococcus lactis* enhances UV resistance and prophage stability. A 5.4-kilobase pIL7 fragment carrying genes coding for both characters was cloned into *S. lactis*, using plasmid pHV1301 as the cloning vector. The recombinant plasmid was subsequently transferred to three other *S. lactis* strains by transformation or protoplast fusion. Cloned genes were expressed in all tested strains.

Almost all lactococcal strains (group N) are lysogenic (4, 8) and even multilyssogenic (3, 6). The prophages may be induced by UV light or mitomycin C, but a significant liberation of temperate bacteriophages also occurs spontaneously. Reyrolle et al. (15) found that over 25% of strains tested showed a spontaneous liberation of temperate phages active on other strains. Moreover, it is possible that virulent phages derive from these spontaneously induced phages. A demonstration of such prophage origin of virulent phage appearing on fermentation has been seen for a *Lactobacillus casei* strain (16, 17). As pointed out by Davies and Gasson (4), this was possible because the study involved a single-strain lactococcal starter used in the manufacture of a fermented milk drink. The demonstration is not so simple in cheese manufacture because of the complexity of the starter systems in use. Despite the fact that some similarities in the morphology and lytic spectra of temperate and virulent phages have been established (4, 8), the contribution of temperate phages to the appearance of lytic bacteriophages during cheese manufacture is still contested (7, 21). Nevertheless, Lawrence et al. (10) have found that strains not readily lysed by UV light gave more stable starters than others. They also observed decreased phage problems when the amount of spontaneously induced phage was reduced. It appears, therefore, that any additional knowledge on temperate phage-host interaction would be useful.

In a previous work, we demonstrated that plasmid pIL7, identified in a *Streptococcus lactis* strain, increases prophage stability and mediates resistance to UV irradiation (2). The influence of pIL7 on prophage induction was studied by following the absorbance curve of the culture. Because of the lack of suitable indicator strains, more detailed study of plasmid interaction with UV and spontaneous prophage induction was not done. Plasmid pIL7 size (33 kilobases [kb]) and lack of markers make it difficult to introduce into strains suitable for prophage induction investigations. In this paper, we report molecular cloning of a pIL7 gene sequence encoding UV resistance directly into an *S. lactis* strain, its subsequent transfer by transformation and protoplast fusion, and its expression in three different lactococcal strains.

MATERIALS AND METHODS

**Bacterial strains and propagation.** The bacterial strains used, their plasmid content, and their relevant properties are listed in Table 1. *S. lactis* strains were grown at 30°C on M17 medium (20) in which lactose was replaced by glucose (M17glc). Bacterial cultures were plated on M17glc agar (1.2% agar [Difco Laboratories]). The *Bacillus subtilis* strain was grown at 37°C on Luria-Bertani medium (12).

**DNA isolation.** Plasmid screening and plasmid DNA preparation for transformation and restriction mapping were performed as previously described (18).

**Restriction enzyme mapping.** All restriction enzymes were obtained from Boehringer Mannheim Biochemicals, and conditions for restriction enzyme reactions were as specified by Maniatis et al. (12). Maps were constructed from double-digest results. In some cases, the order of the restriction fragments was ascertained by partial digestion (12).

**Electrophoresis.** Restriction digests and plasmid preparations were separated on agarose gels (0.5 to 1% Seakem agarose), using Tris-borate-EDTA buffer (12).

**Cloning techniques.** EcoRI-linearized vector molecules and EcoRI pIL7 restriction fragments were mixed at a ratio of 1:3 at a concentration of 100 μg/ml and ligated with T4 DNA ligase (Boehringer Mannheim). *S. lactis* protoplasts were transformed with recombinant DNA as described by Kondo and McKay (9). After transformation, protoplasts were suspended in M17glc with 0.5 M sucrose and incubated for 2 h at 30°C to enable phenotypic expression of erythromycin resistance. Then, they were poured in M17glc agar supplemented with 0.5 M sucrose and 5 μg of erythromycin per ml for direct selection of erythromycin-resistant transformants after 7 days of incubation at 30°C. These erythromycin-resistant transformants were subsequently screened for UV resistance as follows: colonies included in agar medium were dispersed in saline solution by blending with an Ultra-Turrax T18/10 (20,000 rpm) and cultivated in M17glc broth. The culture was then UV irradiated as described below and plated on M17glc agar with erythromycin. UV-surviving colonies were screened for plasmid content and UV resistance.

