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# Campbell-Like Integration of Heterologous Plasmid DNA into the Chromosome of *Lactococcus lactis* subsp. *lactis*

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**Integrable vectors were constructed based on the plasmid pHV60, which is essentially a pBR322 replicon carrying a chloramphenicol resistance marker, by inserting 1.3-kilobase chromosomal fragments of *Lactococcus lactis* subsp. *lactis* MG1363 into this plasmid. Three constructs as well as pHV60 were electroporated to strain MG1363. Transformants were obtained with all constructs, and also with pHV60 (albeit with low frequency). By using Southern hybridizations, it appeared that pHV60 showed homology with the chromosome of MG1363, and that it most probably uses this homology to integrate in a Campbell-like manner. The presence of chromosomal sequences in pHV60 stimulated insertion elsewhere in the chromosome by a factor of 5 to 100. In all cases the integrated plasmids were amplified, at a selective pressure of 5 μg of chloramphenicol per ml, to a level of approximately 15 copies per chromosome. Although the amplification was gradually lost under nonselective conditions, one copy remained stably integrated in the chromosome. The results show that a Campbell-like integration strategy can be used to improve the accessibility of the lactococcal chromosome for genetic analysis and is potentially useful in stabilizing unstable genes in lactococci.**

The development of procedures for DNA-mediated transformation and the construction of plasmid cloning vectors for lactococci (formerly designated lactic streptococci [23]) has greatly accelerated our knowledge of plasmid genetics of these organisms (6, 17). In contrast, our knowledge of the chromosome of these organisms is very slight, because the genetic tools for such investigations are still limited. Recently, it was reported that the related transposons Tn*916* and Tn*919* can be used to inactivate chromosomally located genes (12, 22) and that a prophage harbored by the chromosome of *Lactococcus lactis* subsp. *lactis* IL1403 was inactivated by inserting heterologous DNA (M. C. Chopin, personal communication).

In other organisms such as *Saccharomyces cerevisiae* and *Bacillus subtilis*, integration of plasmid DNA has been successfully used to elucidate at least parts of their chromosomes, by generating mutations (21, 24, 25), by mapping unselectable genes (10, 27, 29), and by cloning genes (21, 28). By analogy, the development of a useful integration strategy based on the use of plasmids could provide an important tool for exploring the lactococcal chromosome. A further advantage of the availability of such a system would be the possibility it offers to introduce and stabilize heterologous as well as homologous genes such as the proteinase, lactose, and citrate permease genes, which are essential for dairy practice. These plasmid-located genes are often found to be unstable through segregational loss of the plasmid (5, 19).

For several organisms it has been shown that a plasmid that is unable to replicate in the recipient strain can integrate efficiently into the host chromosome when it carries chromosomal sequences. The region of homology provided usually stimulates the integration of the plasmid DNA by a Campbell-like mechanism, which leads to duplication of the homologous chromosomal insert as a consequence of this mode of integration. This, in turn, may create a substrate for amplification (31, 32).

To improve the accessibility of the lactotococcal chromosome, we constructed a number of integrable vectors based on the plasmid pHV60; this is essentially a pBR322 replicon carrying a chloramphenicol resistance marker (20), which is expressed in gram-positive bacteria. Replication of this plasmid in gram-positive bacteria has not been described. We report here the integration of these vectors in the chromosome of *L. lactis* subsp. *lactis* by a Campbell-like mechanism, the amplification of the integrated sequences, and the stability of the clones obtained.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** All strains and plasmids used in this study are listed in Table 1. The bacterial strains were grown as described previously (16). Plasmid pHV60 ($Ap^r$ $Tc^r$ $Cm^r$), a 5.5-kilobase (kb) *Escherichia coli* plasmid, carries the $Cm^r$ gene of pC194 (20). The unique *Bam*HI site in the $Tc^r$ gene of pHV60 was used for the cloning of 1- to 1.5-kb chromosomal *Sau*3A fragments of strain MG1363.

**Enzymes.** All DNA modification enzymes were obtained from Boehringer GmbH, Mannheim, Federal Republic of Germany, or New England BioLabs, Inc., Beverly, Mass., and used as specified by the suppliers.

