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Stability of Integrated Plasmids in the Chromosome of *Lactococcus lactis*

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Derivatives of plasmids pBR322, pUB110, pSC101, and pTB19, all containing an identical fragment of lactococcal chromosomal DNA, were integrated via a Campbell-like mechanism into the same chromosomal site of *Lactococcus lactis* MG1363, and the transformants were analyzed for the stability of the integrated plasmids. In all cases the erythromycin resistance gene of pE194 was used as a selectable marker. Transformants obtained by integration of the pBR322 derivatives contained a head-to-tail arrangement of several plasmid copies, which most likely was caused by integration of plasmid multimers. Single-copy integrations were obtained with the pSC101 and pTB19 derivatives. In all of these transformants no loss of the erythromycin gene was detected during growth for 100 generations in the absence of the antibiotic. In contrast, transformants containing integrated amplified plasmid copies of pUB110 derivatives were unstable under these conditions. Since pUB110 appeared to have replicative activity in *L. lactis*, we suggest that this activity destabilized the amplified structures in *L. lactis*.

A substantial number of genes that are important for dairying appear to be plasmid located, and because of segregational instability the traits specified by these genes are often lost (10, 22). A clear trend in the dairy industry is the scaling-up and automation of fermentations with strains having predictable properties under the fermentation conditions used; the success of this approach ultimately depends on the availability of genetically stable strains, especially with respect to the traits which are important for dairy practice.

Basically, the following two ways may be considered to achieve this goal: (i) the stabilization of the plasmid-encoded genes in the chromosome by replacement recombination, and (ii) the stabilization of these genes by incorporation of the plasmids in the chromosome by Campbell-like integration, which has the additional advantage of possible gene copy effects if the integrated plasmids become amplified.

Both approaches seem to be feasible (7, 20). We previously reported Campbell-like integration of a pBR322 replicon into the chromosome of *Lactococcus lactis* subsp. *lactis* in which a chloramphenicol resistance gene was used as a selectable marker (20). Although amplification of the plasmid in the chromosome was observed, integrated plasmid copies were gradually lost under nonselective growth conditions. However, in all cases examined at least one copy remained stably integrated. Loss of amplified integrated heterologous DNA in *L. lactis* subsp. *lactis* has also been reported by Chopin et al. (7).

The fact that the use of pBR322-derived vectors and selection for resistance to chloramphenicol resulted in unstable amplification prompted us to extend our investigation with a number of different replicons and selection for a different marker to examine whether these variables could be used in a more successful way. In this paper we describe the results of integrations with plasmid vectors pBR322 (4) and pSC101 (9), both of which originated from gram-negative bacteria, and with vectors pUB110 (13) and pTB19 (16), which originated from gram-positive bacteria. We found that, with selection for the erythromycin resistance gene obtained from pE194 (15), stable integration of pSC101- and pTB19-based vectors and stable multicopy integration of pBR322-derived vectors could be obtained. Although transformation with pUB110-derived plasmids initially yielded multicopy integrations, under nonselective conditions deamplification was observed, ultimately resulting in the complete loss of the plasmid from the chromosome. We describe results which suggest that the unstable integration of the pUB110-derived plasmids was related to residual replicative activity of the replicon in the lactococcal host.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are shown in Table 1. *Bacillus subtilis* and *Escherichia coli* were grown on TY broth and agar (26). *L. lactis* was cultured and plated onto glucose-M17 broth and agar (28). After electrotransformation of *L. lactis*, cells were plated onto glucose-M17 agar plates containing 0.3 M sucrose. Erythromycin and chloramphenicol were used at final concentrations of 5 μg/ml for both *B. subtilis* and *L. lactis*. Ampicillin, erythromycin, and chloramphenicol were used for *E. coli* at final concentrations of 100, 100, and 20 μg/ml, respectively.

DNA isolation and manipulation. Plasmid DNA was isolated from *E. coli* and *B. subtilis* as described by Ish-Horowicz and Burke (17) or by using the method of Birnboim and Doly (3). The method used to obtain minipreparations of plasmids from *L. lactis* has been described previously (20). *L. lactis* chromosomal DNA was isolated by using a mini-isolation procedure. After overnight growth, 5 ml of a culture was centrifuged. The resulting pellet was washed once with 1 ml of distilled water and suspended in 1 ml of lysis solution (25 mM Tris hydrochloride [pH 8], 50 mM EDTA, 50 mM glucose) containing 5 mg of lysozyme per ml and 30 U of mutanolysin per ml. The suspension was incubated for 30 min at 37°C, after which 20 μl of proteinase K (20 mg/ml) and 50 μl of 10% sodium dodecyl sulfate were added. Incubation was continued at 60°C for at least 1 h. The lysate was extracted five times with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and twice with

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TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant properties	Source or reference(s)
Bacteria		
E. coli JM101		29
B. subtilis PSL1		25
L. lactis subsp. *lactis* strains		
MG1363	Em^s, plasmid free	11
MG10A	Em^r, MG1363 carrying (three to five) copies of pKL10A in the chromosome	This work
MG10B	Em^r, MG1363 carrying two copies of pKL10B in the chromosome	This work
MG200	EM^r, MG1363 carrying amplified pKL200 in the chromosome	This work
MG203A	Em^r, MG1363 carrying amplified pKL203A in the chromosome	This work
MG203B	Em^r, MD1363 carrying amplified pKL203B in the chromosome	This work
MG301B	Em^r, MG1363 carrying one copy of pKL301B in the chromosome	This work
MG400B	Em^r, MG1363 carrying one copy of pKL400B in the chromosome	This work
Plasmids		
pBR322	Ap^r Tc^r	4
pUC19E	Ori pBR322, Ap^r Em^r	Laboratory collection
	pUC19 carrying the *erm* gene of pE194 in the *Sma*I site	14
pHV60	Ori pBR322, Ap^r Cm^r Tc^r	22
pGI404	pHV60 carrying 1.3-kb chromosomal insertion A	19
pGI406	pHV60 carrying 1.3-kb chromosomal insertion B	28
pKL10	Ori pBR322, Em^r	This work
pKL10A	pKL10 with chromosomal insertion A	This work
pKL10B	pKL10 with chromosomal insertion B	This work
pUB110	Km^r	13, 18
pKL200	pUB110, Em^r Km^s	Laboratory collection
pKL202	Ori pUB110, Em^r	This work
pKL203A	pKL202 with chromosomal insertion A, Em^r, Cm^r	This work
pKL203B	pKL202 with chromosomal insertion B, Em^r Cm^r	This work
pLG339	Ori pSC101, Km^r Tc^r	27
pKL300	Ori pSC101, Km^r Cm^r Tc^r	This work
pKL301B	pKL300 with chromosomal insertion B, Em^r Km^r Tc^s	This work
pHV1436	Ori pTB19, *ori* pBR322, Cm^r Tc^r	17a
pKL400	Ori pTB19, Ap^r Em^r	This work
pKL400B	pKL400 with chromosomal insertion B	This work

