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## A System To Generate Chromosomal Mutations in *Lactococcus lactis* Which Allows Fast Analysis of Targeted Genes

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**A system for generating chromosomal insertions in lactococci is described. It is based on the conditional replication of lactococcal pWV01-derived Ori<sup>+</sup> RepA<sup>-</sup> vector pORI19, containing *lacZ* $\alpha$  and the multiple cloning site of pUC19. Chromosomal *AluI* fragments of *Lactococcus lactis* were cloned in pORI19 in RepA<sup>+</sup> helper strain *Escherichia coli* EC101. The frequency of Campbell-type recombinants, following introduction of this plasmid bank into *L. lactis* (RepA<sup>-</sup>), was increased by combining the system with temperature-sensitive pWV01 derivative pVE6007. Transformation of *L. lactis* MG1363(pVE6007) with the pORI19 bank of lactococcal chromosomal fragments at the permissive temperature allowed replication of several copies of a recombinant plasmid from the bank within a cell because of the provision in *trans* of RepA-Ts from pVE6007. A temperature shift to 37°C resulted in loss of pVE6007 and integration of the pORI19 derivatives at high frequencies. A bank of lactococcal mutants was made in this way and successfully screened for the presence of two mutations: one in the monocistronic 1.3-kb peptidoglycan hydrolase gene (*acmA*) and one in the hitherto uncharacterized maltose fermentation pathway. Reintroduction of pVE6007 into the Mal<sup>-</sup> mutant at 30°C resulted in excision of the integrated plasmid and restoration of the ability to ferment maltose. The integration plasmid (pMAL) was rescued by using the isolated plasmid content of a restored Mal<sup>+</sup> colony to transform *E. coli* EC101. Nucleotide sequencing of the 564-bp chromosomal fragment in pMAL revealed an internal part of an open reading frame of which the translated product showed significant homology with ATP-binding proteins MalK of *E. coli*, *Salmonella typhimurium*, and *Enterobacter aerogenes* and MsmK of *Streptococcus mutans*. This combined use of two types of conditional replicating pWV01-derived vectors represents a novel, powerful tool for chromosomal gene inactivation, targeting, cloning, and sequencing of the labelled gene.**

Considerable effort in recent years has focussed on the development of gene tagging and targeting techniques by insertions in the lactococcal genome to facilitate chromosomal gene analysis and gene cloning. Chromosomal integration and gene inactivation in *Lactococcus lactis* have been achieved by using the conjugative transposable elements Tn916, Tn919, and Tn1545 (9). A gene targeting and cloning system based on the Tn919 and Tn916 family of transposons was used for the cloning of streptococcal genes (11, 12, 38). However, it was subsequently shown (17) that this system could not be successfully applied to lactococci. Although chromosomal mutations in the genes involved in citrate metabolism have been obtained in *L. lactis* subsp. *lactis* biovar diacetylactis 18-16 by using Tn919, the activity of this transposon is apparently strain dependent and transposition appears to be site specific in *L. lactis* MG1363 (17). Recently, a potentially useful system based on the lactococcal insertion sequence IS946 was studied and random integration in *L. lactis* was demonstrated (35). Le Bourgeois et al. (24) used the insertion sequence ISS1 to construct a chromo-

somal map of strains IL1403 and MG1363. Maguin et al. (31) described a temperature-sensitive (Ts) derivative of the pWV01 replicon. By propagating *L. lactis* carrying this Ts plasmid at the permissive temperature and a subsequent shift to the nonpermissive temperature, chromosomal insertion occurred because of transposition or because of recombination events when homology was provided (2, 32).

Despite the merits of the above-mentioned systems, their use either is strain dependent, requires continuous growth of the transformants at a nonpermissive temperature, or requires laborious cloning steps for initial analysis of the targeted gene. Therefore, a widely applicable system combining efficient chromosomal mutagenesis with fast analysis of the labelled gene is needed. Recently, we designed pWV01-derived vectors whose conditional replication in helper strains of *Escherichia coli*, *Bacillus subtilis*, and *L. lactis* depends on the presence on the chromosomes of those strains of *repA*, the gene for the pWV01 replication protein (25, 28, 29). The availability of these strains enabled the development of pWV01-derived vectors devoid of their own *repA* gene. These Ori<sup>+</sup> vectors cannot replicate in the absence of RepA and, if endowed with lactococcal chromosomal DNA fragments, undergo homologous recombination in *L. lactis* (RepA<sup>-</sup>). This report describes a strategy in which the combined use of two pWV01 derivatives, namely, Ts plasmid pVE6007 (31) and a new Ori<sup>+</sup> derivative, pORI19, results in stable integrant formation with high efficiencies, allowing identification of mutants at optimal growth temperatures. Moreover, the system allows easy recovery of the integrated mutation plasmid from the chromosome. The developed strategies are potentially useful in a large number of bacteria.

