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Published in:
Journal of Bacteriology

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1991

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Replacement Recombination in *Lactococcus lactis*

**KEES J. LEENHOUTS,† JAN KOK, AND GERARD VENEMA**

Institute of Genetics, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

Received 30 January 1991/Accepted 28 May 1991

In the pUC18-derived integration plasmid pML336 there is a 5.3-kb chromosomal DNA fragment that carries the X-prolyl dipeptidyl aminopeptidase gene (*pepXP*). The gene was inactivated by the insertion of an erythromycin resistance determinant into its coding sequence. Covalently closed circular DNA of pML336 was used for the electrot transformation of *Lactococcus lactis*. In 2% of the erythromycin-resistant transformants the *pepXP* gene was inactivated by a double-crossover event (replacement recombination) between pML336 and the *L. lactis* chromosome. The other transformants in which the *pepXP* gene had not been inactivated carried a Campbell-type integrated copy of the plasmid. Loss of part of the Campbell-type integrated plasmid via recombination between 1.6-kb non tandem repeats occurred with low frequencies that varied between <2.8 × 10<sup>-6</sup> and 8.5 × 10<sup>-6</sup>, producing cells with a chromosomal structure like that of cells in which replacement recombination had taken place.

A convenient way to examine chromosome-located genes is to mark such genes by insertional mutagenesis. Among other systems, integration of (parts of) nonreplicating heterologous plasmids by means of Campbell-type integration or replacement recombination is a powerful tool to obtain mutants, and this methodology has been used in *Escherichia coli* and *Bacillus subtilis* (6, 20, 22, 25). An additional advantage of plasmid integration strategies is that they can also be used to stabilize plasmid-borne genes in the chromosom es of bacterial species (1, 10, 11, 24).

Recently, it was demonstrated that heterologous nonreplicating plasmids can also integrate in the chromosome of *Lactococcus lactis* via Campbell-type integration and replacement recombination (4, 13–15). In principle, Campbell-type integrated plasmids can recombine between the generated non tandem repeats that flank the integrated plasmid. However, in previously described experiments the frequency of recombination between the generated non tandem repeats could not be determined accurately (15). So far, just one case of apparent replacement recombination in *Lactococcus* has been reported (4). The experimental approach in this study was such that it could not be excluded that the observed replacement integration had been the result of loss of part of a Campbell-type integrated plasmid. These uncertainties prompted us to determine the frequency of recombination between non tandemly repeated sequences and to examine whether replacement recombination operates in lactococci.

To this purpose we used the recently cloned and sequenced chromosomal X-prolyl dipeptidyl aminopeptidase (X-PDAP) gene (*pepXP*) of *L. lactis* (18). The activity of the *pepXP* gene product in colonies of *L. lactis* is readily detectable in a plate assay, thus facilitating the selection of strains with an X-PDAP<sup>−</sup> phenotype. A pUC18-derived plasmid was constructed that carried the 2.3-kb *pepXP* gene on a 5.3-kb chromosomal fragment. The gene had been inactivated by the insertion of an erythromycin resistance gene. By using this plasmid we were able to demonstrate that the *pepXP* gene was inactivated by a replacement recombination event between the plasmid and the lactococcal chromosome and, for Campbell-type integrations, to determine the frequency of recombination between 1.6-kb nontandem repeats in the *pepXP* region.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *E. coli* JM101 (28) was grown in TY medium (23), and *L. lactis* was grown in glucose-M17 medium (26) as described before (15). The *L. lactis* strains used are listed in Table 1.

**Plasmids.** Plasmids pGK1, pGK12, and pBM33 have been described elsewhere (12, 18). Plasmid pML336 was constructed by inserting the erythromycin resistance gene of pE194 (7), located on a 1-kb *EcoRI-HindIII* fragment in pUC19E (laboratory collection), into the *BglII* site within the coding region of the *pepXP* gene in plasmid pBM33. Before ligation, the 5′ protruding ends of the *EcoRI-HindIII* fragment were made blunt by treatment with Klenow enzyme. To recess the 3′ protruding ends of the *BglII* site, the DNA was treated with T4 DNA polymerase before ligation.