**Transformation and electroporation.** *E. coli* was transformed as described by Mandel and Higa (18). Electroporation of *L. lactis* subsp. *lactis* was performed with a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) essentially as described by Van der Lelie et al. (26) with the following modification: after being resuspended in 0.8 ml of electroporation buffer and mixed with DNA, the cells were exposed to one pulse of 6,250 V/cm, capacitance (C) = 25 μF. The cells were plated on SM17-glucose (glc) agar plates containing 5 μg of chloramphenicol per ml.

**Isolation of DNA.** Plasmid DNA of *E. coli* was prepared by CsCl-ethidium bromide centrifugation (13) or by the method of Birnboim and Doly (3). For small-scale isolations of plasmid DNA (5-ml cultures of *L. lactis*) the method of Birnboim and Doly was modified by adding 10 μl of mutanolysin (1,500 U/ml) and lysozyme (5 mg/ml) to the lysis solution. Total DNA of *L. lactis* was extracted as follows. An overnight culture was diluted 100-fold in 500 ml of

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TABLE 1. Plasmids and strains used

| Plasmid or strain | Characteristics | Reference or source |
|---|---|---|
| pGK12 | ori pWV01; $Cm^r$ $Em^r$ | 15 |
| pHV60 | ori pBR322; $Cm^r$ $Ap^r$ $Tc^r$ | 20 |
| pG1401 | pHV60 with 1.3-kb chromosomal insert | This work |
| pG1404 | pHV60 with 1.3-kb chromosomal insert | This work |
| pG1406 | pHV60 with 1.3-kb chromosomal insert | This work |
| *E. coli* JM101 | *supE thi* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*$^q$ZΔM15] | 30 |
| *L. lactis* subsp. *lactis* | | |
| MG1363 | $Cm^s$, plasmid free | 9 |
| MG60 | $Cm^r$, MG1363 carrying amplified pHV60 in the chromosome | This work |
| MG401 | $Cm^r$, MG1363 carrying amplified pGI401 in the chromosome | This work |
| MG404 | $Cm^r$, MG1363 carrying amplified pGI404 in the chromosome | This work |
| MG404-I | $Cm^{r/s}$, mixed culture obtained after nonselective growth of MG404[a] | This work |
| MG404-Ia | $Cm^{r/s}$, MG1363 carrying one copy of pGI404 in the chromosome[a] | This work |
| MG406 | $Cm^r$, MG1363 carrying amplified pGI406 in the chromosome | This work |

[a] $Cm^{r/s}$, Mixture of chloramphenicol-resistant and -sensitive CFU.

M17-glc medium supplemented with 20 ml of 1 M DL-threonine. The culture was grown at 30°C until an optical density at 660 nm of 0.5 was reached. The culture was centrifuged, washed once with 20 ml of TE (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA), and suspended in 5 ml of lysis solution (25 mM Tris hydrochloride [pH 8], 50 mM EDTA, 50 mM glucose). After 5 mg of lysozyme per ml and 750 U of mutanolysin were added, the suspension was incubated for 30 min at 37°C; 1 ml of 10% sodium dodecyl sulfate followed by 50 μl of proteinase K (20 mg/ml) were added. Incubation was carried out at 60°C until the suspension was clear. The lysate was extracted five times with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and twice with an equal volume of chloroform-isoamyl alcohol (24:1). Sodium acetate (3 M) was added (1/10 volume), and the DNA was precipitated with 2 volumes of 96% ethanol (−20°C). The isolate was dissolved in 5 ml of TE supplemented with 20 μg of RNase per ml. This mixture was incubated for 30 min at 37°C and subsequently extracted once more with 1 volume of chloroform-isoamyl alcohol. DNA was again precipitated as described above and dissolved in 3 ml of TE. The concentration was determined spectrophotometrically (at 260 nm).

**Southern hybridization.** Alkaline transfer of DNA on 0.8% agarose gels to GeneScreen Plus membranes was done as described in a protocol of Chomczynski and Qasba (4). Appropriate probes were labeled with [α-$^{32}$P]dCTP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) by using the Random Primed Labeling kit of Boehringer GmbH. The filters were hybridized and washed as instructed by the suppliers of GeneScreen Plus (Du Pont, NEN Research Products). Fuji RX or Kodak XAR-5 films were used for autoradiography.