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TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant properties	Source or reference
<b>Bacteria</b>		
<i>E. coli</i> JM101	<i>supE thi (lacproAB)</i> (F' <i>traD36 proAB lacI<sup>q</sup></i> $\Delta$ M15)	43
<i>E. coli</i> EC101	<i>E. coli</i> JM101 with <i>repA</i> from pWV01 integrated in chromosome	This work
<i>L. lactis</i> MG1363	Plasmid-free derivative of <i>L. lactis</i> subsp. <i>cremoris</i> NCDO712	10
<i>L. lactis</i> MG1363 <i>acmA</i> $\Delta$ I	MG1363 with deletion in <i>acmA</i>	4
<i>L. lactis</i> MG1363(pVE6007)	Cm <sup>r</sup> derivative of MG1363 harboring pVE6007 in replicative form	31
<i>L. lactis</i> MG1363 ( <i>mal-1</i> )	Em <sup>r</sup> Mal <sup>-</sup> derivative of MG1363 containing pMal integrated in chromosome	This work
<b>Plasmids</b>		
pKVB2	Tc <sup>r</sup> Km <sup>r</sup> containing internally deleted <i>E. coli glgB</i> gene, 11.7 kb	23
pUC23rep3	Ap <sup>r</sup> , pUC23 carrying <i>repA</i> of pWV01 under control of promoter P23	28
pEC1/pEC2	<i>repA</i> of pWV01 cloned into pKVB2	This work
pUC19	Ap <sup>r</sup> <i>lacZ'</i>	43
pORI28	Em <sup>r</sup> Ori <sup>+</sup> RepA <sup>-</sup> derivative of pWV01	29
pORI19	Em <sup>r</sup> Ori <sup>+</sup> RepA <sup>-</sup> <i>lacZ'</i> derivative of pORI28	This work
pVE6007	Cm <sup>r</sup> Ts derivative of pWV01	31
pMAL	Em <sup>r</sup> Ori <sup>+</sup> RepA <sup>-</sup> <i>lacZ'</i> derivative of pORI19 containing 564-bp <i>AluI</i> lactococcal chromosomal fragment cloned into <i>SmaI</i> site of MCS <sup>a</sup>	This work

<sup>a</sup> MCS, multiple cloning site.

It overcomes the limitations imposed on chromosomal gene analysis by uncoupling of transformation and recombination.

## MATERIALS AND METHODS

**Bacteria and plasmids.** The strains and plasmids used in this study are shown in Table 1. *E. coli* was cultured in TY broth (36). TY agar plates contained 1.5% agar and 40  $\mu$ g of 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal) per ml and 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) when required. M17 medium with 0.5% glucose was used for *L. lactis* (41). Erythromycin (EM), ampicillin, tetracycline, and kanamycin were used at final concentrations of 100, 100, 12.5, and 50  $\mu$ g/ml, respectively, for *E. coli*, and EM and chloramphenicol were used at a final concentration of 5  $\mu$ g/ml for *L. lactis*.

**Chemicals.** Restriction enzymes, T4 DNA ligase, and T4 DNA polymerase were obtained from Boehringer GmbH (Mannheim, Germany) and used as recommended by the manufacturer. All chemicals were of analytical grade and were from Merck (Darmstadt, Germany) or BDH (Poole, United Kingdom).

**DNA preparation, molecular cloning, and transformation.** Chromosomal and plasmid DNAs were isolated from *E. coli* by the methods of Kiel et al. (23) and Ish-Horowitz and Burke (22), respectively. Chromosomal and plasmid DNAs were isolated from *L. lactis* as described by Leenhouts et al. (26, 27). Minipreparations of plasmid DNA from *E. coli* and *L. lactis* were made by the method of Birnboim and Doly (1), with minor modifications for *L. lactis* (26).

Routine DNA manipulations were performed as described by Maniatis et al. (33). DNA restriction enzyme fragments were isolated from agarose gels with the Qiagen kit (Qiagen GmbH, Hilden, Germany) as recommended by the manufacturer.

Electrotransformation of *E. coli* and *L. lactis* was performed by the methods of Dower et al. (8) and Holo and Nes (19), respectively, with a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.).

**Estimation of average insert size.** PCRs were performed directly with plasmid DNA from 100 transformants (21) by using the universal and reverse primers supplied by Pharmacia (Uppsala, Sweden). PCR DNA from each colony was run on 2% agarose gels, and insert size was determined by comparison with sized *EcoRI* DNA fragments of *B. subtilis* bacteriophage SPPI.

**Southern blot hybridizations.** Transfer of DNA from 0.8% agarose gels to GeneScreen Plus membranes (Du Pont, NEN Research Products, Boston, Mass.) was performed by the method of Southern as modified by Chomczynski and Qasba (5). DNA probe labelling, hybridization conditions, washing steps, and film development were as described in the instructions of the manufacturer of the ECL gene detection system (Amersham International, Amersham, United Kingdom).

**Stocking of lactococcal chromosomal insertion mutants.** Approximately 30,000 colonies were harvested from 150 plates with 2 ml of M17 medium per plate. The mixture was pooled, centrifuged at 8,000  $\times$  g for 10 min, and resuspended in 100 ml of GM17 containing 29% glycerol. Aliquots of 2 ml of this suspension were stored at  $-80^{\circ}\text{C}$ .

**Bioassays.** To initiate glycogen production, *E. coli* was plated on TY plates supplemented with 0.5% glucose. Following overnight incubation at 37°C, the colonies were flooded with an iodine solution (0.01 M I<sub>2</sub>, 0.03 M KI) and stained for 1 min as described by Govons et al. (14). Brown-staining colonies had

wild-type glycogen branching enzyme activity, while yellow colonies were identified as putative mutants (7).

Cell wall-hydrolyzing activity of *L. lactis* was visible as a halo around colonies grown for 36 to 48 h at 30°C on glucose-M17 plates containing 0.2% autoclaved, lyophilized *Micrococcus lysodeikticus* ATCC 4698 cells (Sigma Chemical Co., St. Louis, Mo.).

Colonies containing chromosomal inserts were diluted 10<sup>-6</sup>-fold and spread plated onto M17 containing 0.5% maltose, 0.005% bromocresol purple, and 5  $\mu$ g of EM per ml. Maltose-positive colonies appear yellow on this medium, while colonies unable to metabolize maltose are white.