an equal volume of chloroform-isoamyl alcohol (24:1). Sodium acetate (3 M) was added (0.1 volume), and the DNA was precipitated with 2 volumes of 96% ethanol (−20°C). The DNA was dissolved in 150 μl of TE (10 mM Tris hydrochloride [pH 8], 1 mM EDTA) containing 20 μg of RNase. The DNA concentration was determined spectrophotometrically.

Restriction enzymes, endonuclease S1, T4 ligase, and Klenow enzyme were obtained from Boehringer GmbH, Mannheim, Federal Republic of Germany, or New England BioLabs, Inc., Beverly, Mass., and were used according to the instructions of the suppliers.

Construction of integration plasmids. The compositions of integration plasmids pKL10A, pKL10B, pKL203A, pKL 203B, pKL301B, and pKL400B are shown in Fig. 1. *L. lactis* subsp. *lactis* MG1363 chromosomal fragments A and B corresponded to the chromosomal fragments present in previously described plasmids pGI404 and pGI406, respectively (20). Vectors pKL10A and pKL10B carried the origin of replication of pBR322 and were constructed as described below. A *Dra*I digest of pBR322 was ligated with *Eco*RI linkers. This ligation mixture was then digested with *Eco*RI and *Pvu*II. Plasmid pKL10 was obtained by ligating the *Eco*RI-*Pvu*II origin-carrying fragment with an *Eco*RI-*Pvu*II fragment carrying the erythromycin resistance gene of pUC19E. Chromosomal fragment A was isolated as an *Sph*I fragment from pGI404 and was ligated in the *Sph*I site of pKL10, resulting in plasmid pKL10A. Plasmid pKL10B was constructed by inserting chromosomal fragment B as a *Bam*HI-*Sph*I fragment from pGI406 in the corresponding restriction sites of pKL10.

Plasmids pKL203A and pKL203B containing the origin of replication of pUB110 were constructed as described below. *Acc*I-digested pUB110 was treated with Klenow enzyme to fill in the recessed ends and was subsequently cut with *Hae*III. The blunt end fragment containing the origin of replication was isolated. The erythromycin resistance gene was obtained as an *Eco*RI-*Hin*dIII fragment from pUC19E, the recessed ends of which were filled in with Klenow enzyme. These two fragments were ligated, which resulted in plasmid pKL202. Chromosomal fragments A and B were inserted into pKL202 as *Sph*I-*Pst*I fragments from pGI404 and pGI406, respectively. These fragments also contained the chloramphenicol resistance marker.

To obtain pKL301B, which contained the origin of replication of pSC101 (8, 9), pKL300 was constructed first. Plasmid pHV60 (23) was digested with *Eco*RI and *Sal*I, and the fragment containing the chloramphenicol resistance marker was then ligated with *Eco*RI-*Sal*I-digested pLG339 (27). Plasmid pKL10B was digested with *Eco*RI, and the recessed ends were filled in with Klenow enzyme; this was followed by digestion with *Sph*I. The fragment containing chromosomal fragment B, as well as the erythromycin resistance gene, was isolated and inserted into the *Eco*RV and *Sph*I sites of pKL300, resulting in plasmid pKL301B.

Plasmid pKL400B contained the following two origins of replication: the pBR322 origin and the pTB19 origin (16). Plasmid pKL400 was constructed by inserting the pTB19

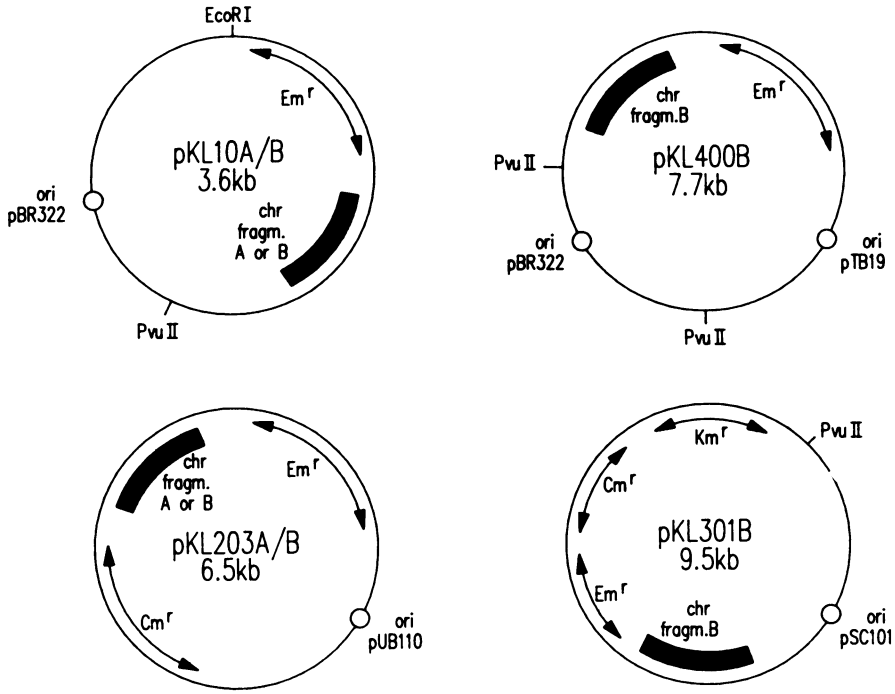

FIG. 1. Schematic representation of integration plasmids pKL10A, pKL10B, pKL203A, pKL203B, pKL301B, and pKL400B. Sizes (in kilobases) are indicated. The plasmids are not drawn to scale, and only relevant restriction enzyme sites are shown. chr fragm., Chromosomal fragment.

origin as an *Eco*RI fragment from pHV1436 (comprising the *repA* replication region of pTB19) into the *Eco*RI site of pUC19E. Chromosomal fragment B was isolated as a *Bam*HI-*Sph*I fragment from pGI406 and then ligated with *Bam*HI- and *Sph*I-digested pKL400, which resulted in pKL400B.

