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Construction of Specific Erythromycin Resistance Mutations in the Temperate Lactococcal Bacteriophage TP901-1 and Their Use in Studies of Phage Biology

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A method for the construction and isolation of specifically designed mutations of the temperate lactococcal phage TP901-1 has been developed. Two different *erm*-labeled mutants were isolated. One was shown to be defective in lysogenization and excision. The other, showing normal lysogenization, was used for host range studies.

The amount of *Lactococcus* phage sequence information is growing fast (2, 13, 15, 17). Unfortunately, low sequence similarity to genes with known functions makes the assignment of gene products difficult. Therefore, we have developed a method for constructing specifically designed phage mutants, and in this work, we describe their use in the study of phage biology and gene function, using the temperate lactococcal phage TP901-1 as a model.

Construction and verification of mutant phages. Lysates of TP901-1 were obtained by UV induction from *Lactococcus lactis* subsp. *cremoris* 901-1 (3). Mutants of TP901-1 were isolated by screening lysates, grown lytically on the indicator strain *L. lactis* subsp. *cremoris* 3107 (3) containing a pCI372 shuttle vector derivative (9). pCI372 carried a phage fragment into which the *erm* gene from pUC7,*erm* (3) had been introduced at a chosen location. Phage mutants were expected to arise at low frequency as a result of homologous recombination between the phage genome and the mutated phage fragment on the plasmid.

The recombinant bacterial host was grown at 30°C in M17 broth (16) containing 0.5% glucose, 5 mM $CaCl_2$, and 1 μg of erythromycin per ml. At an optical density at 600 nm of 0.1 to 0.3, cultures were infected by wild-type TP901-1 at a multiplicity of infection (MOI) of 0.001 to 0.01 (10^5 to 10^6 phage per ml) and incubated for 20 h. Resulting phage lysates were filter sterilized (0.45-μm-pore-size filter). Plaque-forming recombinant phages were detected by DNA hybridization to plaque lifts (18) by using an [α-^{32}P]dATP (Amersham) randomly labeled *erm* gene probe. Recombinant phage frequency was determined to be between 1×10^{-4} and 7×10^{-4} in several independent experiments using the two plasmids described below. Two phage mutants, TP901-BK2 and TP901-BC1034, were constructed. TP901-BK2 was obtained by recombination between TP901-1 and plasmid pBK2, which contains a 2.8-kb *Eco*RI-*Nsi*I phage fragment, with the *erm* gene introduced into the central *Hin*dIII site after treatment of the phage fragment with Klenow enzyme. Sequencing revealed a 448-bp deletion (Fig. 1). The deletion plasmid pBK2 was used to construct the mutation. Phage TP901-BC1034 was constructed by recombination with the plasmid pBC197, containing *Eco*RI fragment 2 (EI2), with the *erm* gene inserted into the blunt-ended *Cla*I site separating *Cla*I fragment 3 (C3) and C4 (Fig. 1). Both mutant phages were able to form plaques on 3107, and yields were similar to those of wild-type phage, showing that lytic growth of the phage mutants was not impaired. DNA was extracted as described for phage λ (14) and analyzed by PCR and by restriction endonuclease digestion (Fig. 2). As expected, EI2 showed altered mobility in both mutant phages. In TP901-BC1034, C3 and C4 had disappeared. The resultant *Cla*I fragment migrates with C2. Similarly, the *Eco*RV fragment 3 (EV3) is no longer present in TP901-BC1034; the resultant fragment migrates with EV2. As expected for TP901-BK2, the C2 fragment shows altered mobility. EV4 and EV9 are not present, due to the deletion in pBK2 spanning the *Eco*RV site between EV4 and EV9. The resulting *Eco*RV fragment migrates above EV3. The fragment sizes were consistent with the expected pattern. All other fragments corresponded to the fragments of wild-type TP901-1, with the exception of EI4, which is very weak or missing in both mutant phages. The intensity of EI4 varies, and additional bands (EI4s, EI6s, and EI9s [Fig. 2]), possibly derived from EI4, appear when TP901-1 is propagated lytically (10a).

PCR products from phage-specific primers encompassing the *erm* insertions were of the expected size (data not shown). Thus, both phage mutants result from homologous recombination events between the phage genome and the phage sequences carried on their respective plasmids.