**Nucleotide sequence analysis.** Nucleotide sequences were determined by employing the universal sequencing primers on double-stranded plasmid DNA with the T7 DNA polymerase sequencing kit (Pharmacia) in the dideoxy-chain termination method (39). Microgenie software (Beckman, Palo Alto, Calif.) was used for computer-assisted sequence analysis.

**Computer analysis.** Predicted amino acid sequences were compared to the EMBL and SwissProt databases by using the FASTP (30) and TFasta (34) programs. Protein alignments were performed by using the CLUSTAL (16) program of PC/GENE Release 6.5 (IntelliGenetics Inc.).

**Nucleotide sequence accession number.** The nucleotide sequence identified in this study has been assigned GenBank accession number U37409.

## RESULTS

**Introduction of *repA* from pWV01 into the chromosome of *E. coli* JM101.** pKVB2 is a Tc<sup>r</sup> Km<sup>r</sup> plasmid of 11.7 kb containing the origin of replication of pBR322. It carries the *E. coli* chromosomal *glgB* gene in which an internal 1.2-kb *Bam*HI fragment was replaced with the gene for Km<sup>r</sup> from *Streptococcus faecalis* plasmid pJH1 (42) and has been used before as an *E. coli* integration plasmid (23). The *repA* gene from pWV01, driven by lactococcal consensus promoter P23 (28), was taken as a *Hind*III-*Pvu*II fragment from pUC23rep3 and used to replace a 700-bp *Sma*I fragment within *glgB* without interrupting the gene for Km<sup>r</sup>. The resultant plasmids, pEC1 and pEC2, differ solely in the orientation of *repA*.

Plasmids pEC1 and pEC2 were used to transform *E. coli* JM101. Before plating on kanamycin-containing plates, the transformation mixtures were transferred for 30 generations in the absence of antibiotic. Colonies were tested for glycogen production. Approximately 600 non-glycogen-producing colonies were transferred onto plates containing kanamycin and tetracycline and onto plates containing kanamycin alone. Fifteen colonies were Km<sup>r</sup> Tc<sup>s</sup>, and on analysis only three of these were plasmid free and contained *repA* integrated at the specific site on the chromosome. Confirmation of the RepA<sup>+</sup> nature of

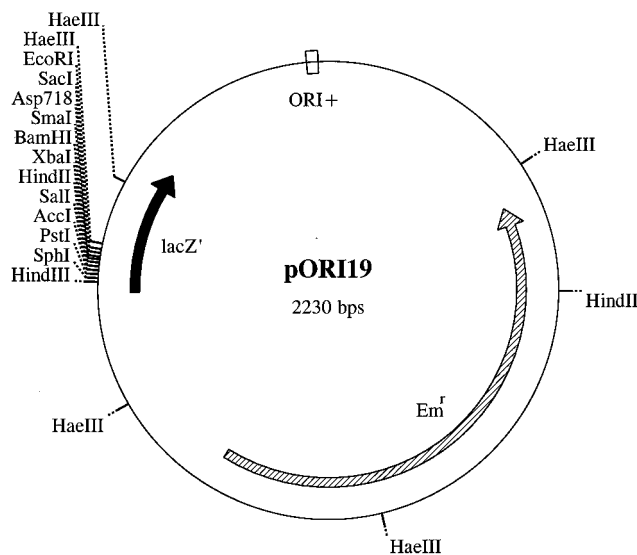


FIG. 1. Schematic representation of ORI<sup>+</sup> RepA<sup>-</sup> integration plasmid pORI19. ORI<sup>+</sup>, positive origin of replication of pWV01. lacZ', *lacZ'* gene from pUC19. Em<sup>r</sup>, Em<sup>r</sup>-encoding gene. Only relevant restriction enzyme sites are shown.

one of the strains (*E. coli* EC101) was obtained by successful transformation of this strain with an ORI<sup>+</sup> RepA<sup>-</sup> plasmid. The orientation of the *repA* gene in the chromosome did not influence the copy number of the RepA<sup>-</sup> plasmids, as judged from agarose gels (results not shown).

**Use of pORI19 for construction of a lactococcal chromosomal DNA bank in *E. coli* EC101.** The *lacZ* $\alpha$  gene from pUC19 was cloned as a 633-bp *SnoI*-*AflIII* fragment in the *BglIII*-*XbaI* restriction sites of Ori<sup>+</sup> RepA<sup>-</sup> vector pORI28 (25, 29), resulting in plasmid pORI19 (Fig. 1). *E. coli* JM101 derivative strain EC101 allows rapid assessment of the efficiency of construction of a plasmid bank of chromosomal DNA in pORI19 by  $\alpha$  complementation.

Several partial *AluI* digests of *L. lactis* MG1363 chromosomal DNA were mixed, and fragments ranging in size from 100 to 1,500 bp were ligated into the dephosphorylated *SmaI* site of pORI19. The ligation mixture was used to transform *E. coli* EC101. More than 90% of the transformants were white or pale blue on agar plates containing X-Gal. All of the white colonies analyzed contained inserts, as did several of the blue and pale blue colonies, indicating that in-frame insertions had occurred in the latter. By PCR, the estimated average insert size in pORI19 of 100 randomly picked colonies was 650 bp. The number of colonies required for 99.9% certainty that all 0.65-kb fragments of the *L. lactis* MG1363 chromosome were cloned is 18,000 (33). The plasmid bank was isolated from approximately 30,000 *E. coli* colonies without further propagation of the cells.