**DNA amplification.** Total DNAs from strain MG1363 and from the transformants grown on M17-glc with 5 μg of chloramphenicol per ml were digested with *Pvu*II. Starting with 2.5 μg of DNA for all strains, decreasing amounts of DNA from the transformants were applied to a 0.8% agarose gel. After electrophoresis, blotting, and hybridization with the appropriate probes, the intensity of the amplified band was compared with that of the DNA of the parental strain MG1363. The copy number of the amplifiable unit (AU) was estimated densitometrically (Microdensitometer MK IIIc; Joyce, Loebl and Co., Ltd, Gateshead-on-Tyne, England) by assuming that the homologous chromosomal region in strain MG1363 occurred only once per chromosome. Furthermore, corrections were made for the differences in the sizes of the homologous regions between the probe and the hybridizing bands in the chromosomal DNA of strains MG1363, MG401, MG404, and MG406. The copy number of pHV60 in strain M660 was determined by comparing the intensities of hybridizations of dilutions of its chromosomal DNA with those of the DNAs from strains MG401, MG404, and MG406.

**Stability of transformants.** To examine the stability of the $Cm^r$ phenotype of the transformants, overnight cultures of strains MG1363, MG1363(pGK12), MG60, and MG404 grown in M17-glc with 5 μg of chloramphenicol per ml were diluted in fresh M17-glc broth and grown for 100 generations under nonselective conditions. After 100 generations, dilutions were spread on selective (5 μg of chloramphenicol ml) and nonselective M17-glc plates to determine the percentage of $Cm^r$ cells in the cultures. In addition, 50 colonies of each strain growing on the nonselective plates were transferred with toothpicks onto selective and nonselective plates.

## RESULTS

**Analysis of a transformant obtained with pHV60.** Several independent electroporation experiments were carried out to establish whether the *E. coli* plasmid pHV60 was able to replicate in *L. lactis* subsp. *lactis* MG1363. Unexpectedly, we obtained $Cm^r$ transformants at low frequencies (Table 2). One such transformant, MG60, was used for further analysis. Total DNA as well as plasmid DNA (miniprep procedure) was isolated from strains MG1363, MG60, and MG1363(pGK12). The DNAs obtained were used in a Southern hybridization experiment with pHV60 as a probe. In addition, the total DNAs of strains MG60 and MG1363 were digested with *Eco*RI and *Pvu*II, which have unique restriction sites on pHV60 (Fig. 1A). The results are presented in

TABLE 2. Electroporation of strain MG1363 with pHV60 and derivatives

| Plasmid | Insert size (kb) | No. of $Cm^r$ colonies/μg of DNA[a] |
|---|---|---|
| pHV60 | 0 | 1 |
| pGI401 | 1.3 | 4 |
| pGI404 | 1.3 | 127 |
| pGI406 | 1.3 | 5 |

[a] The values are the means of three independent electroporations.