Lysogenization of *L. lactis* subsp. *cremoris* 3107 by phage mutants. TP901-BK2 was expected to be integration defective due to disruption of open reading frame 1 (ORF1), which is necessary for integration (4). Furthermore, in TP901-BK2 the first 90 amino acids of ORF1 and the last 39 amino acids of ORF2 are also deleted. The ability of the mutant phages to lysogenize *L. lactis* subsp. *cremoris* 3107 was analyzed by screening for Em^r colonies. Cultures were infected at an optical density at 600 nm of 0.1 to 0.3 and an MOI of 1 to 3. The mixtures were incubated for 1 h at 30°C to allow phenotypic expression of Em^r before plating. Transductants were selected after 2 days at 30°C on GM17 agar containing erythromycin (1 μg/ml). The transduction frequency of TP901-BC1034 was 10^{-2}, corresponding to that of TP901-1 (3). TP901-BK2 formed transductants at a frequency of only 10^{-7}. As expected, Em^r clones from TP901-BC1034 resulted from site-specific

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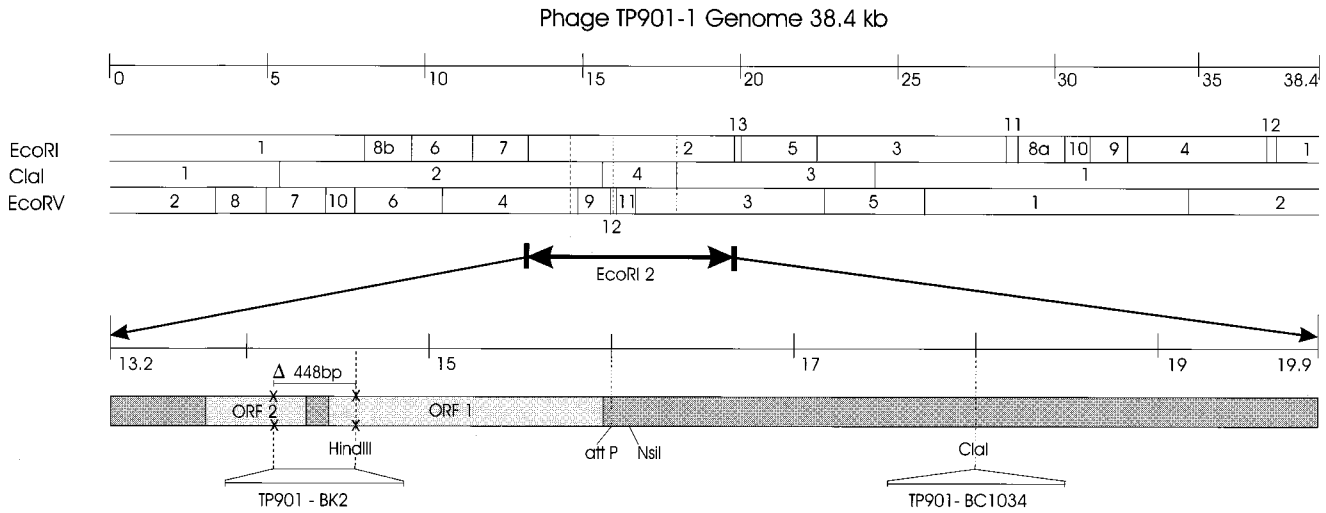

FIG. 1. Restriction map of TP901-1 (3), with enlarged 6.5-kb EI2, carrying ORF1, which encodes the integrase and *attP*. TP901-BK2 and TP901-BC1034, respectively, indicate the positions of the *erm* gene. The solid line terminating at *Hin*dIII represents the 448-bp deletion in TP901-BK2.

integration. Conversely, the Em^r clones from TP901-BK2 did not contain the phage at the usual *attB* site (the PCR and DNA hybridization data are not shown). The inability of TP901-BK2 to integrate into *attB* agrees with the designation of ORF1 as the TP901-1 integrase (4). However, when *L. lactis* subsp. *cremoris* 3107 was infected with a mixture of TP901-1 and TP901-BK2 (MOIs of 6×10^{-2} and 60, respectively), Southern blot analysis showed that the Em^r colonies were mostly tandem, double lysogens of TP901-BK2, with few single lysogens. All were integrated into *attB* (data not shown), indicating that the gene product(s) necessary for integration can be delivered in *trans.*