**Integration of the plasmid bank in *L. lactis* MG1363.** Transformation of *L. lactis* (RepA<sup>-</sup>) with the plasmid bank resulted in approximately 10<sup>3</sup> Em<sup>r</sup> colonies per  $\mu$ g of DNA. pORI19 alone (1  $\mu$ g), i.e., without chromosomal DNA inserts, did not yield any Em<sup>r</sup> colonies. Southern hybridization analysis of chromosomal DNAs of several of the transformants revealed that all carried an integrated copy of pORI19 at different locations (results not shown). To increase the number of integrants, the transformation event was separated from the integration event by using the Ts replication property of pVE6007 (31). A schematic representation of the transformation and

subsequent integration of the pORI19 plasmid bank is presented in Fig. 2. The temperature shift from 30 to 37°C causes loss of pVE6007 and integration of the pORI19 recombinants at the sites on the chromosome where their inserts originated. A 1- $\mu$ g sample of the pORI19 chromosomal DNA bank was used to transform *L. lactis* MG1363(pVE6007) (Cm<sup>r</sup>). Immediately following transformation, the transformation mixture was incubated at 30°C for 90 min in the presence of 50 ng of EM per ml to induce expression of the Em<sup>r</sup>-encoding gene. Subsequently, the EM concentration was increased to 5  $\mu$ g/ml and incubation was continued at 30°C for a further 90 min to ensure proper replication of the plasmid bank in *L. lactis* prior to the temperature shift. Plating of the cells at this point and incubation overnight at the nonpermissive temperature for pVE6007 (37°C) did not cure the plasmid, as 40% of the colonies at this stage were Em<sup>r</sup> Cm<sup>r</sup> and thus harbored both plasmids. To ensure total curing of pVE6007, it was necessary to incubate the transformation mixture at 37°C for at least 3 h following the 180-min period at 30°C before plating on GM17-EM agar plates and incubation overnight at 37°C. Overnight incubation at 30°C following this 6-h treatment reduced the percentage of colonies harboring both replicating plasmids to 10%, while at 37°C all Em<sup>r</sup> colonies, recovered at a frequency of 10<sup>4</sup>/ $\mu$ g of DNA, were Cm<sup>s</sup>.

Twenty Em<sup>r</sup> Cm<sup>s</sup> colonies were tested for the presence of a replicating plasmid, and in all cases no plasmid was present. The chromosomal DNAs from 11 of these colonies were digested with *HindII* and *EcoRI* and hybridized with linear pORI19. The combined results of the *HindII* and *EcoRI* digests indicated chromosomal integration of pORI19 at different sites. The results of the *HindII* digests are shown in Fig. 3.

**Mutant isolation among the bank of *L. lactis* chromosomal mutants. (i) Selection of an autolysin-negative mutant of *L. lactis*.** To assess the potential of the system for generation of stable chromosomal mutations in *L. lactis*, the bank was screened for a mutation in the cell wall-hydrolyzing system. The target gene, *acmA*, was selected because (i) it is nonessential, (ii) a simple bioassay is available, and (iii) the gene is monocistronic and of average size (1.3 kb) (4). The size of the target fragment is even smaller if it is taken into account that three repeats located in the C terminus of the hydrolase can be removed without loss of enzyme activity (3). Therefore, integration within the first 700 bp of the gene is required to inactivate it. About 5,000 *L. lactis* transformants were spread onto glucose-M17 plates with autoclaved *M. lysodeikticus* cells, allowing approximately 40 colonies per plate. One transformant without a halo was detected (Fig. 4B). Its chromosomal DNA and those of wild-type strain *L. lactis* MG1363 and the *acmA* deletion mutant MG1363*acmA* $\Delta$ I described earlier (4) were isolated and digested with *HaeIII*. This enzyme cuts once in *acmA*, approximately in the middle. A 4.1-kb *Sau3A* fragment with the entire *acmA* gene was used as a probe for Southern hybridization. Figure 4A shows that, as expected, the 1,599-bp MG1363 chromosomal *HaeIII* fragment carrying the 5' end of *acmA* has disappeared in MG1363*acmA* $\Delta$ I. In the selected integration mutant, the 1,599-bp *HaeIII* fragment has been replaced with two new fragments. This result indicates that pORI19 had integrated within the 5' end of *acmA* in such a way that it interrupted its expression.

**(ii) Identification of a mutant unable to ferment maltose.** The maltose metabolic pathway was chosen for study since identification of mutations in this pathway with another insertional system had previously been shown to be unsuccessful (18). The bank of lactococcal colonies was plated on maltose-M17 agar containing 0.005% bromocresol purple as a pH indicator. Following 24 h of incubation, 1 in 10,000 colonies was