**DNA techniques.** Plasmid DNA was isolated from *E. coli* by the method of Ish-Horowicz and Burke (8). Chromosomal DNA was isolated from *L. lactis* as previously described (15). Restriction enzymes, Klenow enzyme, T4 DNA polymerase, and T4 ligase were obtained from Boehringer GmbH, Mannheim, Germany, and used according to the instructions of the supplier. Linear pML336 was made single stranded by heating the DNA at 95°C for 10 min. Recombinant DNA techniques were essentially as described by Maniatis et al. (17).

**Transformation.** *E. coli* was transformed as described by Mandel and Higa (16). Electrot transformation of *L. lactis* was as described before (15).

**X-PDAP plate assay.** The X-PDAP plate assay was performed as follows. *L. lactis* colonies were covered with 3 ml of 0.5% agarose dissolved in water and 0.2 ml of H-Gly-Pro-β-naphthylamide (Bachem, Bubendorf, Switzerland) dissolved in dimethylformamide (10 mg/ml) was used as a substrate, together with 5 ml of Fast Garnet GBC Salt (Sigma, St. Louis, Mo.) dissolved in 0.2 M Tris (pH 7.4) (2 mg/ml) to detect activity (19). In this assay, X-PDAP<sup>−</sup> colonies stain red, whereas X-PDAP<sup>+</sup> colonies remain white.

**Blot hybridizations.** Transfer of DNA (3 μg of chromosomal DNA per lane in each case) from 0.8% agarose gels to

* Corresponding author.
GeneScreen Plus membranes (Du Pont, NEN Research Products, Boston, Mass.) was performed by using the protocol of Southern as modified by Chomczynski and Qasba (3). Probe labeling, hybridization conditions, and washing steps were done according to the instructions of the manufacturers of the ECL gene detection system (Amersham International, Amersham, United Kingdom). Labeled phage SPP1 DNA was added to the hybridization mixtures to enable the determination of the sizes of the hybridizing fragments in the chromosomal digests.

**RESULTS**

**Inactivation of the chromosomal pepXP gene.** Plasmid pML336 (Fig. 1C), which is unable to replicate in L. lactis, is a pUC18 derivative containing a 5.3-kb XbaI chromosomal DNA fragment of L. lactis P8-2-47. This fragment carries an erythromycin resistance gene that disrupts the coding region of the pepXP gene. Electrotransformation of the plasmid-free L. lactis strain MG1363 with covalently closed circular pML336 DNA resulted in approximately 60 erythromycin-
TABLE 2. Electrottransformation of strain MG1363 with pGK12 and various forms of pML336

<table>
<thead>
<tr>
<th>Plasmid and shape</th>
<th>Amt (µg)</th>
<th>No. of CFU</th>
<th>Tested for X-PDAP activity</th>
<th>X-PDAP− CFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGK12 Circular (replicating)</td>
<td>5 x 10^6</td>
<td>700</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>pML336 Circular</td>
<td>315</td>
<td>242</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Linear (double stranded)</td>
<td>10</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Linear (single stranded)</td>
<td>10</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

resistant (Em') CFU/µg of DNA. The expected modes of integration with plasmid pML336 are schematically represented in Fig. 1C. Integration of the plasmid via a single crossover (Campbell-type integration; Fig. 1C) results in the original X-PDAP phenotype (X-PDAP+), whereas integration via a double crossover (gene replacement; Fig. 1C) produces an X-PDAP− phenotype. To examine the effect of the physical form of the nonreplicating plasmid pML336 on the production of transformants, electrottransformation experiments were carried out with the following plasmid forms: covalently closed circular DNA and double stranded and single stranded linear DNA. Restriction endonuclease BglII was used to linearize pML336. The results of the electrottransformation experiments are presented in Table 2. The electrottransformation procedure did not detectably produce mutations in the pepXP gene, since none of the screened transformants obtained with pGK12 showed an X-PDAP− phenotype. In contrast, 2% of the Em' transformants obtained with circular pML336 had acquired an X-PDAP− phenotype. The electrottransformation frequency of linear double- or single-stranded pML336 DNA was extremely low, and the transformants had the parental X-PDAP+ phenotype.