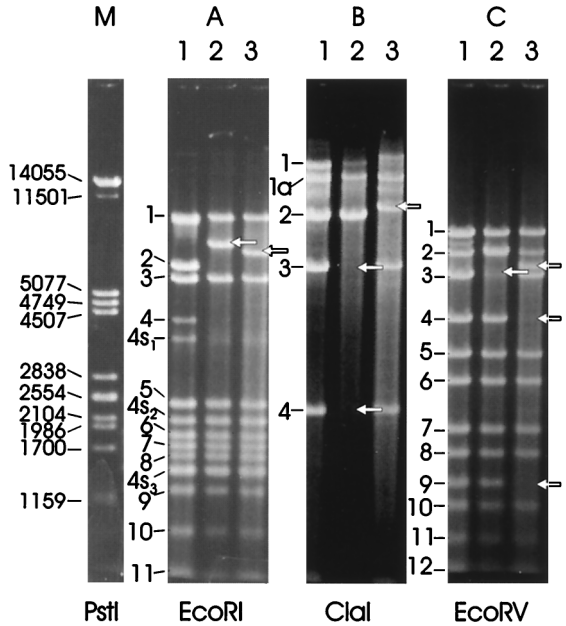

FIG. 2. Restriction endonuclease digestion of phage DNA. (M) *Pst*I digestion of λ DNA; (A) *Eco*RI digestion of TP901-1 (lane 1), TP901-BC1034 (lane 2), and TP901-BK2 (lane 3); (B) *Cla*I digestion of TP901-1 (lane 1), TP901-BC1034 (lane 2), and TP901-BK2 (lane 3); (C) *Eco*RV digestion of TP901-1 (lane 1), TP901-BC1034 (lane 2), and TP901-BK2 (lane 3). Fragments with changed mobility are indicated with arrows. Numbers to the left of panels A to C are fragment numbers; molecular weight are indicated to the left of panel M.

Typically, overnight cultures of *L. lactis* subsp. *cremoris* 3107 lysogenized with TP901-1 contain from 10^5 to 10^6 PFU/ml (3). Similar numbers were obtained not only from *L. lactis* subsp. *cremoris* 3107 lysogenized with TP901-BC1034 but also from double lysogens of TP901-BK2 integrated into *attB* and single lysogens of TP901-BK2 not integrated into *attB*. However, when TP901-BK2 was integrated as a single lysogen in *attB*, phage liberation was not detected. In double lysogens, this is probably due to the detachment of one complete phage genome by a recombination event anywhere along the length of the two prophages, as seen for Int$^-$ λ (8). This could also be the case for single TP901-BK2 lysogens integrated elsewhere than *attB*, if the integration has taken place in homologous regions of TP3107, a related defective prophage of *L. lactis* subsp. *cremoris* 3107 (11). The absence of phage liberation in single lysogenic TP901-BK2 derivatives inserted in *attB* suggests the lack of a necessary excision factor, indicating that ORF1 and/or ORF2 is involved in excision.

Host range determination by transduction. Plaque assays, used to determine the host ranges of phages, are not reliable assays for lactococcal temperate phages. The ability of TP901-BC1034 to transduce Em^r provided the means for a sensitive assay for determining the host range of TP901-1. The transducing ability was tested, as described for transduction of Em^r into 3107, in *L. lactis* F7/2 (1) and C10 (1) and *L. lactis* subsp. *cremoris* MG1363 (7), NCDO712 (7), LM0230 (6), BC101 (19), IMN-C17 (12), IMN-C18 (12), 40-3 (1), 4843 (5), H2 (10), BK-5 (10), TP901-1 indicator strain Wg2 (1), and host strain 901-1 (1), as well as two TP901-1-lysogenized derivatives of 3107, ES41 and ES46 (3). Of the strains tested, Em^r could be transduced to Wg2, ES41, ES46, and IMN-C17, an indicator strain for ϕLC3 (12). IMN-C17, ES41, and ES46 could not support plaque formation by TP901-1 or TP901-BC1034. PCR analysis of IMN-C17 with primers for amplification of *attB*, *attR*, and *attL* (3) indicated that the *attB* region is conserved and that TP901-BC1034 integrates site specifically (data not shown). Phage was detected in very low numbers in overnight cultures (10 to 30 PFU/ml) and mitomycin C (1 μg/ml)-induced cultures (10^3 to 10^4 PFU/ml) of IMN-C17 lysogenized

with TP901-BC1034, indicating that IMN-C17 is a poor host for TP901-1. We have previously shown that TP901-1 would not form plaques on *L. lactis* subsp. *cremoris* 3107 and Wg2 derivatives lysogenized by TP901-1 (3). However, TP901-BC1034 was able to transduce Em^r to the lysogenized derivatives ES41 and ES46. PCR analysis of chromosomal DNA from superinfected strains showed that in some strains both TP901-1 and TP901-BC1034 are present, while other strains have lost TP901-1 (data not shown). The ability of TP901-BC1034 to transduce ES41 and ES46 demonstrated that the resident phage was able to inhibit lytic growth but not integration. In contrast, *L. lactis* subsp. *cremoris* 901-1 could not be transduced by TP901-BC1034. Nor were MG1363 and LM0230, plasmid-free and prophage-cured strains in which a TP901-1-derived integration system functions (3, 4), transduced. Since it can be assumed that TP901-1 is able to integrate in the chromosomes of 901-1, MG1363, and LM0230, absence of transduction indicates that TP901-1 is unable to carry out an early step(s) of the infection.

In conclusion, we have developed a simple method for the construction of specific mutations in lactococcal phages, based on homologous recombination between the wild-type phage and a plasmid containing a phage DNA fragment harboring the desired mutation. We have demonstrated that such mutants can be used to study phage biology and assign functions to putative genes. Finally, we have demonstrated that transduction is a sensitive assay for host range determinations of temperate lactococcal phages.

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