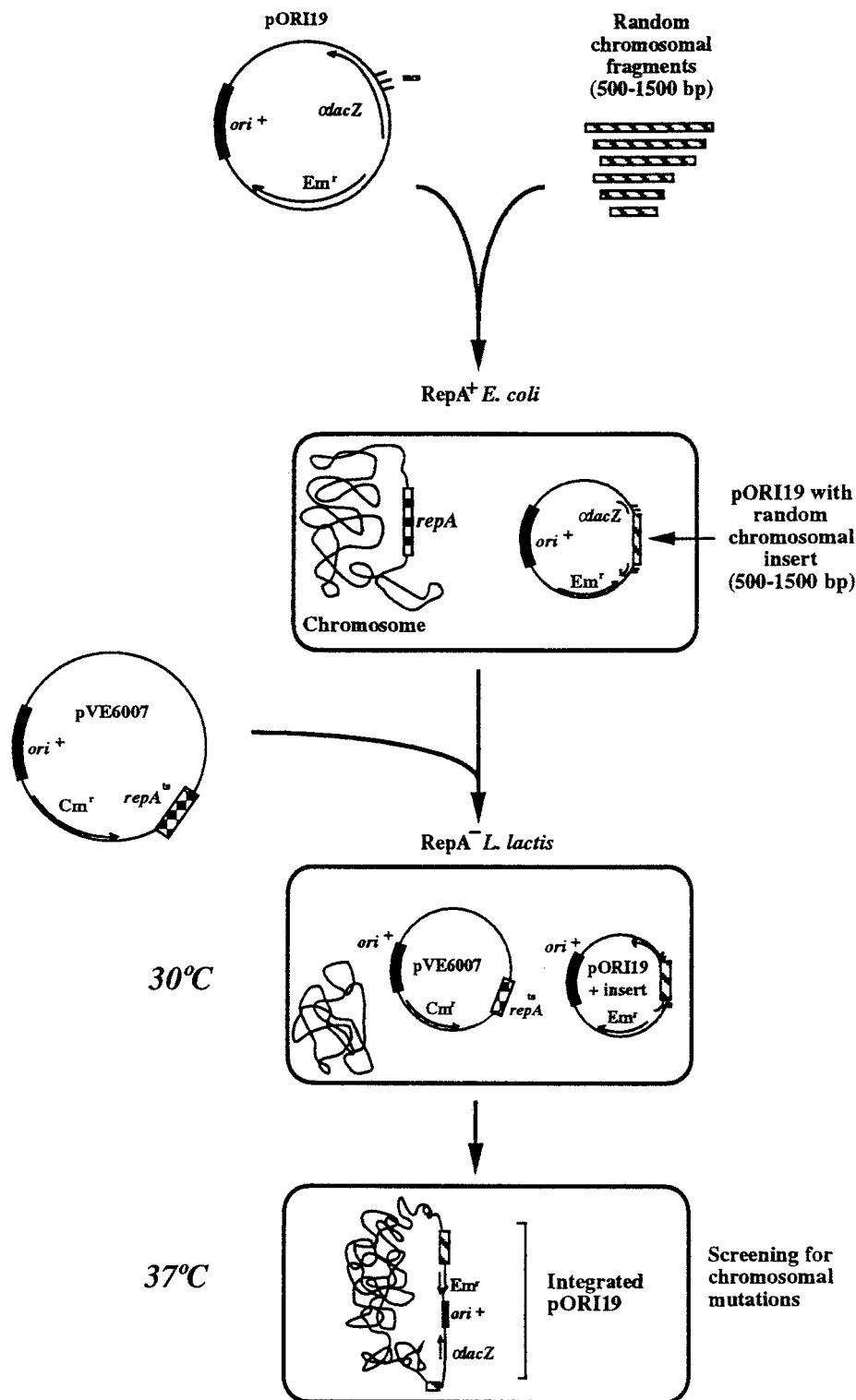


FIG. 2. Schematic representation of the system for generating random chromosomal insertions in *L. lactis* by combined use of pORI19 and pVE6007, the latter of which contains the pWV01 positive origin of replication and encodes a Ts pWV01 RepA protein. For details, see the text.

found to be unable to ferment maltose (approximately 30,000 colonies were screened). One of the *L. lactis* Mal<sup>-</sup> colonies [*L. lactis* (*mal-1*)] was streaked for single colonies on maltose selective agar and after 3 days of incubation at 30°C, all of the

colonies were still white, indicating that the mutation was stable. A culture of this strain maintained its maltose-negative phenotype even after overnight incubation at 30°C in GM17 without EM and subsequent plating on maltose selective agar.

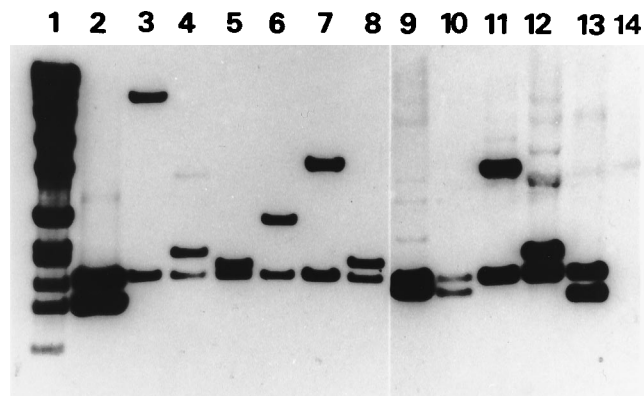


FIG. 3. Southern hybridization analysis of *Hind*II-digested chromosomal DNAs of MG1363 (lane 14) and of 11 transformants (lanes 3 to 13) obtained with the pORI19 plasmid bank. Lane 1, bacteriophage SPP1 DNA cleaved with *Eco*RI (fragment sizes (from the top), 8,545, 7,425, 6,150, 4,905, 3,590, 2,840, 1,970, 1,910, 1,560, 1,450, 1,160, 999, and 760 bp). Lane 2, pORI19 DNA cleaved with *Hind*II, which was also used as the probe.