**UV irradiation.** An early exponential-phase culture was centrifuged, suspended in saline solution, and irradiated under constant stirring with a 247-nm UV lamp as previously described (2). UV fluences were measured with a Black-Ray...
**TABLE 1.** Strain characteristics and designations

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Phenotype</th>
<th>Reference source, or derivation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. lactis</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL1403</td>
<td>Plasmid-free</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>IL1403(pHV1301)</td>
<td>pHV1301</td>
<td>Em'</td>
<td>18</td>
</tr>
<tr>
<td>IL1403(pIL31)</td>
<td>pHV1301, pIL7</td>
<td>Em' UV r'^/</td>
<td>18</td>
</tr>
<tr>
<td>IL1403(pIL31)</td>
<td>pIL31 (pHV1301 carrying EcoRI B fragment of pIL7)</td>
<td>Em' UV r'^</td>
<td>This paper</td>
</tr>
<tr>
<td>IL562</td>
<td>Several cryptic plasmids</td>
<td></td>
<td>Wild-type strain from our collection</td>
</tr>
<tr>
<td>IL562(pHV1301)</td>
<td>Same as IL562 + pHV1301</td>
<td>Em'</td>
<td>This paper</td>
</tr>
<tr>
<td>IL562(pIL31)</td>
<td>Same as IL562 + pIL31 (pHV1301 carrying EcoRI B fragment of pIL7)</td>
<td>Em' UV r'^</td>
<td>This paper</td>
</tr>
<tr>
<td>IL413</td>
<td>Several cryptic plasmids</td>
<td></td>
<td>Wild-type strain from out collection</td>
</tr>
<tr>
<td>IL1377</td>
<td>Same as IL413</td>
<td>Str'^</td>
<td>This paper</td>
</tr>
<tr>
<td>IL1377(pHV1301)</td>
<td>Same as IL1377 + pHV1301</td>
<td>Str' Em'^</td>
<td>This paper</td>
</tr>
<tr>
<td>IL1377(pIL31)</td>
<td>Same as IL1377 + pIL31 (pHV1301 carrying EcoRI B fragment of pIL7)</td>
<td>Str' Em' UV'^</td>
<td>This paper</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>pHV1301</td>
<td>Em'</td>
<td>S. D. Ehrlich</td>
</tr>
</tbody>
</table>

* r'^/m'^. Restriction '/modification".

UVX radiometer (Ultraviolet Products Inc.). After irradiation, samples were diluted in 10% M17 broth and plated as described above. For prophage induction, 5 ml of irradiated bacteria in saline solution was mixed with 5 ml of double-strength M17glc broth. After clearing up during subsequent incubation at 30°C, the culture was filter sterilized through a 0.45-μm-pore size membrane filter (type HA; Millipore Corp.). A nonirradiated culture served as the control. Phages were enumerated by a double agar layer method (20).

**Fusion experiments.** The donor strains were spontaneous mutants of IL1403(pHV1301) or IL1403(pIL31) resistant to fusidic acid and rifampin. The recipient strain was IL1377, a spontaneous mutant of IL413 resistant to streptomycin. Fusion experiments were performed as described by Gasson (5). Before plating on M17 supplemented with Fus (25 μg/ml), Rif (100 μg/ml), Str (200 μg/ml), or Em (5 μg/ml) or appropriate combinations of antibiotics, protoplasts were suspended in M17glc with 0.5 M sucrose and incubated for 1.5 h at 30°C.

**RESULTS**

**Construction of a restriction endonuclease map of pIL7.**

Covalently closed circular pIL7 DNA from *S. lactis* was subjected to single and double digestions with restriction endonucleases, and the resultant fragments were sized on agarose gels. The positions of the restriction sites on pIL7 (Fig. 1) were deduced from the sizes of fragments liberated by appropriate combinations of enzymes. No sites for restriction endonuclease *XhoI, SalI*, or *AvaI* were found. Multiple fragments were liberated from pIL7 by digestion with *HindIII, XbaI, EcoRI, BamHI, EcoRV, KpnI, PvuII, Clal, BglII*, and *BclI*.

**Characterization of the cloning vector pHV1301.**

pHV1301 was isolated from *B. subtilis* M1119. This strain, received from S. D. Ehrlich (Institut Jacques Monod, Paris, France), had been transformed to erythromycin resistance with pAM81. Plasmid analysis revealed that *B. subtilis* M1119 contained a single plasmid with a molecular size of 13 kb which was designated pHV1301. A restriction endonuclease map of pHV1301 was constructed by single and double digestions with restriction endonucleases, and the resultant fragments were sized on agarose gels. Comparison of the restriction map of pHV1301 (Fig. 2) and those of pAM81 (11) indicated that pHV1301 derives from pAM81 by a single deletion event.

**Cloning and expression of the pIL7 UV resistance determinant in *S. lactis* IL1403.**

EcoRI restriction digestion of pIL7 yielded seven fragments of 1.7, 2.6, 2.8, 3.0, 3.4, 5.4, and 15 kb (Fig. 1). These fragments were inserted into the single EcoRI site of pHV1301. Recombinant DNA molecules formed were used in transformation of *S. lactis* IL1403. The transformation efficiency was 10^2 transformants per μg of DNA.

![FIG. 1. Restriction endonuclease cleavage map of pIL7.](http://aem.asm.org/... Downloaded from http://aem.asm.org/ on September 29, 2017 by guest)
recombinant DNA, i.e., two orders of magnitude lower as compared with those obtained with covalently closed circular pHV1301 DNA (data not shown). Ten randomly isolated UV-surviving, erythromycin-resistant transformants were screened for plasmid content. Nine of them harbored plasmid pHV1301 carrying the EcoRI B fragment (5.4 kb) of pIL7, designated pIL31 (Fig. 3). One clone harbored pHV1301 without an insertion fragment.

