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Replacement Recombination in *Lactococcus lactis*

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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 electrotransformation 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 nontandem repeats occurred with low frequencies that varied between $<2.8 \times 10^{-6}$ and 8.5×10^{-6} , 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 chromosomes 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 nontandem repeats that flank the integrated plasmid. However, in previously described experiments the frequency of recombination between the generated nontandem 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 nontandemly 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⁻ 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 *BglI* 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 *BglI* 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). Electrotransformation 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⁺ colonies stain red, whereas X-PDAP⁻ colonies remain white.

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

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TABLE 1. Strains of *L. lactis* used

Strain	Relevant properties	Source or reference
MG1363	Em ^s X-PDAP ⁺ , plasmid free	5
MG33611 through 33615	Em ^r X-PDAP ⁺ , MG1363 carrying an Em ^r marker in the coding sequence of the <i>pepXP</i> gene	This work
MG3360 through 3364	Em ^r X-PDAP ⁺ , MG1363 carrying one Campbell-type integrated copy of pML336 in the <i>pepXP</i> region	This work
MG3360a	Em ^r X-PDAP ⁺ , deletion derivative of MG3360	This work

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.

Determination of recombination frequencies. Recombination between the duplicated 1.6-kb 3' ends of the *pepXP* gene in strain MG3360 was determined by inoculating a 5 to 10 MG3360 cells in 5 ml of antibiotic-free medium. After the cultures had reached the stationary phase (about 10⁹ cells per ml, i.e., approximately 35 generations), appropriate dilutions were plated. About 10⁴ colonies were then tested for their X-PDAP phenotype by means of the plate assay described above. The frequency of recombination per generation (*p*) was calculated with the formula $p = F/n$ (27), where *F* and *n* are the proportion of X-PDAP⁺ cells and the number of generations of growth, respectively.

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 *Xba*I 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-

FIG. 1. (A) Southern hybridization analysis of *Eco*RI-*Hind*III-digested chromosomal DNAs of the following strains (lanes): 1, MG1363; 2, MG33611; 3, MG33612; 4, MG33613; 5, MG33614; 6, MG33615. Other lanes: 7, phage SPP1 DNA cleaved with *Eco*RI; 8, pML336 cleaved with *Eco*RI-*Hind*III. The labeled internal 1.2-kb *Eco*RI-*Hind*III fragment of *pepXP* was used as a probe. Sizes (in kilobases) are indicated in the margins. (B) Southern hybridization analysis of *Eco*RI-*Hind*III-digested chromosomal DNAs of the following strains (lanes): 1, MG1363; 2, MG3360; 3, MG3360a; 4, MG33611. Other lanes: 5, phage SPP1 DNA cleaved with *Eco*RI; 6, pML336 cleaved with *Eco*RI-*Hind*III. The probe used is described in the legend to panel A. (C) Schematic representation of the chromosomal structures after a double crossover between pML336 and the chromosome of MG1363, after Campbell-type integration of pML336, and after recombination between the 1.6-kb nontandem repeats of the structure obtained after Campbell-type recombination. Symbols: □, 5.3-kb chromosomal fragment cloned in pML336; ▢, *pepXP* gene; ▨, erythromycin resistance gene; ▴, 1.6-kb nontandem repeats.

TABLE 2. Electrotransformation of strain MG1363 with pGK12 and various forms of pML336

Plasmid and shape	Amt (μ g)	No. of CFU		
		Em ^r	Tested for X-PDAP activity	X-PDAP ⁻ CFU
pGK12				
Circular (replicating)	1	5×10^5	700	0
pML336				
Circular	5	315	242	5
Linear (double stranded)	10	2	2	0
Linear (single stranded)	10	1	1	0