**Rapid isolation of integrated plasmid pMAL from *L. lactis* (*mal*-1).** To isolate the integrated plasmid from *L. lactis* (*mal*-1), pVE6007 was used to transform the strain (Fig. 5) at 30°C. The transformation mixture was plated on maltose selective agar plates containing chloramphenicol and EM. After 24 h of incubation at 30°C, all of the colonies were white, whereas after 48 h approximately 8% exhibited faint yellowing, indicating that cells in these colonies had reverted to the wild type. After streak plating of the faintly yellow colonies and overnight incubation at 30°C, it was found that 20% of the single colony isolates had regained maltose-fermenting ability, most probably because of precise excision of the integrated plasmid.

A *Mal*<sup>+</sup> *L. lactis* single colony isolate contained two plasmids, pVE6007 and pORI19 containing an insert (pMAL). The plasmid mixture was used to transform *E. coli* EC101 (*RepA*<sup>+</sup>) at 37°C with selection for *Em*<sup>r</sup> only, resulting in separation of pVE6007 and pMAL. Upon introduction of pMAL into *L. lactis* MG1363 (*RepA*<sup>-</sup>), all transformants were white on maltose selective agar, as expected. Southern hybridization analysis of five transformants revealed that pMAL had integrated at the same site on the lactococcal chromosome as in *L. lactis* (*mal*-1) (data not shown).

**The insert in pMAL encodes part of MalK.** The nucleotide sequence of the *Alu*I insert in pMAL was determined. A continuous open reading frame is present on this fragment, and its deduced amino acid sequence of 189 amino acids shows 75.5% identity to part of the *Streptococcus mutans* MsmK protein (37). Identities of 49.7 and 51.3% with the inner membrane MalK proteins of *Enterobacter aerogenes* and *E. coli* (6), respectively, were found (Fig. 6); these are known members of the family of ATP-binding proteins (20). Consequently the targeted gene in *L. lactis* was designated *malK*.

## DISCUSSION

In the present study, a plasmid library of lactococcal chromosomal DNA fragments established in a *RepA*<sup>+</sup> *E. coli* helper strain was used to construct a library of integrants in *L. lactis* by using two conditionally replicating pWV01 derivatives. It can be argued that this procedure lowered the randomness of the library since certain chromosomal DNA fragments of *L. lactis* may not be clonable in *E. coli*. The *RepA*<sup>+</sup> *E. coli* strain could merely be used to determine the efficiency of cloning in

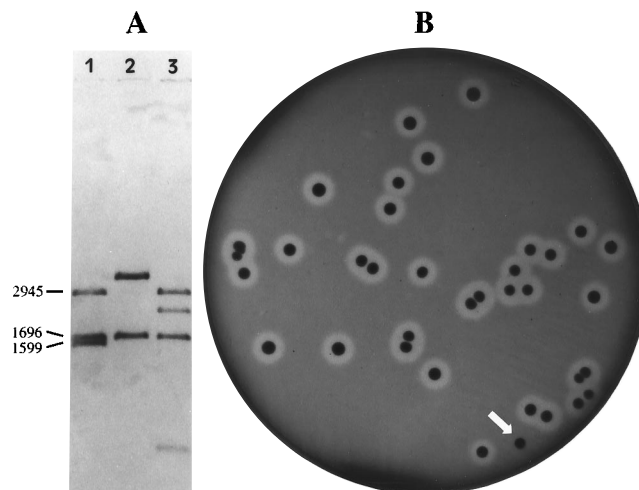


FIG. 4. (A) Southern hybridization analysis of *Hae*III-digested chromosomal DNAs of *L. lactis* MG1363 (lane 1), *L. lactis* MG1363*acmA*Δ1 (lane 2), and the transformant lacking halo formation (lane 3). The sizes (in base pairs) of the bands in the chromosomal DNA of MG1363 hybridizing with a 4.1-kb *Sau*3A fragment used as the *acmA* probe are shown on the left. (B) Analysis of halo formation by *L. lactis* MG1363 integrants on a glucose M17 agar plate containing 0.2% autoclaved, lyophilized *M. lysodeikticus* cells. Arrow, *acmA* interruption mutant.

pORI19 by the  $\alpha$  complementation strategy, after which the ligation mixture could be used to make a library in either or both of the other two cloning hosts, *L. lactis* (*RepA*<sup>+</sup>) and *B. subtilis* (*RepA*<sup>+</sup>) (25, 28), to ensure cloning of a maximum number of lactococcal fragments. The latter procedure was not followed in this work, as we reasoned that a high percentage of those fragments which are unclonable in *E. coli* probably contain (strong) promoters or complete (lethal) genes. Such fragments do not result in mutants, since the cloned fragments need to be internal to a transcriptional unit to result in a mutant phenotype. Constructing the library in *E. coli* (*RepA*<sup>+</sup>) may increase the percentage of plasmids containing an internal gene fragment, in which case the mutation efficiency of the library may be enhanced. The number of mutations screened in this work does not allow conclusions to be drawn about the efficiency of the library used. An *AcmA*<sup>-</sup> mutant was obtained at a frequency ( $5 \times 10^{-3}$ ) slightly higher than that expected. In contrast, mutants defective in the maltose fermentation route, probably a multigenic pathway, were observed at a lower frequency ( $10^{-4}$ ) than expected. It should be noted that screening for *Mal*<sup>-</sup> mutants was done by using agar plates with a high colony density (approximately 1,000 CFU per plate), possibly underestimating the actual number of *Mal*<sup>-</sup> colonies. Nevertheless, it is our view that the library of integration plasmids is efficient enough to generate many different mutations, even in average-size monocistronic genes, as was convincingly demonstrated by the isolation of the *AcmA*<sup>-</sup> mutant.