The five X-PDAP− transformants obtained with circular pML336 were designated MG33611 through MG33615 and were analyzed by means of Southern hybridizations. Their chromosomal DNAs and that of the recipient strain MG1363 were digested with both EcoRI and HindIII. An EcoRI-HindIII fragment of approximately 1.2 kb, located in the coding region of the pepXP gene, was used as a probe. The erythromycin resistance gene was located within this EcoRI-HindIII fragment on pML336. The fragment hybridizing at 1.2 kb in Fig. 1A, lane 1, containing the chromosomal DNA of MG1363, was replaced by a fragment at 2.2 kb in all transformants analyzed (Fig. 1A, lanes 2 through 6). This fragment was the same size as the 2.2-kb EcoRI-HindIII chromosomal fragment of pML336 carrying the erythromycin resistance gene (Fig. 1A, lane 8). The vector part of pML336 was not present in the DNAs of strains MG33611 through MG33615, since no hybridization was observed in lanes 2 to 6 when labeled pUC18 was used as a probe, whereas a clearly hybridizing band at 2.7 kb was present in pML336 digested with EcoRI-HindIII (data not shown). These results strongly suggest that the pepXP gene in the strains MG33611 through MG33615 had been inactivated by the insertion of the Em' gene via a replacement mechanism.

Inactivation of the pepXP gene is the result of a double-crossover event. Although the results suggest that strains MG33611 through MG33615 resulted from a double-crossover event between pML336 and the chromosome of strain MG1363, they might also have resulted from the Campbell-type integration of the complete plasmid pML336 followed by recombination between the 1.6-kb nontandem repeats, characterized in Campbell-type recombinations (Fig. 1C). This would eliminate the vector part of the integrated plasmid, thus mimicking replacement recombination. To distinguish between these two possibilities the following questions should be answered: (i) whether the Campbell-type integrations can produce structures that are indistinguishable from those produced by replacement recombination and (ii) whether the frequency of such events is sufficiently high to account for the frequency with which X-PDAP+ Em' transformants were produced (5 of 242 [2%]).

To that purpose, the chromosomal DNAs of five Em' X-PDAP+ transformants obtained with pML336, designated MG3364 through MG3366, were analyzed by Southern hybridizations to verify that a Campbell-type integration of the plasmid had occurred in these strains. The Em' gene in pML336 is flanked by 3.7 and 1.6 kb of chromosomal DNA. The hybridization analysis showed that one copy of pML336 had integrated via the 3.7-kb fragment in a Campbell-type manner in all five transformants. Again, no hybridizing fragment was detected in duplications in the pepXP gene region, generating 3.7- and 1.6-kb nontandem repeats. The chromosomal structures of strains MG3360 through MG3364 were depicted schematically in Fig. 1C.

Recombination between the 1.6-kb nontandem repeats produces one copy of a nonfunctional pepXP gene because of the Em' marker in the coding sequence. The recombination frequency between these 1.6-kb nontandem repeats was determined in cultures of strain MG3360 grown for 35 generations in the absence of erythromycin. The cultures were plated, and from each culture the X-PDAP phenotype of approximately 10^6 colonies was determined. The numbers of X-PDAP− colonies (F x 10^9) and the recombination frequencies per generation (p; see above) in each culture were as follows: for F x 10^9 values of 1, 3, 1, 2, and 0 CFU, p values of 2.8 x 10^-6, 8.5 x 10^-6, 2.8 x 10^-6, 5.7 x 10^-6, and <2.8 x 10^-6, respectively, were obtained. All X-PDAP− colonies were still Em'. The chromosomal DNA of one of the X-PDAP− colonies, designated MG3360a, was analyzed by Southern hybridizations with the 1.2-kb internal EcoRI-HindIII pepXP gene fragment as a probe (Fig. 1B). Lane 2 of Fig. 1B contained EcoRI-HindIII-digested chromosomal DNA of strain MG3360. As expected on the basis of Campbell-type integration, two hybridizing fragments were present, a 1.2-kb fragment also present in EcoRI-HindIII-digested chromosomal DNA of the recipient strain MG1363 (Fig. 1B, lane 1) and a 2.2-kb fragment like that in the EcoRI-HindIII-digested pML336 DNA (Fig. 1B, lane 6). Since the 1.2-kb hybridizing fragment was absent and the 2.2-kb fragment was still present in the EcoRI-HindIII chromosomal digest of strain MG3360a (Fig. 1B, lane 3), the chromosomal structure of this strain had been produced as the result of recombination between the 1.6-kb nontandem repeats in strain MG3360. Thus the chromosomal structures equivalent to those obtained by a double-crossover event can, indeed, be produced by recombination of a Campbell-type integrated plasmid containing two noncontiguous chromosomal DNA fragments in L. lactis.