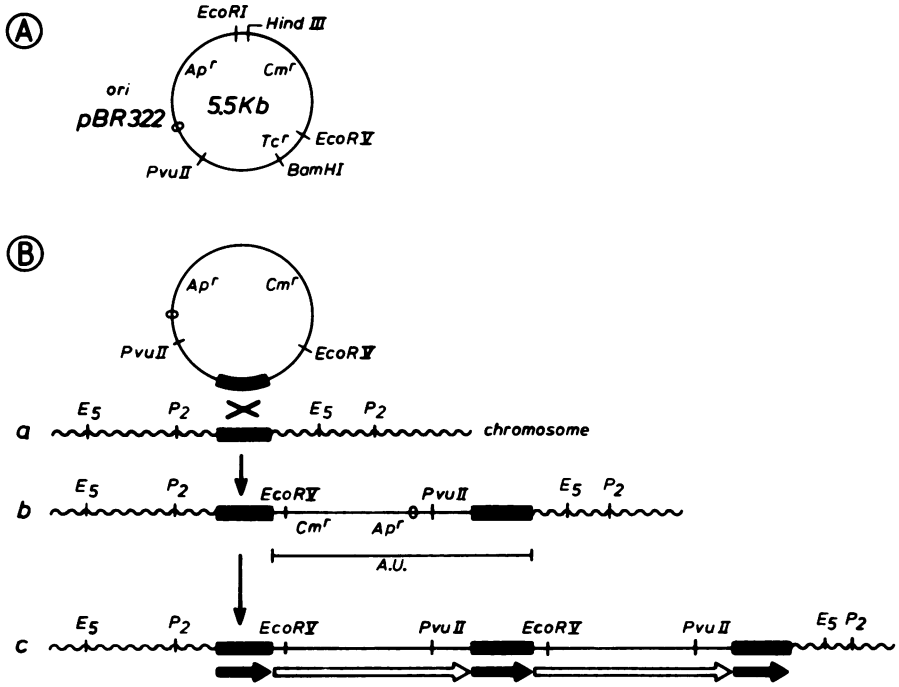


FIG. 1. Schematic representation of plasmid pHV60 (A) and integration of this plasmid or its derivatives into the chromosome of strain MG1363 via a Campbell-like mechanism (B). Symbols: ▬, either the unidentified region of homology of pHV60 with the chromosome or cloned chromosomal DNA; $E_5$ and $P_2$, unknown positions of restriction sites for *Eco*RV and *Pvu*II on the chromosome, respectively; a, host chromosome; b, chromosome after the integration event; c, chromosome after amplification.

Fig. 2 and show that in the total DNA of strain MG60 a hybridization signal comigrated with the undigested chromosomal DNA (Fig. 2, lanes 2). No bands were present which comigrated with pHV60 (compare with lanes 1). This observation suggests that pHV60 was not present as a free plasmid in the transformant but, instead, had integrated into the chromosome. The *Eco*RI and *Pvu*II digests of MG60 DNA (lanes 3 and 4) showed one intense signal corresponding to the linear form of pHV60 (lanes 5) and two additional bands. These two additional bands may represent restriction frag-