The *Mal*<sup>-</sup> mutant was used to develop a protocol for rapid isolation of the integrated plasmid. Introduction of pVE6007 into the mutant resulted in excision of the integrated plasmid because of provision of *RepA*-Ts in *trans*. Although the excision is likely to be precise, sequencing of the chromosome before and after the insertion-excision event is required to confirm this assumption.

Hill et al. (18) described the isolation of *L. lactis* subsp. *lactis* biovar diacetylactis mutants defective in the maltose metabolic pathway by use of conjugative transposable element Tn919. These mutants reverted at a frequency too high to allow clon-

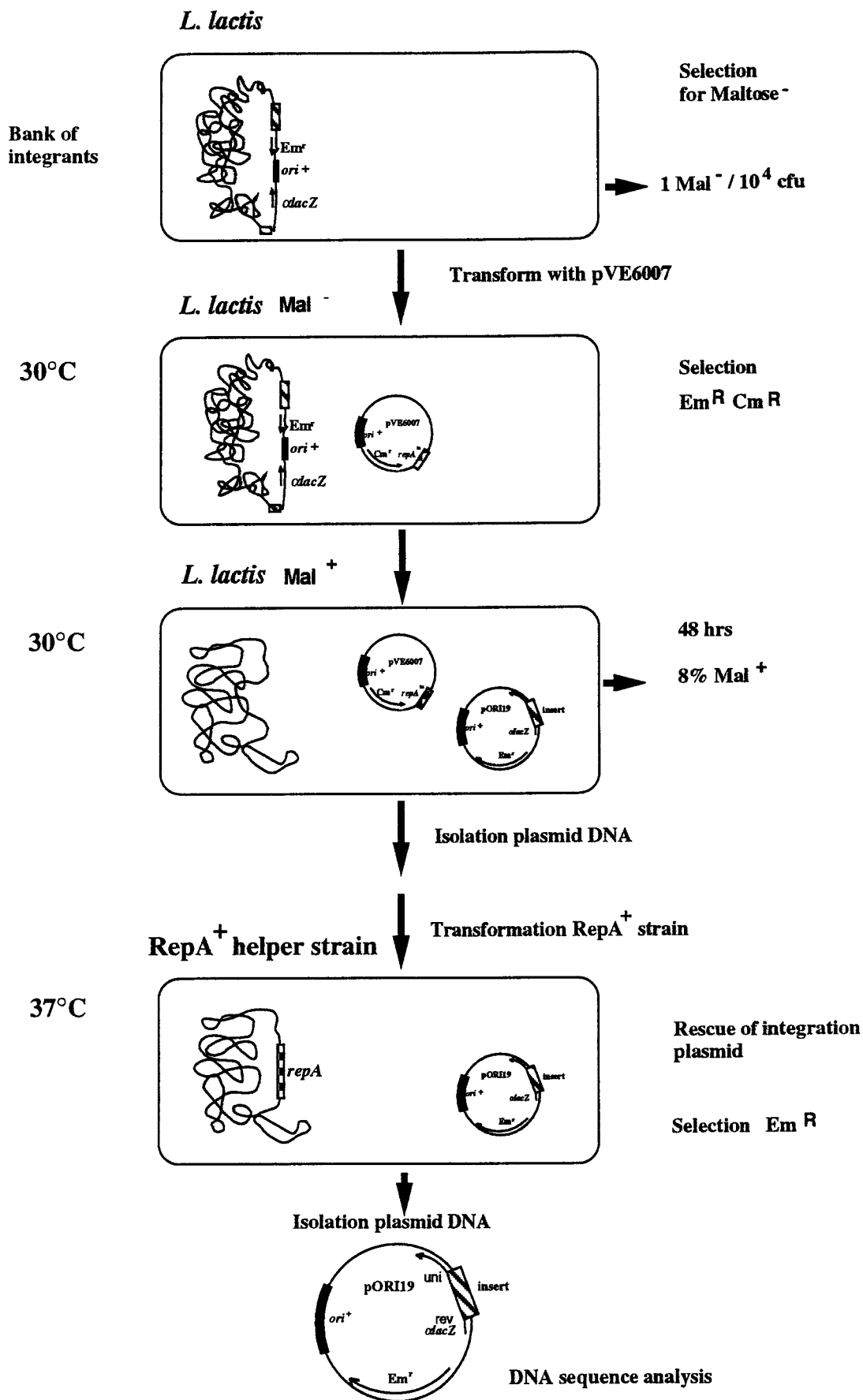


FIG. 5. Scheme of the rescue of an integration plasmid from an identified mutant, as illustrated by the recovery of pMAL from *L. lactis* (*malI*) (see the text for a detailed description).