Transformation. *E. coli* was transformed as described by Mandel and Higa (21). Protoplasts of *B. subtilis* were prepared and transformed by using the method of Chang and Cohen (5). *L. lactis* was transformed by a modification of the electroporation method developed by Harlander (14), using a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.). Cells were grown in 25 ml of glucose-M17 medium supplemented with 40 mM DL-threonine to an optical density at 660 nm of 0.2 to 0.3. The cultures were centrifuged, and the resulting pellets were washed twice with 1 ml of ice-cold distilled water, once with 1 ml of 50 mM EDTA (pH 8), once with 1 ml of distilled water, and finally with 1 ml of 0.3 M sucrose. The cell pellets were suspended in 0.2 ml of a 0.3 M sucrose solution. After the DNA solutions were mixed with the cell suspensions, electroporation was carried out in a precooled (0°C) 2-mm cuvette at 12,500 V/cm. The capacitance was set at 25 μF, and the pulse controller (Bio-Rad) was set at 200 Ω. Immediately after the electrical discharge the cell suspensions were transferred to 10 ml of glucose-M17 medium containing 0.3 M sucrose and 50 ng of erythromycin per ml and left for 2 h at 30°C to allow expression of the erythromycin marker. Suitable dilutions of the suspensions were subsequently plated onto glucose-M17 agar plates containing 0.3 M sucrose and selective antibiotics.

Southern hybridization. After electrophoresis in 0.8% agarose gels, the DNA (3 μg of chromosomal DNA per lane in each case) was transferred to GeneScreen Plus filters (Du Pont, NEN Research Products, Boston, Mass.) by using the protocol of Southern, as modified by Chomczynski and Qasba (6). DNA was labeled either with digoxigenin-dUTP by using a Nonradioactive DNA Labeling and Detection Kit (Boehringer) or with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) by using a Random Primed DNA Labeling Kit (Boehringer). The nonradioactive hybridization, washing, and staining steps were done according to the instructions of the supplier. The hybridizations with the radioactive probes and the subsequent washing steps were carried out as instructed by the manufacturers of GeneScreen Plus (Du Pont). Labeled phage SPP1 DNA or labeled phage lambda DNA or both were added to the hybridization mixtures to facilitate easy determination of the sizes of the hybridizing fragments in the chromosomal digests.

Determination of the stability of transformants. To assess the stability of the Em^r phenotype of the transformants, strains MG10A, MG10B, MG203A, MG203B, MG301B, and MG400B were grown in the absence of the antibiotic. After 100 generations (indicated by the suffix -I) the erythromycin gene was induced by incubating the cultures for 2 h with 50 ng of erythromycin per ml. Dilutions were plated onto selective and nonselective plates. In addition, 50 randomly chosen colonies were transferred from the nonselective plates to selective and nonselective plates. The chromosomal structures of the strains after nonselective growth were examined by using Southern hybridization.

RESULTS

Chromosomal structures and stabilities of transformant strains MG10A and MG10B. pBR322-derived plasmids pKL10A and pKL10B were used in several electrotransformation experiments (with transformation frequencies varying from 1 to 10 transformants per μg of DNA) to transform plasmid-free *L. lactis* strain MG1363; the erythromycin-

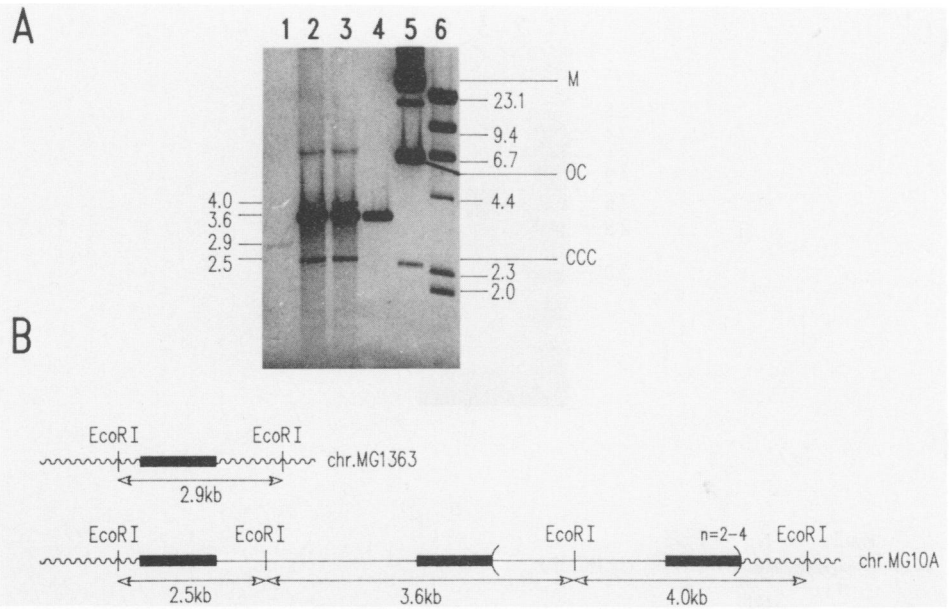

FIG. 2. (A) Southern hybridization analysis of chromosomal DNAs of strains MG1363, MG10A, and MG10A-I digested with *Eco*RI and hybridized with digoxigenin-dUTP-labeled pKL10A. Lane 1, Strain MG1363; lane 2, strain MG10A; lane 3, strain MG10A-I; lane 4, pKL10A cleaved with *Eco*RI; lane 5, undigested pKL10A; lane 6, phage lambda DNA cleaved with *Hin*dIII. Sizes (in kilobases) are indicated on the sides. M, Multimers; OC, open circular DNA; CCC, covalently closed circular DNA. (B) Structure of the relevant parts of the chromosomes of host strain MG1363 and transformant strain MG10A. The solid boxes indicate the locations of chromosomal fragment A.