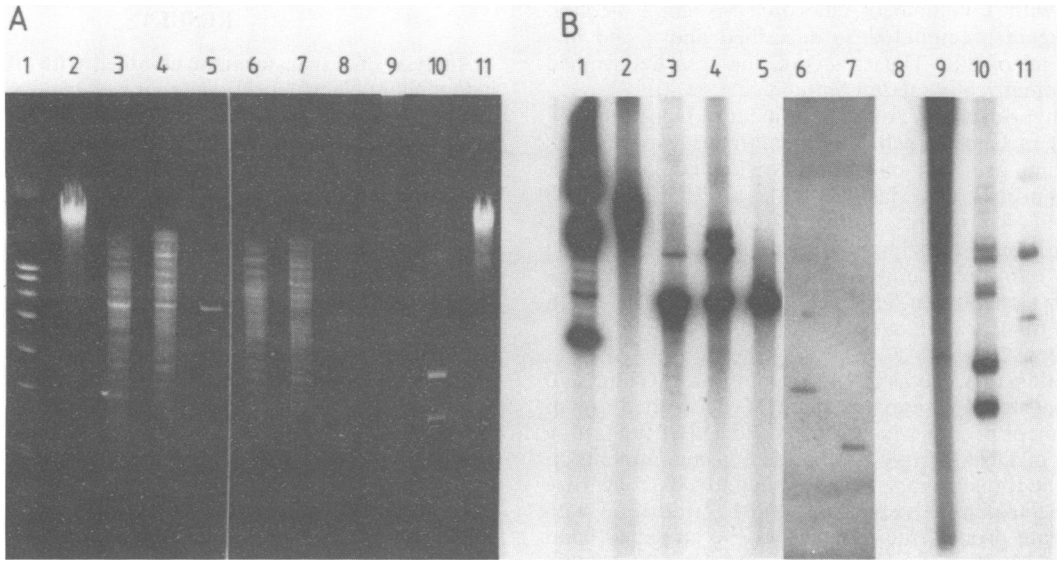


FIG. 2. Southern hybridization of DNAs of strains MG1363, MG60, and MG1363(pGK12). (A) DNA samples on a 0.8% agarose gel. Lanes: 1, pHV60 and marker DNA (phage SPP1 DNA digested with *Eco*RI); 2 to 4, total DNA of strain MG60, undigested, cleaved with *Eco*RI, and cleaved with *Pvu*II, respectively; 5, pHV60 digested with *Pvu*II; 6 and 7, total DNA of strain MG1363 cleaved with *Eco*RI and *Pvu*II, respectively; 8 to 10, small-scale plasmid isolation of strains MG1363, MG60, and MG1363(pGK12), respectively (the equivalent of 2.5 ml of culture was used); 11, total DNA of strain MG1363(pGK12). (B) Autoradiogram of a Southern blot of the gel shown in panel A, hybridized with $^{32}$P-labeled pHV60 (lanes 6 and 7 were subjected to an extended exposure time).

ments in which both plasmid sequences and chromosomal sequences flanking the integrated DNA were present. This would be consistent with the integration of pHV60 into the chromosome by a Campbell-like mechanism. Subsequent amplification has to be postulated to explain the hybridization signal at the position of linear pHV60. If this interpretation is correct, one would expect homology between pHV60 and the chromosome of strain MG1363. Lanes 6 and 7 show that this is indeed the case: a clear signal was present when the chromosome of strain MG1363 was probed with pHV60 under stringent conditions of hybridization and washing. Apparently, this homology provides a substrate for integration of pHV60. Interestingly, in small-scale plasmid isolations from strain MG60, traces of plasmid DNA were observed (lanes 9; the equivalent of 2.5 ml of culture). The smear of hybridization in Fig. 2B, lane 9, is most probably caused by a background of degraded chromosomal DNA. For comparison, a Southern hybridization was carried out on a pGK12 minipreparation from strain MG1363(pGK12). Plasmid pGK12 is partly homologous with pHV60 because they share the $Cm^r$ gene (15, 20). The results (Fig. 2, lanes 10) show that the intensity of the signal in lane 10 is much stronger than in lane 9, although the segment of pGK12 which is homologous with pHV60 is small. The copy number of pWV01-based vectors in *L. lactis* subsp. *lactis*, including pGK12, is about 4 (15). The intensity of the signals in lanes 2, 3, and 4 is too strong to be accounted for by assuming that pHV60 is able to replicate in strain MG1363. Moreover, a replicating low-copy-number plasmid such as pGK12 can also be visualised by Southern hybridization in total DNA extracts (lanes 11), which is obviously not the case in transformants obtained with pHV60 (lanes 2). Although most of the plasmid DNA isolated from MG1363(pGK12) was present in the multimeric form, open- and closed-circular monomers were present. Taking all these observations together, this strongly suggests that pHV60 cannot replicate in strain MG1363, but integrates via a region of homology into the chromosome in a Campbell-like manner and is then amplified. The very small amount of free plasmid DNA might be generated by recombination in the direct repeats flanking the integrated plasmid DNA or by recombination in the directly repeated amplified plasmid sequences (Fig. 1B).

**Analysis of transformants obtained with pHV60 carrying chromosomal inserts.** Sized chromosomal *Sau*3A fragments (1 to 1.5 kb) of strain MG1363 were inserted into the *Bam*HI site of pHV60, and the ligation mixture was used to transform *E. coli* JM101. Three recombinant plasmids, designated pGI401, pGI404, and pGI406, each carrying a different insert of approximately 1.3 kb, were chosen for use in electroporations of strain MG1363. The results are listed in Table 2. Electroporations with pGI401 and pGI406 showed only a slight increase in the number of transformants over that with pHV60. However, with pGI404, the number of transformants increased more than 100-fold. One transformant obtained with each of the plasmids pGI401, pGI404, and pGI406 (designated MG401, MG404, and MG406, respectively) was analyzed in Southern hybridizations. Total DNA was extracted from each transformant and from strain MG1363 and digested with several restriction enzymes for which a unique site was present on the vector part of the plasmid but not on the chromosomal DNA insert. In a Southern hybridization of chromosomal DNA obtained from the nontransformed host strain MG1363, probed with the plasmids carrying the chromosomal inserts, the corresponding DNA fragments on the recipient chromosome were