However, the recombination values in the five cultures of strain MG3360, ranging between <2.8 x 10^-6 and 8.5 x 10^-6 per generation, are far too low to account for the frequency of the X-PDAP− phenotypes produced by pML336 (approximately 2% of the Em' transformants produced). Therefore, the chromosomal structures of strains MG3361 through
MG33615 must have resulted from a double-crossover event between pML336 and the chromosome of strain MG1363.

DISCUSSION

To our knowledge the work described here provides the first example on the inactivation of a chromosomal L. lactis gene by a mechanism of gene replacement. The inactivation of the pepXP gene with circular pML336 might have resulted from either a double cross-over event between the integration plasmid and the chromosome or the resolution of a Campbell-type integrated copy of pML336. The experimental approach chosen showed that the latter process can, indeed, operate by recombination between identical non tandem repeats in the L. lactis chromosome. However, the recombination frequencies between the 1.6-kb repeats in the pepXP gene region, which vary between $<2 \times 10^{-6}$ and $8.5 \times 10^{-6}$ per generation, were far too low to account for the frequency of X-PDA$^+$ Em$^+$ transformants obtained after electrottransformation of strain MG1363 with pML336. This frequency of loss by precise excision of part of the Campbell-type structure was less than that observed in 3.4-kb repeats generated in the B. subtilis chromosome, which varied between $1.2 \times 10^{-5}$ and $4 \times 10^{-4}$ per generation (27). This difference may relate to the difference in lengths of the repeats and would be in accordance with the observation in B. subtilis that large repeats recombine more efficiently than small repeats (9, 21).

In previous work we described the stable Campbell-type integration of pBR322-, pTB19-, and pSC101-derived plasmids at chromosomal location A or B of L. lactis MG1363 (15). In that work a lower limit of the recombination frequency between the generated 1.3-kb non tandem repeats was estimated to be less than $10^{-4}$ per generation. This value is in the range of recombination frequencies determined in this work. For B. subtilis it has been established that the location of non tandem repeats in the chromosome affects the stability of the intervening DNA. Up to 33-fold differences in recombination frequency were observed between the same repeats generated at 12 different chromosomal locations (27). Further work will be needed to establish whether similar location-specific recombination differences exist in the L. lactis chromosome.

Replacement recombination was only obtained with covalently closed circular pML336. The few X-PDA$^+$ transformants produced most probably resulted from Campbell-type integration of circular pML336, which was still present in trace amounts in the plasmid digests. The extremely low transformation frequency obtained with linear pML336 may have resulted from exonuclease activity in L. lactis.

In the inactivated pepXP gene of strains MG33611 through MG33615 and MG3360a, no X-PDA$^+$ activity in enzyme assays of crude cell extracts was detectable (2), whereas mutations in the pepXP gene obtained via chemical mutagenesis usually showed residual X-PDA$^+$ activity, which could be as high as 25% of the wild-type level (18). Thus, inactivation of genes by replacement recombination produces a clear-cut phenotype in L. lactis. It is expected that the pepXP mutant obtained by replacement recombination will be of help in the elucidation of the role of X-PDA$^+$ in the complex proteolytic system of L. lactis.

ACKNOWLEDGMENTS

This work was supported by Unilever Research, Vlaardingen, The Netherlands.

We thank Henk Mulder for preparing the figures.

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