resistant transformants obtained were designated strains MG10A and MG10B, respectively. Two independently obtained transformants of strain MG10A and three independently obtained transformants of strain MG10B were used for further analysis. None of these transformants contained free plasmid DNA when they were analyzed by standard procedures (data not shown). The chromosomal structures of the two strain MG10A transformants were identical, as judged by the results of Southern hybridization when pKL10A was used as a probe; the results of the analysis of one of these hybridizations are shown in Fig. 2. In Fig. 2A, lane 1 contained *Eco*RI-digested chromosomal DNA of strain MG1363, and one hybridizing fragment was present, at 2.9 kilobase (kb). In lane 2, which contained an *Eco*RI digest of the chromosomal DNA of the transformant MG10A strain, three fragments were present, one at 4.0 kb, one at 2.5 kb, and one intensely hybridizing band at 3.6 kb, which was the same size as linear pKL10A (lane 4). A fourth band, at approximately 7.2 kb, was most likely due to a minor fraction of undigested chromosomal DNA. Because of some smearing of the hybridizing bands, the absence of the 2.9-kb fragment could not be determined with absolute certainty. Nevertheless, the pattern in lane 2 indicates that there was Campbell-like integration of pKL10A into fragment A on the chromosome of strain MG1363. The absence of free plasmid DNA and the presence of the 3.6-kb band in lane 2 strongly suggest that there was a tandem arrangement of multiple copies of pKL10A on the chromosome (Fig. 2B). The number of copies was determined densitometrically to be three to five. The tandem arrangement of multiple copies may have been caused either by amplification of the plasmid in the chromosome or by the integration of multimeric forms of the plasmid, as we noticed that the plasmid preparation which we used contained considerable amounts of multimers (Fig. 2A, lane 5).

Strain MG10A was grown in the absence of erythromycin for 100 generations. The resulting culture was designated strain MG10A-I. In a plate assay no loss of the Em^r phenotype was detected. In addition, 50 colonies that were randomly picked from nonselective plates were all resistant to erythromycin (Table 2). The stability of the Em^r phenotype was confirmed by our analysis of the chromosomal structure of strain MG10A-I (Fig. 2A, lane 3), which was identical to that of strain MG10A (lane 2).

The chromosomes of the three strain MG10B transformants were also examined by using Southern hybridization, and all were found to be structurally identical. In the experiment shown in Fig. 3, labeled pKL10B was used as a probe. In Fig. 3A, lanes 2 to 4 contained *Pvu*II digests of chromosomal DNA from strain MG1363, chromosomal DNA from one of the strain MG10B transformants, and DNA from plasmid pKL10B, respectively. An approximately 3.6-kb fragment hybridized in the chromosomal digest of strain MG1363 (lane 2). In case of a Campbell-like integration event, this fragment should have been absent in the chromosomal digest of strain MG10B (lane 3). However, in addition to two new fragments at 4.0 and 3.2 kb, as

TABLE 2. Percentage of Em^r colony-forming units after growth in the absence of selective pressure[a]

Strain	% of Em^r CFU
MG1363	0
MG10A-1	100
MG10B-I	100
MG203A-I	25
MG203B-I	13
MG301B-I	100
MG400B-I	100

[a] Values were determined by transferring 100 colonies from nonselective plates to selective plates after approximately 100 generations of nonselective growth.

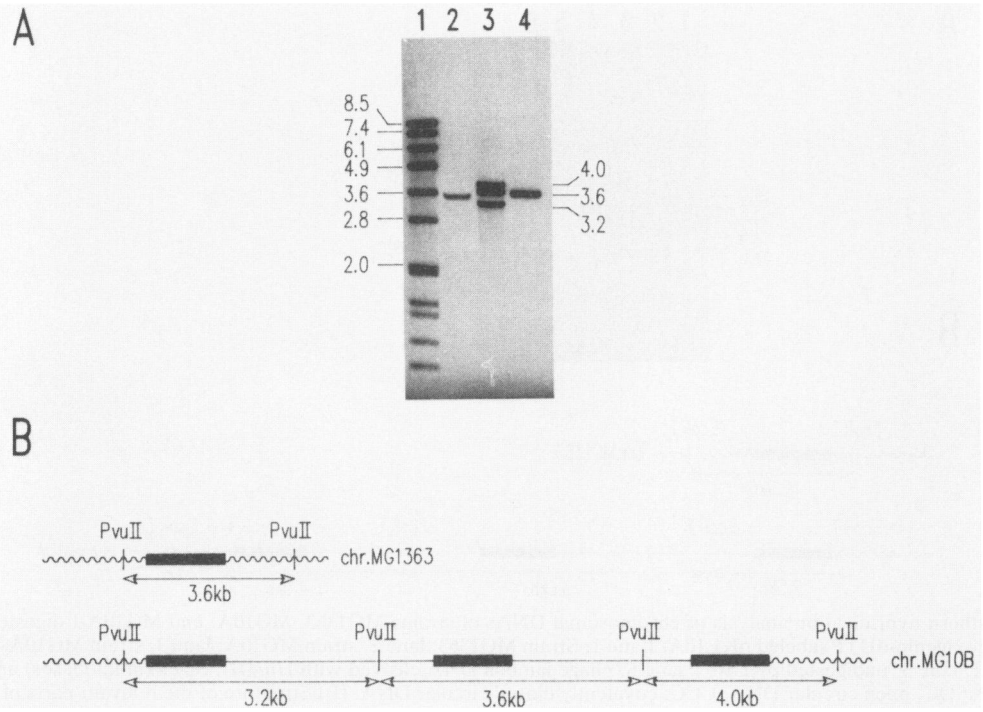

FIG. 3. (A) Southern hybridization analysis of chromosomal DNAs of strains MG1363 and MG10B digested with *Pvu*II and hybridized with digoxigen-dUTP-labeled pKL10B. Lane 1, Phage SPP1 DNA cleaved with *Eco*RI; lane 2, strain MG1363; lane 3, strain MG10B; lane 4, pKL10B cut with *Pvu*II. Sizes (in kilobases) are indicated on the sides. (B) Proposed structure of the relevant parts of the chromosomes of host strain MG1363 and transformant strain MG10B. The solid boxes indicate the locations of chromosomal fragment B.