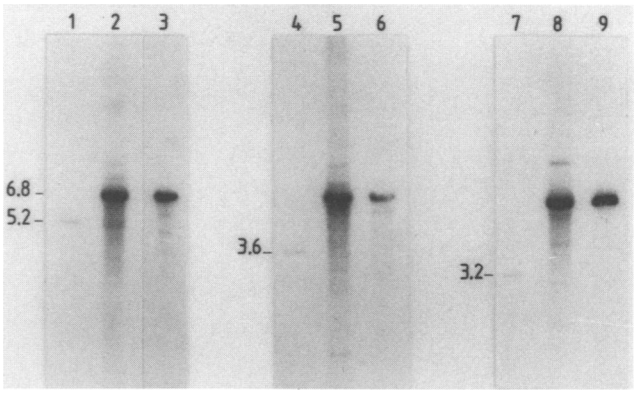


FIG. 3. Southern hybridization with $^{32}P$-labeled pGI401 (lanes 1 to 3), pGI404 (lanes 4 to 6), and pGI406 (lanes 7 to 9) as the probes. Lanes: 1, 4, and 7, total DNAs of strain MG1363 (2.5 μg); 2, 5, and 8, total DNAs of strains MG401, MG404, and MG406 (1 μg), respectively; 3, 6, and 9, pGI401, pGI404, and pGI406, respectively. DNAs in lanes 1 to 3 and 7 to 9 were digested with *Pvu*II; DNAs in lanes 4 to 6 were digested with *Eco*RV. Indicated in the margin are the sizes (in kilobases) of the various DNA bands mentioned in the text.

readily identified (Fig. 3, lanes 1, 4, and 7). After a Campbell-type integration, these hybridizing bands in the digests of MG1363 total DNA should be absent in the digests of the total DNAs of the transformants (for explanation, see Fig. 1B). To verify this prediction, total DNA of the three transformants obtained were analyzed by Southern hybridizations. In the experiment presented in Fig. 3, 2.5-fold less DNA of the transformed strains was used than DNA of strain MG1363 to prevent the strong radiation of the amplified plasmid band masking the single-copy band (the target for the integrating plasmid) in the same lane. Figure 3, lanes 1 to 3, shows *Pvu*II digests of total DNA of the strains MG1363 and MG401 and of plasmid pGI401. $^{32}P$-labeled pGI401 was used as a probe. The hybridizing *Pvu*II fragment (lane 1) of approximately 5.2 kb was still present in the transformant MG401 (lane 2). This observation indicated either that the plasmid had not integrated or that integration had occurred at a location not determined by the homologous insert of the plasmid.

Southern hybridization of *Eco*RV-digested total DNA extracted from strains MG1363 and MG404, with pGI404 as a probe, is presented in Fig. 3, lanes 4 and 5, respectively. A 3.6-kb fragment in the host DNA (lane 4) has disappeared in the DNA of the transformant (lane 5). This result indicates that pGI404 had integrated into this *Eco*RV fragment via the homology provided by the cloned chromosomal DNA. Since an intense signal in lane 5 was located at the position of *Eco*RV-digested linear pGI404 (compare with lane 6), the integrated plasmid DNA was apparently amplified in the transformant.

Data supporting Campbell-like integration, followed by amplification of the integrated plasmid DNA, were also obtained from a transformant produced by pGI406. *Pvu*II digestion of total DNA extracts of strains MG1363 and MG406 and probing with pGI406 showed that the 3.2-kb band hybridizing in the MG1363 host DNA (Fig. 3, lane 7) had disappeared in the DNA of transformant MG406 (lane 8). The most intense signal in lane 8 corresponded to linear pGI406 DNA (lane 9). Because the original hybridizing fragment in the chromosomal DNA of strain MG1363 was absent in the transformant DNA, this result indicates that