resistant (Em^r) CFU/ μ g 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, electrotransformation experiments were carried out with the following plasmid forms: covalently closed circular DNA and double stranded and single stranded linear DNA. Restriction endonuclease *Bgl*I was used to linearize pML336. The results of the electrotransformation experiments are presented in Table 2. The electrotransformation 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^r transformants obtained with circular pML336 had acquired an X-PDAP⁻ phenotype. The electrotransformation 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 *Eco*RI and *Hind*III. A *Eco*RI-*Hind*III 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 *Eco*RI-*Hind*III 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 *Eco*RI-*Hind*III 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 *Eco*RI-*Hind*III (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^r 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, characteristically produced 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 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^r transformants were produced (5 of 242 [2%]). To that purpose, the chromosomal DNAs of five Em^r X-PDAP⁺ transformants obtained with pML336, designated MG3360 through MG3364, were analyzed by Southern hybridizations to verify that a Campbell-type integration of the plasmid had occurred in these strains. The Em^r 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 (data not shown), resulting 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 as 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^r 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 10^4 colonies was determined. The numbers of X-PDAP⁻ colonies ($F \times 10^4$) and the recombination frequencies per generation (p ; see above) in each culture were as follows: for $F \times 10^4$ values of 1, 3, 1, 2, and 0 CFU, p values of 2.8×10^{-6} , 8.5×10^{-6} , 2.8×10^{-6} , 5.7×10^{-6} , and $<2.8 \times 10^{-6}$, respectively, were obtained. All X-PDAP⁻ colonies were still Em^r. The chromosomal DNA of one of the X-PDAP⁻ colonies, designated MG3360a, was analyzed by Southern hybridizations with the 1.2-kb internal *Eco*RI-*Hind*III *pepXP* gene fragment as a probe (Fig. 1B). Lane 2 of Fig. 1B contained *Eco*RI-*Hind*III-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 *Eco*RI-*Hind*III-digested chromosomal DNA of the recipient strain MG1363 (Fig. 1B, lane 1) and a 2.2-kb fragment like that in the *Eco*RI-*Hind*III-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 *Eco*RI-*Hind*III 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 $<2.8 \times 10^{-6}$ and 8.5×10^{-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^r transformants produced). Therefore, the chromosomal structures of strains MG33611 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 nontandem repeats in the *L. lactis* chromosome. However, the recombination frequencies between the 1.6-kb repeats in the *pepXP* gene region, which vary between $<2.8 \times 10^{-6}$ and 8.5×10^{-6} per generation, were far too low to account for the frequency of X-PDAP⁺ Em^r transformants obtained after electrotransformation 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 1.2×10^{-5} and 4×10^{-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 nontandem 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 nontandem 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-PDAP⁺ 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-PDAP 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-PDAP 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-PDAP in the complex proteolytic system of *L. lactis*.