predicted on the basis of Campbell-like integration, a 3.6-kb fragment was clearly present (lane 4). This fragment could have been either the original 3.6-kb chromosomal *Pvu*II fragment or linear pKL10B if amplification occurred. When vector pKL10 without the chromosomal insertion was used as a probe, the 3.6-kb fragment in Fig. 3A, lane 3, still hybridized, whereas the 3.6-kb fragment in lane 2 did not (data not shown), indicating that the 3.6-kb fragment in lane 3 was linear pKL10B. Therefore, since no free plasmid DNA was detected in strain MG10B, we propose the chromosomal structure for strain MG10B shown in Fig. 3B, in which two tandemly integrated plasmid copies generate, after cleavage with *Pvu*II, fragments having the sizes and intensities of hybridization shown in lane 3. Such a structure may have been generated either by integration of a monomer with subsequent duplication of the plasmid in the chromosome or by integration of a dimer.

The chromosomal structure was very stable; in the stability tests no loss of the Em^r phenotype was detected in the strain MG10B-I culture (Table 2). The chromosomal structure of strain MG10B-I was analyzed and found to be identical to the chromosomal structure of strain MG10B (data not shown).

Chromosomal structure and stability of transformant strain MG301B. Strain MG1363 was transformed by electrotransformation with pSC101-based plasmid pKL301B (transformation frequency 1 to 10 transformants per μg of DNA). Two randomly chosen transformants, designated strain MG301B, were analyzed. Free plasmid DNA was not detected in either of the two transformants. Chromosomal analysis showed that both transformants had the same structure. The results of a Southern hybridization analysis of *Pvu*II-digested chromosomal DNA from strain MG301B are shown in Fig. 4A, lane 3. Labeled pKL301B was used as the probe. Two fragments hybridized. The sizes of these fragments, (6.2 and 7.0 kb) were in agreement with the expectation after Campbell-like integration of one copy of 9.6-kb plasmid pKL301B (lane 6) into the 3.6-kb *Pvu*II fragment of the chromosome of strain MG1363 (lane 2). The relevant part of the chromosome of strain MG1363 is shown schematically in Fig. 4B.

The stability of the integrated plasmid in strain MG301B was examined by growing the organism under nonselective conditions. The strain obtained, strain MG301B-I, did not shown any loss of the Em^r phenotype in the plating assays (Table 2). Southern hybridization analysis revealed no differences between the hybridization patterns of strains MG301B and MG301B-I (Fig. 4, lane 4).

Chromosomal structure and stability of transformant strain MG400B. Transformant strain MG400B was constructed by electrotransformation of pTB19-derived plasmid pKL400B to strain MG1363 (transformation frequencies varied between 1 and 10 transformants per μg of DNA). Two transformants were analyzed. Both lacked free plasmid DNA and had the same chromosomal structure. The chromosomal structure of one of the strains was analyzed by comparing *Pvu*II digests of strain MG1363 and MG400B chromosomal DNAs and pKL400B; the results of a Southern hybridization analysis of these digests, in which labeled pKL400B was used as a probe, are shown in Fig. 5, lanes 2, 3, and 6, respectively. The hybridization pattern of strain MG400B showed fragments with sizes which were in agreement with what was expected after Campbell-like integration of only one copy of pKL400B into homologous fragment B on the chromosome of strain MG1363. A schematic drawing of the

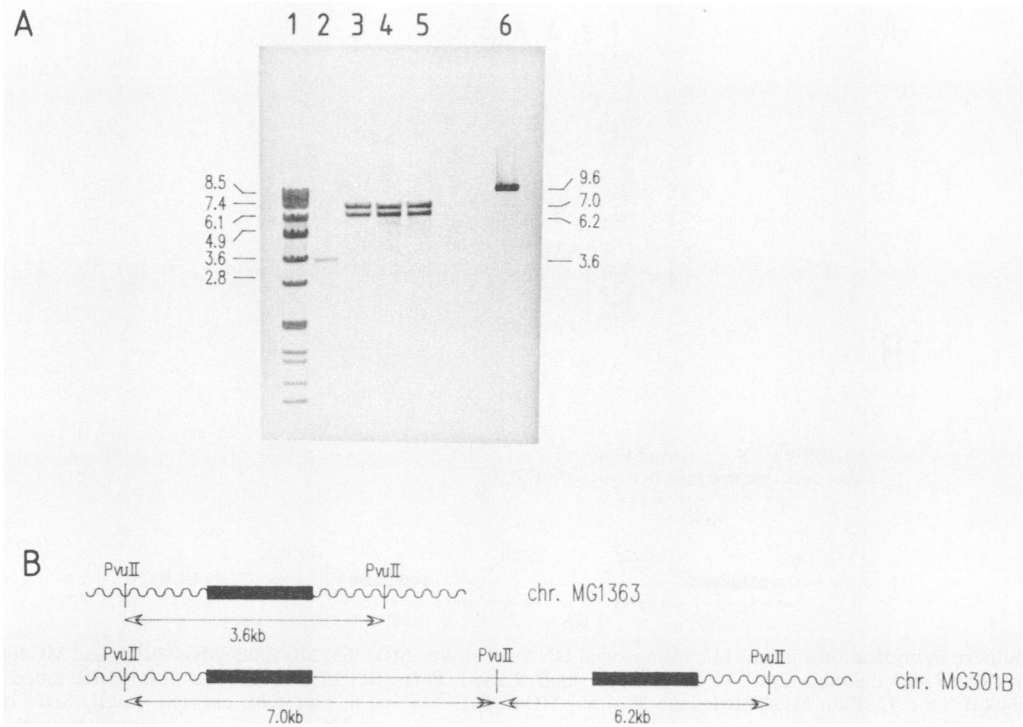

FIG. 4. (A) Southern hybridization analysis of chromosomal DNAs of strains MG1363, MG301B, MG301B-I, and MG301B(E^{100}) cleaved with *Pvu*II and hybridized with digoxigenin-dUTP-labeled pKL301B. Lane 1, Phage SPP1 DNA cleaved with *Eco*RI; lane 2, strain MG1363; lane 3, strain MG301B; lane 4, strain MG301B-I; lane 5, strain MG301B(E^{100}); lane 6, pKL301B cut with *Pvu*II. Sizes (in kilobases) are indicated on the sides. (B) Structure of the relevant parts of the chromosomes of host strain MG1363 and transformant strain MG301B. The solid boxes indicate the locations of chromosomal fragment B.