The UV sensitivities of IL1403(pHV1301), IL1403(pHV1301, pIL7), and IL1403(pIL31) were compared (Fig. 4). IL1403(pHV1301, pIL7) and IL1403(pIL31) showed similar and markedly increased resistance to UV irradiation. This result suggests that the EcoRI B fragment of pIL7 contains the UVr genes. This fragment possesses three EcoRV sites. We tried to subclone the EcoRI B fragment in EcoRV C and D fragments (Fig. 1). Neither of these two EcoRV fragments conferred enhanced UV resistance to the IL1403 strain. Thus UVr genes are probably located on both sides of the median EcoRV site in the EcoRI B fragment from pIL7.

Effect of the pIL7 UV resistance determinant on prophage stability in *S. lactis* IL562 and *S. lactis* IL1377. A high level of temperate phages active on suitable indicator strains were spontaneously liberated from *S. lactis* IL562 and IL1377. In an aim to study the influence of the EcoRI B fragment of pIL7 on prophage induction, we tried to transfer pHV1301 and pIL31 into these two *S. lactis* strains. Plasmids pHV1301 and pIL31 were introduced into IL562 by transformation with a very low efficiency (20 transformants per μg of DNA). Under the same conditions, no transformants were obtained from IL1377 (<0.5 transformants per μg of DNA). Nevertheless, transfer of pHV1301 and pIL31 into IL1377 occurred during protoplast fusion at a frequency of 2.5 × 10⁻³, with recombination of chromosomal markers occurring at a frequency of <4 × 10⁻⁷. In each case, transfer of the plasmid was confirmed by physical analysis (data not shown). The number of temperate phages liberated either spontaneously or after UV irradiation from IL562 and IL1377 harboring pHV1301 or pIL31 is represented in Fig. 5 and 6. In these two strains, for a given UV fluence of <60 J/m², prophage induction was higher in the parental lysogen than in a derivative carrying pIL31. The highest difference (two orders of magnitude) was observed at about 10 J/m² in strain IL1377 (Fig. 6). From 60 J/m², prophage induction was
**FIG. 5.** Prophage induction in *S. lactis* IL562(pHV1301) (○) and IL562(pIL31) (●). Values are means of three independent experiments. Vertical bars indicate the dispersion of the data.

maximum and similar in strains with or without pIL31. In the case of strain IL1377 (Fig. 6), pIL31 also decreased spontaneous prophage induction, which was one order of magnitude lower.

**DISCUSSION**

The present work describes molecular cloning of a 5.4-kb plasmid DNA fragment containing genes conferring to *S. lactis* both UV resistance and enhanced prophage stability. This is the second report of such a cloning operation involving direct recombinant DNA transformation into *S. lactis*. The first one was reported by Kondo and McKay (9), who have cloned a 17.9-kb plasmid DNA fragment containing genes coding for lactose assimilation.

The use of transformation to introduce the cloned genes in two other *S. lactis* strains succeeded in only one strain and with a very low efficiency. No transformants could be obtained from the second strain. These variations in the transformation ability from one strain to another are in agreement with the observations made by Kondo and McKay (9). These authors tried to transform several strains of *S. lactis* and succeeded in transforming only three strains which are probably genetically related. Our results and those obtained by Simon et al. (18) indicate that at least three other genetically unrelated strains can be transformed, though the efficiencies are variable.

Cloned genes were successfully introduced by protoplast fusion into the nontransformable *S. lactis* strain as well as into another strain (data not shown). Protoplast fusion had previously been used to transfer plasmid and chromosomal markers between isogenic strains of *S. lactis* (5, 14). In this study, protoplast fusion was used to transfer a recombinant plasmid between heterologous strains of *S. lactis*. Plasmid transfer occurred at a relatively high frequency when chromosomal marker transfers were undetectable under the same conditions. Similar results were recently obtained by Smith (19), who transferred plasmid pAMa1 between heterologous strains of *S. faecalis* by protoplast fusion. This suggests that the method could be of general use in introducing recombinant DNA into a variety of wild-type *S. lactis* strains.

Cloned genes were expressed in three unrelated *S. lactis* strains where they markedly decreased the amount of UV-induced temperate phages. pIL7-like plasmids could be present in other lactic streptococcal strains and would contribute to explaining the differences observed in the response of strains to UV induction (13). In addition, these genes seemed to decrease spontaneous prophage induction in one *S. lactis* strain. Although this effect is too limited to have a direct practical application, a further study of these genes would contribute to a better knowledge of DNA repair and prophage induction mechanisms in *S. lactis*.

The cloning experiment reported here indicates that it is possible to clone plasmid-linked genes from lactic streptococci into *S. lactis* and to subsequently transfer them into nontransformable wild-type strains by protoplast fusion.

**LITERATURE CITED**

CLONING OF PLASMID-MEDIATED UV IN S. LACTIS

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