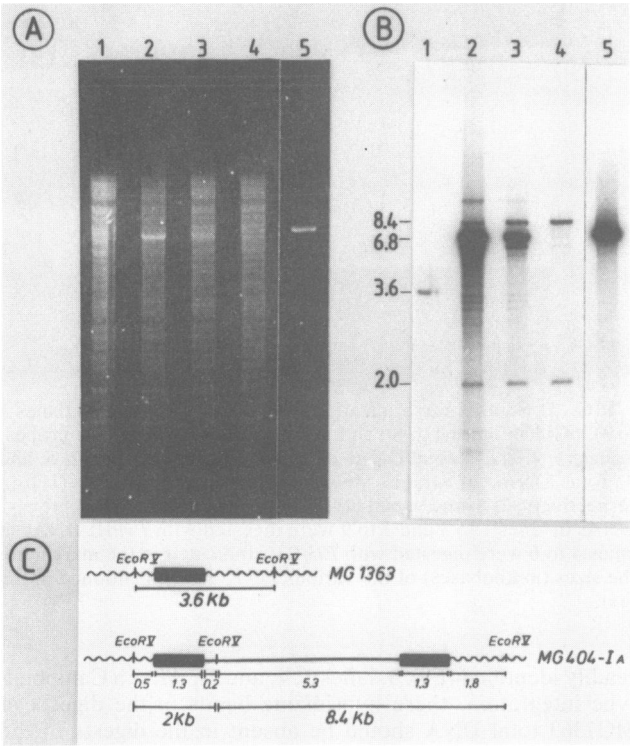


FIG. 4. Southern hybridization of total DNA extracted from strains MG1363, MG404, MG404-I, and MG404-Ia digested with *Eco*RV. (A) DNA samples on a 0.8% agarose gel. Lanes: 1, MG1363 (2.5 μg); 2, MG404 (1 μg); 3, MG404-I (1 μg); 4, MG404-Ia (1 μg); 5, pGI404. (B) Autoradiogram of a Southern blot of the gel shown in panel A, hybridized with $^{32}$P-labeled pGI404. (C) Structure of the relevant part of the chromosomes of host MG1363 and of the transformant MG404-Ia. Symbol: ▬, cloned chromosomal *Sau*3A fragment.

integration had taken place via the chromosomal insert present in pGI406.

**Extent of amplification.** In all transformed strains examined, amplification of the integrated DNA was detected in cells grown in the presence of 5 μg of chloramphenicol per ml. Amplification was visible directly on ethidium bromide-stained agarose gels (Fig. 2 and 4). The extent of amplification was determined by Southern hybridization experiments as described in Materials and Methods; values of approximately 15 copies of the AU in each strain were found, assuming that the homologous chromosomal region occurred only once per chromosome (data not shown).

**Stability of the Cm$^r$ phenotype of strains MG60 and MG404.** The percentage of chloramphenicol-resistant cells in cultures of strains MG1363, MG1363(pGK12), MG60, and MG404 after growth for more than 100 generations under nonselective conditions was determined as described in Materials and Methods. The results are listed in Table 3. The number of Cm$^r$ CFU as determined by direct plating of the control strains MG1363 and MG1363(pGK12) was in agreement with that obtained after transfer with toothpicks. In contrast, a clear discrepancy existed between the two methods when the transformant strains MG60 and MG404 were examined (Table 3). Induction of the Cm$^r$ gene prior to plating of the cells, either overnight with 0.1 μg of chloramphenicol per ml or for 1 h with 0.5 μg of chloramphenicol per ml, did not significantly change these results (data not shown). These observations indicate that the discrepancy between direct plating and transfer with toothpicks was not related to possible inducibility of the chloramphenicol gene. To further analyze this discrepancy, two colonies (MG404-Ia and MG404-Ib) of strain MG404, which had grown for 100 generations in the absence of selective pressure (designated as MG404-I), were randomly chosen from nonselective plates and grown overnight in liquid medium under nonselective conditions. After plating, strain MG404-Ia showed a substantially lower percentage of Cm$^r$ CFU than strain MG404-I, whereas the other (MG404-Ib) showed the same percentage as strain MG404-I. Fifty colonies from strains MG404-Ia and MG404-Ib were transferred from nonselective plates to selective plates by using toothpicks; again, all colonies showed resistance to chloramphenicol (Table 3). The loss of the Cm$^r$ phenotype after cultivation in the absence of chloramphenicol in the direct-plating assay prompted us to examine the chromosomal DNA of strains MG404-I and MG404-Ia. Figure 4 shows a Southern hybridization experiment of *Eco*RV-digested DNAs from these transformants as well as from the original transformant MG404 and the recipient MG1363. From this figure it is clear that the number of copies of the AU had decreased in MG404-I (lanes 3) as compared with that in MG404 (lanes 2). The decrease in signal intensity might be due to a reduction of the copy number of the AU in each cell in the culture or, alternatively, to the generation of cells in which the copy number of the AU was widely different. Because two different colonies from the MG404-I culture, both subjected to the same plating and growing strategy (see Materials and Methods), gave different numbers of Cm$^r$ CFU after plating (Table 3, MG404-Ia and MG404-Ib), we prefer the latter possibility.