relevant part of the chromosome of strain MG400B is shown in Fig. 5B.

Strain MG400B was grown in the absence of erythromycin to examine the stability of the integrated plasmid. The stability tests showed that strain MG400B-I was still able to grow in the presence of erythromycin (Table 2). The stable maintenance of the integrated plasmid was confirmed by Southern hybridization analysis (Fig. 5, lane 4).

Attempts to amplify single-copy integrations. Strains MG301B and MG400B harbored only one integrated plasmid copy in their chromosomes. The integrated sequences were potentially amplifiable, since, as a result of Campbell-like integration, the chromosomal structures of these strains contained a duplicated sequence. We attempted to amplify the plasmid sequences by growing strains MG301B and MG400B in the presence of concentrations of erythromycin ranging from 5 to 400 μg/ml. The plating efficiencies of these cultures did not change significantly. Chromosomal DNAs were extracted from strains MG301B and MG400B which had been grown in the presence of an erythromycin concentration of 100 μg/ml (E^{100}). The results of Southern hybridization did not reveal any amplification in either strain (Fig. 4A, lane 5, and Fig. 5A, lane 5). We also attempted to obtain amplification in these strains by using clindamycin as described by Chopin et al. (7). However, as with erythromycin, no difference in plating efficiencies was observed when we used concentrations of clindamycin ranging from 5 to 300 μg/ml, and no amplification was detected in these strains (data not shown).

Chromosomal structures and stabilities of transformant strains MG203A and MG203B. Transformant strains MG203A and MG203B were obtained by electrotransformation of strain MG1363 with pUB110-derived plasmids pKL203A and pKL203B, respectively. Two colonies of both types of transformants were analyzed in Southern hybridization experiments. In Fig. 6A, lane 1, the chromosomal 3.6-kb *Pvu*II fragment from strain MG1363 was hybridized to pKL203B, which was used as a probe. Lane 2 shows the hybridization pattern of a *Pvu*II digest of one of the strain MG203B transformants, and only a weakly hybridizing 3.6-kb band was present, together with a strongly hybridizing band corresponding to a size larger than 10 kb. Figure 6B shows the expected chromosomal structure of strain MG203B after Campbell-like integration of pKL203B and predicts the absence of the 3.6-kb fragment but the presence of a 10.1-kb fragment if single-copy integration in the chromosome of strain MG203B occurred. As pKL203B did not contain *Pvu*II sites, fragments larger than 10.1 kb were expected if amplification of the plasmid occurred. Since such fragments were clearly present in the chromosomal digest of strain MG203B and the intensity of the 3.6-kb chromosomal fragment had clearly decreased, the plasmid integrated in all probability via a Campbell-like mechanism. However, the presence of a weakly hybridizing band at 3.6 kb suggests that strain MG203B consisted of a mixed population in which part of the cells had lost the amplified structure through precise excision. If this is correct, the presence of free plasmid DNA in strain MG203B might be anticipated. Indeed, we detected small amounts of free plasmid DNA in strain MG203B, as well as in the transformants obtained with pKL203A (data not shown).

To test the stability of the integrated plasmids, strains MG203A and MG203B were grown under nonselective conditions for 100 generations. The cultures obtained, strains

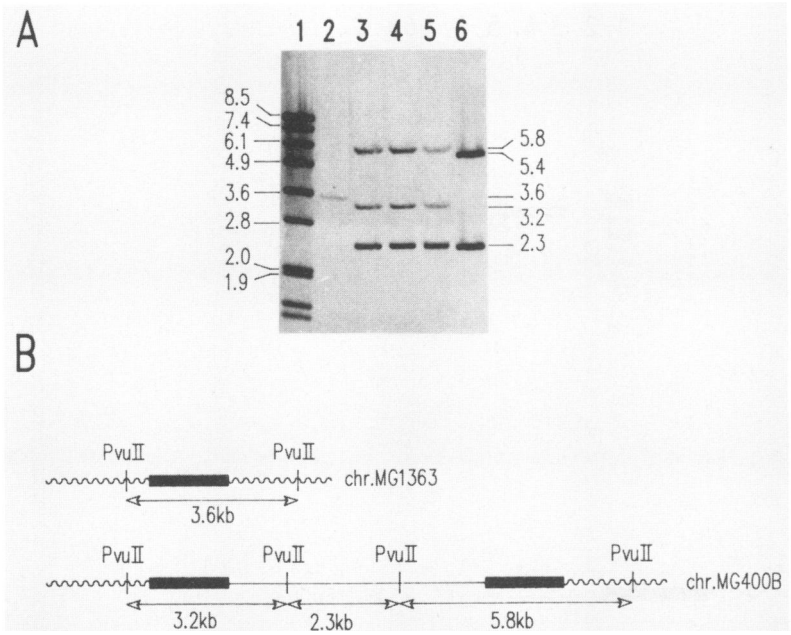

FIG. 5. (A) Southern hybridization analysis of chromosomal DNAs of strains MG1363, MG400B, MG400B-I, and MG400B(E^{100}) cleaved with *Pvu*II and hybridized with digoxigenin-dUTP-labeled pKL400B. Lane 1, Phage SPP1 DNA digested with *Eco*RI; lane 2, strain MG1363; lane 3, strain MG400B; lane 4, strain MG400B-I; lane 5, strain MG400B(E^{100}); lane 6, pKL400B cut with *Pvu*II. Sizes (in kilobases) are indicated on the sides. (B) Structure of the relevant parts of the chromosomes of strain MG1363 and transformant strain MG400B. The solid boxes indicate the locations of chromosomal fragment B.