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REFERENCES

1. Albertini, A. M., and A. Galizzi. 1985. Amplification of a chromosomal region in *Bacillus subtilis*. *J. Bacteriol.* **162**:1203-1211.
2. Bockelmann, W. Personal communication.
3. Chomeczynski, P., and P. K. Qasba. 1984. Alkaline transfer of DNA to plastic membrane. *Biochem. Biophys. Res. Commun.* **122**:340-344.
4. Chopin, M. C., A. Chopin, A. Rouault, and N. Galleron. 1989. Insertion and amplification of foreign genes in the *Lactococcus lactis* subsp. *lactis* chromosome. *Appl. Environ. Microbiol.* **55**:1769-1774.
5. Gasson, M. J. 1983. Plasmid complements of *Streptococcus lactis* NCD0712 and other lactic streptococci after protoplast-induced curing. *J. Bacteriol.* **154**:1-9.
6. Guttererson, N. I., and D. E. Koshland, Jr. 1983. Replacement and amplification of bacterial genes with sequences altered *in vitro*. *Proc. Natl. Acad. Sci. USA* **80**:4894-4898.
7. Horinouchi, S., and B. Weisblum. 1982. Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibiotics. *J. Bacteriol.* **150**:801-814.
8. Ish-Horowicz, D., and F. J. Burke. 1981. Rapid and efficient cosmid cloning. *Nucleic Acids Res.* **9**:2989-2999.
9. Jannire, L., and S. D. Ehrlich. 1987. Recombination between short repeated sequences is more frequent in plasmids than in the chromosome of *Bacillus subtilis*. *Mol. Gen. Genet.* **210**:116-121.
10. Jannire, L., B. Niaudet, E. Pierre, and S. D. Ehrlich. 1985. Stable gene amplification in the chromosome of *Bacillus subtilis*. *Gene* **40**:47-55.
11. Kallio, P., A. Palva, and I. Palva. 1987. Enhancement of α -amylase production by integrating and amplifying the α -amylase gene of *Bacillus amyloliquefaciens* in the genome of *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.* **27**:64-71.
12. Kok, J., J. M. B. M. van der Vossen, and G. Venema. 1984. Construction of plasmid cloning vectors for lactic streptococci which also replicate in *Bacillus subtilis* and *Escherichia coli*. *Appl. Environ. Microbiol.* **48**:726-731.
13. Leenhouts, K. J., J. Gietema, J. Kok, and G. Venema. 1991. Chromosomal stabilization of the proteinase genes in *Lactococcus lactis*. *Appl. Environ. Microbiol.*, in press.
14. Leenhouts, K. J., J. Kok, and G. Venema. 1989. Campbell-like integration of heterologous plasmid DNA into the chromosome of *Lactococcus lactis* subsp. *lactis*. *Appl. Environ. Microbiol.* **55**:394-400.
15. Leenhouts, K. J., J. Kok, and G. Venema. 1990. Stability of integrated plasmids in the chromosome of *Lactococcus lactis*. *Appl. Environ. Microbiol.* **56**:2726-2735.
16. Mandel, M., and A. Higa. 1970. Calcium-dependent bacteriophage DNA infection. *J. Mol. Biol.* **53**:159-162.
17. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. Mayo, B., J. Kok, K. Venema, W. Bockelmann, M. Teuber, H. Reinke, and G. Venema. 1991. Molecular cloning and sequence of an X-prolyl dipeptidyl aminopeptidase. *Appl. Environ. Microbiol.* **57**:38-44.
19. Miller, C. G., C. Heiman, and C. Yen. 1976. Mutants of *Salmonella typhimurium* deficient in an endoprotease. *J. Bacteriol.* **127**:490-497.
20. Niaudet, B., A. Goze, and S. D. Ehrlich. 1982. Insertional mutagenesis in *Bacillus subtilis*: mechanism and use in gene cloning. *Gene* **19**:277-284.
21. Niaudet, B., L. Jannire, and S. D. Ehrlich. 1984. Recombination between repeated DNA sequences occurs more often in plasmids than in the chromosome of *Bacillus subtilis*. *Mol. Gen. Genet.* **197**:46-54.
22. Niaudet, B., L. Jannire, and S. D. Ehrlich. 1985. Integration of linear, heterologous DNA molecules into the *Bacillus subtilis* chromosome: mechanism and use in induction of predictable rearrangements. *J. Bacteriol.* **163**:111-120.
23. Rottlander, E., and T. A. Trautner. 1970. Genetic and transfection

- tion studies with *Bacillus subtilis* phage SP50. Mol. Gen. Genet. **108**:47–60.
24. Scheirlinck, T., J. Mahillon, H. Joos, P. Dhaese, and F. Michiels. 1989. Integration and expression of α -amylase and endoglucanase genes in the *Lactobacillus plantarum* chromosome. Appl. Environ. Microbiol. **55**:2130–2137.
25. Stahl, M. L., and E. Ferrari. Replacement of the *Bacillus subtilis* subtilisin structural gene with an in vitro-derived deletion mutant. J. Bacteriol. **158**:411–418.
26. Terzaghi, B. E., and W. E. Sandine. 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. **29**:807–813.
27. Vagner, V., and S. D. Ehrlich. 1988. Efficiency of homologous recombination varies along the *Bacillus subtilis* chromosome. J. Bacteriol. **170**:3978–3982.
28. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.