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\(^{+}\) Rep\(^{+}\) vector pORI19, containing lacZ\(_{a}\) and the multiple cloning site of pUC19. Chromosomal AluI fragments of Lactococcus lactis were cloned in pORI19 in Rep\(^{+}\) helper strain Escherichia coli EC101. The frequency of Campbell-type recombinants, following introduction of this plasmid bank into L. lactis (Rep\(^{+}\)), 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\(^{+}\) 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\(^{+}\) 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 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 conjugal 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 biowar diacylactis 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 chromosomal 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\(^{+}\) vectors cannot replicate in the absence of RepA and, if endowed with lactococcal chromosomal DNA fragments, undergo homologous recombination in L. lactis (Rep\(^{+}\)). This report describes a strategy in which the combined use of two pWV01 derivatives, namely, Ts plasmid pVE6007 (31) and a new Ori\(^{+}\) 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.
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. L. lactis was cultured in TY broth (36). TY agar plates contained 1.5% agar and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml and 1 mM isopropyl-β-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 μg/ml, respectively, for E. coli, and EM and chloramphenicol were used at a final concentration of 5 μg/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 Kiefer et al. (23) and Ish-Horovitz and Burke (22), respectively. Chromosomal and plasmid DNAs were isolated from L. lactis, as described by Leenhout 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 SP12.

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 Sacchi (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 (Amerham 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 × g for 10 min, and resuspended in 100 ml of M17 containing 29% glycerol. Aliquots of 2 ml of this suspension were stored at -80°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 inoculation solution (0.01 M I₃, 0.03 M KI) and stained for 1 min as described by Govorns et al. (14). Brown-staining colonies had wild-type glycogen branching enzyme activity, while yellow colonies were identified as putative mutants (7).

RESULTS

Introduction of repA from pWV01 into the chromosome of E. coli JM101. pKV2 is a Te Km’ 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 BamHI fragment was replaced with the gene for Km’ 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 HindIII-PvuII fragment from pUC23rep3 and used to replace a 700-bp Smal fragment within glgB without interrupting the gene for Km’. 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’ Te’, 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+ nature of
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-μg sample of the pORI19 chromosomal DNA bank was used to transform L. lactis MG1363(pVE6007) (Cm'). 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' encoding gene. Subsequently, the EM concentration was increased to 5 μg/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' Cm' 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' colonies, recovered at a frequency of 10^9/μg of DNA, were Cm'.

Twenty Em' Cm' 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 HindIII and EcoRI and hybridized with linear pORI19. The combined results of the HindIII and EcoRI digests indicated chromosomal integration of pORI19 at different sites. The results of the HindIII digests are shown in Fig. 3.

**Mutant isolation among the bank of L. lactis chromosomal integrants.** (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 autochaved 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 MG1363acmaΔ1 described earlier (4) were isolated and digested with HaeIII. This enzyme cuts once in acmA, approximately in the middle. A 4.1-kb SulfA 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 MG1363acmaΔ1. 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 insertion 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.
found to be unable to ferment maltose (approximately 30,000 colonies were screened). One of the \textit{L. lactis} Mal\textsuperscript{-} colonies [\textit{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 HindIII-digested chromosomal DNAAs of MG1363 (lane 14) and of 11 transformants (lanes 3 to 13) obtained with the pORI19 plasmid bank. Lane 1, bacteriophage SPPI DNA cleaved with EcoR I (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 HindIII, 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+ 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+) at 37°C with selection for Em1 only, resulting in separation of pVE6007 and pMAL. Upon introduction of pMAL into L. lactis MG1363 (RepA-), 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 AalI 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 MsxK 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+ 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+ E. coli strain could merely be used to determine the efficiency of cloning in plORI19 by the α 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+) and B. subtilis (RepA+) (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+) 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- mutant was obtained at a frequency (5 × 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- 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- 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- mutant.

The Mal+ 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 conjugal transposable element Tn917. 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 (mal1) (see the text for a detailed description).
S. mutans to grow on maltose (37). In contrast, was obtained from a maltose-involved in maltose metabolism because the gene fragment was inactivated in their ability to store glycogen. Biochem. Biophys. Res. Commun. 281:236-240.

Reference to the figure: FIG. 6. Alignment of the amino acid sequences encoded by the 564-bp fragment of the putative malK gene of L. lactis (MALPR1), the Msk protein of S. mutans (MSSM), and the MalK protein of E. coli (MAEC) and E. 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.

such a sugar transport system in L. lactis. Further analysis of the Mal − 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 + 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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