MG203A-I and MG203B-I, showed a considerable reduction in the number of Em^r colony-forming units. Only 25 and 13% of the cells of strain MG203A-I and MG203B-I cultures had retained their Em^r phenotype, respectively (Table 2). Chromosomal analysis of strain MG203B-I showed that there was a decrease in the intensity of fragments larger than 10.1 kb and the presence of a 10.1-kb fragment (Fig. 6, lane 3), indicating that some of the cells contained only one integrated plasmid copy (Fig. 6B). The concomitant increase in the intensity of the 3.6-kb band (compared with strain MG203B) was compatible with the assumption that loss of integrated plasmid copies occurred via a mechanism of

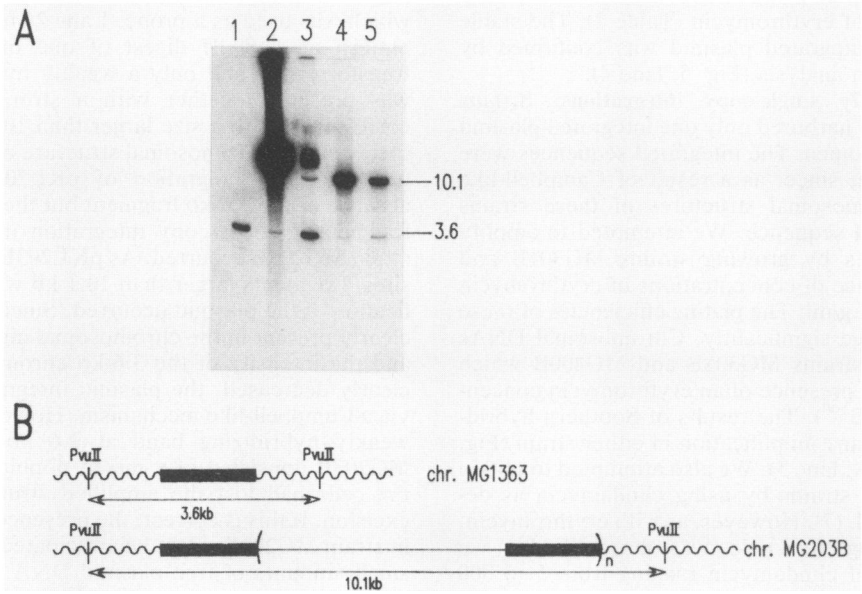

FIG. 6. (A) Southern hybridization analysis of chromosomal DNAs of strains MG1363, MG203B, MG203B-I, MG203B-Ia, and MG203B-IIa cleaved with *Pvu*II and hybridized with ^{32}P-labeled pKL203B. Lane 1, Strain MG1363; lane 2, strain MG203B; lane 3, strain MG203B-I; lane 4, strain MG203B-Ia; lane 5, strain MG203B-IIa. Sizes (in kilobases) are indicated on the right. (B) Structure of the relevant parts of the chromosomes of host strain MG1363 and transformant strain MG203B. The solid boxes indicate the locations of chromosomal fragment B.

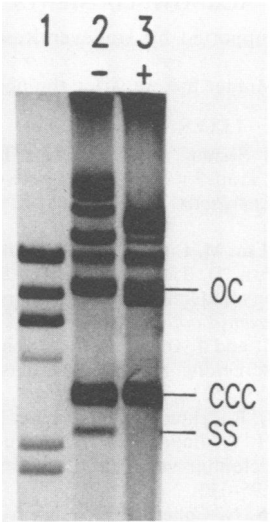

FIG. 7. Southern hybridization analysis of a plasmid preparation obtained from 5 ml of a strain MG200 culture that was treated (lane 3) or not treated (lane 2) with endonuclease S1. Digoxigenin-dUTP-labeled pKL203B was used as a probe. Lane 1, Phage lambda DNA cleaved with *Hin*dIII; lanes 2 and 3, strain MG200. OC, Open circular DNA; CCC, covalently closed circular DNA; SS, single-stranded DNA.

precise excision, resulting in Em^s cells. The strain MG203B-I culture was also a mixed culture, as shown by the isolation and characterization of randomly picked colonies. One of these colonies, designated colony MG203B-Ia, contained only one integrated plasmid copy, as deduced from an analysis of its chromosomal DNA (Fig. 6A, lane 4). Even the presence of only one copy of pKL203B in the chromosome appeared to be unstable, because when colony MG203B-Ia was grown under nonselective conditions for another 100 generations, the intensity of the 10.1-kb band had further decreased in the culture designated MG203B-IIa (lane 5). A concomitant increase in the intensity of the 3.6-kb band was observed, which, as described above, suggests that there was loss of integrated plasmids through precise excision. Similar results were obtained with the strain MG203A transformants (data not shown).

To address the question of whether the instability of integrated pUB110-derived replicons might have been related to possible replicative activity of this replicon in the lactococcal host, pKL200 was constructed. Plasmid pKL200 was essentially the same as pUB110 except that it contained an erythromycin resistance gene instead of a kanamycin resistance gene. Plasmid pKL200 was used to transform strain MG1363 and yielded only approximately 100 Em^r transformants per μg of DNA, a frequency which was 10^3 to 10^4 times less than the frequencies obtained with a plasmid such as pGK12 (19), an autonomously replicating lactococcal plasmid used in these experiments as a control. This result suggests that pUB110 is not capable of maintaining itself in the lactococcal host as a fully functional replicon. This is in agreement with the observation that only very small quantities of free plasmid DNA were present in minipreparations of strain MG200. In fact, DNA extracts of 5 ml of culture were required to visualize plasmid pKL200 on an agarose gel. As Fig. 7 shows, the replicon produced single-stranded DNA in the lactococcal host, which was indicative of replicative activity, because pUB110 replicates via single-stranded intermediates (12).