MALPRT	-----	0
MSSM	MVELNLNHIYKYPNSSHYSVEDFDLDIKNKEFIVFVGPSCGCKSTLLRM	50
MAEC	MASVQLQNVTKAWGEV--VSKDINLDIHEGEFVVFVGPSCGCKSTLLRM	48
MAEA	MASVQLQNVTKAWGEV--VSKDINLEIQDGEFVVFVGPSCGCKSTLLRM	48
MALPRT	-----	0
MSSM	VAGLEDITKGLKIDGEVVDKAPKDRDIAMVFQNYALYPHMSVYDNMAF	100
MAEC	IAGLETITSGDLFI GEKRMNDTPPAERGVGMVFQSYALYPLSVAENMSF	98
MAEA	IAGLETITSGDLFI GDTRMNDVPPAERGI GMVFQSYALYPLSVAENMSF	98
MALPRT	-----AIIGLTDLLDRKPADLGGGQRQVAMGRAI	30
MSSM	GLKLRHYSKEAIDKRVKEAAQILGLTEFLERKPADLGGGQRQVAMGRAI	150
MAEC	GLKPAGAKKEVINQRVQVAEVLQALHLDRKPKALSGGQRQVAI GRTL	148
MAEA	GLKLAGAKKEVINQRVTQVAEVLQALHLLENRKALSGGQRQVAVI-RTL	147
	..*..*..*..*..*..*..*..*..*..*..*..*..*..*..*..*..*..*..*	
MALPRT	VRDAKVFMDPEL SNLDAKLRVSMRTEIAKHRRIGATTIYVTHDQTEAM	80
MSSM	VRDAKVFMDPEL SNLDAKLRVSMRTEIAKHRRIGATTIYVTHDQTEAM	200
MAEC	VAEPSVFLDPEL SNLDAALRVQMR IEISRLHKRLGRTHIYVTHDQVEAM	198
MAEA	VAEPRVFLDPEL SNLDAALRVQMR IEISRLHKRLGRTHIYVTHDQVEAM	197
	*..*	
MALPRT	TLADRIVIMSSPNSDKTIGRVEQIGTPQGLYNEPANKFVAGFIGSPA	130
MSSM	TLADRIVIMSSKTKNEDSGGTIGRVEQVTPQELYNRPANKFVAGFIGSPA	250
MAEC	TLADKIVVLDA-----GRVAQVQKPLELYHPADRFAVAGFIGSPK	238
MAEA	TLADKIVVLDA-----GRVAQVQKPLELYHPADRFAVAGFIGSPK	237
	*****..*	
MALPRT	MNFFNVKVTGGKLTNNEGLNMDLPEGKAKLLKEQGYE--GKEVILGIRP	177
MSSM	MNFFDVTIMDGHVSKDGLTIAVTEGQLKMLKESKGFK--NKMLIFGIRP	297
MAEC	MNFLPVKVTATAI-DOVQVELPMPNRQVQLPVESRDVQVGNANMSLIGIRP	287
MAEA	MNFLPVKVTATAI-EQVQVEL--PNRQVQLPVD SAHVQVGNANMSLIGIRP	284
	***..*	
MALPRT	EDIQASNLAAQA-----	189
MSSM	EDISSLLVQETYPDATVDAEVVSELLGSETMLYLKLG--QTEFAARVD	345
MAEC	EH-----LLPSDIADVILEGEVQVVEQLGNETQIHQIPSIQNLYRQN	332
MAEA	EH-----LLPSDIAD-----	294
	*..*	
MALPRT	-----	189
MSSM	ARDFHEPGEKVSITFNVAKGHFFDAEATAAIR-----	377
MAEC	DVVLVEEGATFAITGLPPERCHLFRDGTACRRLHKEPGV-----	371
MAEA	DVVLVEEGATFAITGLPPERCHLFRDGTACRRLHKEPGV-----	294

FIG. 6. Alignment of the amino acid sequences encoded by the 564-bp fragment of the putative *malK* gene of *L. lactis* (MALPRT), the MsmK protein of *S. mutans* (MSSM), and the MalK proteins of *E. coli* (MAEC) and *A. aerogenes* (MAEA). Conserved amino acids are indicated by dots, and amino acids identical in all four sequences are indicated by asterisks. Dashes indicate gaps introduced to maximize alignment.

ing and characterization of the targeted gene. The mutation described here was superior in stability, probably because of the absence of replication functions in the integration vector.

The highest degree of homology of the putative product of the open reading frame present on the pMAL insert was with the membrane-associated ABC protein MsmK of *S. mutans*. This protein is part of the binding protein-dependent transport system responsible for multiple sugar metabolism. Interestingly, inactivation of *msm* genes had no effect on the ability of *S. mutans* to grow on maltose (37). In contrast, *malK* in *L. lactis* is involved in maltose metabolism because the gene fragment was obtained from a Mal<sup>-</sup> insertion mutant and reinsertion of pMAL into the chromosome of a wild-type strain resulted in a Mal<sup>-</sup> phenotype. In the maltose uptake system of *E. coli*, membrane-associated protein MalK forms a complex with integral membrane proteins MalF and MalG, which interact with maltose-loaded maltose-binding protein MalE to mediate active transport of maltose across the cytoplasmic membrane (15, 40). MalK is believed to energize the system, presumably through ATP hydrolysis (13, 40). On the basis of the similarity of lactococcal *malK* to the *malK* genes from *E. coli* and *E. aerogenes*, we propose that a binding protein-dependent ATP-energized system also operates in *L. lactis* for the transport of maltose. This would be the first indication of the presence of

such a sugar transport system in *L. lactis*. Further analysis of the Mal<sup>-</sup> mutant has to be conducted to confirm this conclusion.

Although the described method is highly efficient as a means of generating chromosomal insertions, the isolation of genes is, like most insertional methods, restricted to nonessential genes and genes that allow detection of phenotypic negatives. Important advantages of the method are that (i) stability of the mutants is high because of the absence of residual activity of transposases or (Ts) replication proteins, (ii) screening of mutants can be performed at optimal growth temperatures, (iii) the integration plasmid can be readily recovered by a simple and rapid procedure, and (iv) the availability of RepA<sup>+</sup> *L. lactis* (25), *B. subtilis* (28), and *E. coli* (this study) helper strains minimizes difficulties (lethality and deletions) in cloning of the targeted gene caused by incompatible host backgrounds. Therefore, we believe that the pWV01-based system described here is an important and valuable tool for analysis of the lactococcal chromosome and may represent a useful tool for use with other bacteria.

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