DISCUSSION

We reported previously that Campbell-like integration into the chromosome of *L. lactis* of a nonreplicative plasmid (pBR322) carrying a chromosomal insertion is possible and results in the duplication of the homologous region provided. Although amplification was also observed, this appeared to be unstable under nonselective conditions (i.e., in the absence of chloramphenicol), ultimately resulting in the stable maintenance of only one integrated plasmid copy (20). In this study we extended the range of replicons that are potentially useful for integration to pUB110, pSC101, and pTB19 derivatives. The pBR322 replicon was included in this study, and in contrast to the previous experiments in which chloramphenicol was used as selective marker, in this study the clones were selected on the basis of resistance to erythromycin. All replicons were integrated into the same site on the lactococcal chromosome via a Campbell type of recombination. Two replicons (a pBR322 derivative and a pUB110 derivative) were also integrated at another chromosomal location by using another chromosomal insertion. The stabilities of the integrated plasmids at the two different chromosomal locations (locations A and B) did not differ significantly.

The stability of the integrated sequences was not influenced by the size of the plasmid used. Strains MG10A, MG10B, MG301B, and MG400B were obtained with plasmids which varied in size from 3.6 to 9.6 kb. No loss of the erythromycin marker was detected in these strains after growth under nonselective conditions. In contrast, strains MG203A and MG203B, which were obtained with 6.5-kb pUB110-derived plasmids, were unstable. The integrated and amplified plasmid copies were apparently lost under nonselective growth conditions via a mechanism of precise excision. We found that free plasmid DNA was present in strains MG203A and MG203B and that pUB110-derived plasmid pKL200 was capable of producing single-stranded replication intermediates in *L. lactis*. It is doubtful whether the lactococcal host is capable of efficiently converting these single-stranded intermediates to fully autonomous replicons, as the frequency of transformation of *L. lactis* when the pUB110 derivative was used was very low compared with the frequency of transformation when we used a functional lactococcal replicon, such as pGK12, which also multiplies according to the rolling circle mode of replication, thus generating single-stranded intermediates (J. Seegers, personal communication). It is even questionable whether the single-stranded intermediates observed in pKL200-mediated transformants were produced from free plasmid DNA, as the plasmid was predominantly present in an integrated form, presumably as the result of illegitimate recombination (unpublished data). It the view that the single-stranded intermediates are formed from integrated plasmid DNA is correct, then the double-stranded pKL200 molecules shown in Fig. 7 represent precisely excised plasmid DNA rather than single-stranded intermediates converted into pUB110 replicons. Replicative activity of integrated plasmids has previously been invoked as a cause of unstable plasmid amplification in the chromosome of *B. subtilis* (24, 30). By analogy, we believe that the instability of the integrated amplified plasmids in *L. lactis* MG203A and MG203B results from replicative activity of the integrated plasmids.

The stable chromosomal structure of the strains obtained with the pBR322 replicon (strains MG10A and MG10B) is interesting for a number of reasons. (i) Hybridization analysis showed that the chromosomes of these strains carried a

tandem arrangement of three to five plasmid copies (strain MG10A) or two plasmid copies (strain MG10B). We believe that it is unlikely that these multiple copies were generated by amplification after the integration of a monomeric plasmid, because no amplification was observed in strains MG301B and MG400B, either by high levels of erythromycin or by clindamycin, showing that a single copy of the erythromycin gene is sufficient to confer resistance to high levels of erythromycin in *L. lactis*. Instead, the tandemly arranged integrated plasmids in strains MG10A and MG10B probably resulted from integration of plasmid dimers or oligomers. It is known that pBR322 derivatives can produce considerable amounts of multimers (2), and, indeed, we observed that the plasmid preparation of pKL10A contained substantial amounts of multimers. The failure to obtain amplification with clindamycin is at variance with the observations of Chopin et al. (7) and may be attributed to differences in the strains used, since we did not observe a decrease in plating efficiencies when we grew the strain MG1363-derived strains in the presence of elevated levels of clindamycin as has been described previously for strain IL1403-derived strains. (ii) The high level of stability of strains MG10A and MG10B seems to contradict the result of our previous findings for strain MG404 (20). This strain, which carried multiple copies of pBR322 derivative pHV60 with chromosomal fragment A, lost integrated sequences under nonselective conditions. pBR322- and pC194-derived fusion plasmids similar to pHV60 have been used to construct duplicated sequences in the chromosome of *B. subtilis*, which were shown to give rise to instability as a result of residual replicative activity of the pC194 moiety of such plasmids (30). The loss of amplified integrated plasmid copies in strain MG404 may well relate to residual replicative activity on the pC194 part of pHV60. Because this part of pC194 is not present on pKL10A and pKL10B, tandemly integrated copies of these plasmids could be stably maintained. However, the specific pC194 part is present on pKL301B and, therefore, also on the chromosome of strain MG301B. In this strain, one fully stable integrated plasmid copy was present. This is in agreement with the observed stability of strain MG404-Ia, which also carried only one integrated copy of the pHV60 derivative (20). Apparently, the residual replicative activity of the pC194 part is compatible with the stable maintenance of just one integrated plasmid copy.

Loss of amplified integrated plasmid copies has also been described by Chopin et al. (7) for strain IL1747. This strain was obtained by Campbell-like integration and subsequent amplification of pE194 in a prophage of strain IL1403. The instability of amplification in this strain may also have been related to the nature of the plasmid used, since it has been reported that pE194 has replicative activity in *L. lactis* (1).

The general outcome of this work is that Campbell-like integration of plasmids which do not contain plasmid replication functions that are active in *L. lactis* can generate stable tandemly arranged multiple plasmid copies in the chromosome of this host. Plasmids pUB110, pE194, and pC194, all of which originated from *Staphylococcus aureus*, seem to have at least residual replicative activity in *L. lactis* and are therefore less suitable for construction of lactococcal strains in which multiple copies of plasmids are to be stably maintained in the chromosome. In contrast, when we used pBR322-derived replicons and selection for erythromycin resistance, stable multicopy integrations were obtained, whereas pSC101- and pTB19-mediated transformation resulted in stable single-copy integrations.

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