TABLE 3. Percentage of CFU after growth in the absence of selective pressure[a]

| Strain | % Cm$^r$ CFU after: | |
|---|---|---|
| | Plating | Transfer with toothpicks |
| MG1363 | 0 | 0 |
| MG1363(pGK12) | 100 | 100 |
| MG60 | 50 | 100 |
| MG404(-I) | 10 | 100 |
| MG404-Ia | $2 \times 10^{-4}$ | 100 |
| MG404-Ib | 10 | 100 |

[a] Values were determined by direct plating onto selective (5 μg of chloramphenicol per ml) and nonselective plates or by transfer with a toothpick from a nonselective plate.

The hybridization pattern of MG404-Ia chromosomal DNA is shown in Fig. 4, lane 4. Since no signal is present at the position of linear pGI404 (lane 5), the amplification was apparently lost. However, two strong signals at the positions of fragments of 8.4 and 2.0 kb were present in the chromosomal DNA of strains MG404 and MG404-I and continued to be present in strain MG404-Ia. Therefore, we conclude that only one copy of pGI404 remained in strain MG404-Ia and that the 2.0- and 8.4-kb hybridizing fragments represent *Eco*RV restriction fragments carrying plasmid sequences in addition to flanking chromosomal DNA sequences. The observed sizes exactly agreed with what was to be expected in this particular case (Fig. 4C). These results convincingly show that plasmid pGI404 became integrated into the chromosome of *L. lactis* subsp. *lactis* MG1363 via a Campbell-like mechanism.

## DISCUSSION

The finding that pHV60, which replicates on the origin of pBR322, was capable of producing $Cm^r$ transformants in *L. lactis* subsp. *lactis* MG1363, albeit with low frequency, was surprising. Two possibilities may be entertained to explain this. First, the plasmid might replicate autonomously. Second, it might have integrated into the chromosome of the recipient, either by illegitimate recombination or by using DNA sequence homology between the plasmid and the chromosome of strain MG1363. Several observations argue in favor of integration of pHV60 by means of a Campbell-like mechanism. In hybridization experiments it was established that (i) no plasmid bands were present in the undigested total DNA of the transformant MG60; (ii) pHV60 showed homology with the chromosome of *L. lactis* subsp. *lactis* MG1363; (iii) additional hybridizing fragments were present in digests of chromosomal DNA of MG60; and (iv) the strong signal in the digests of chromosomal DNA of MG60, which represented approximately 15 copies of the plasmid per chromosome, cannot be accounted for by the extremely small amount of free plasmid DNA observed in small-scale plasmid isolations of strain MG60. The small amount of free plasmid DNA might be generated by recombination in the direct repeats flanking the plasmid DNA when integrated or, alternatively, by recombination in the directly repeated plasmid DNA when amplified.

The insertion of chromosomal fragments in pHV60 did not invariably lead to a substantial increase in the production of transformants. In fact, the number of transformants obtained per microgram of DNA with pGI401 and pGI406 was only slightly higher than that obtained with pHV60 (Table 2). The hybridization results indicated that pGI401 did not integrate via the chromosomal insert. It is conceivable that pGI401 became integrated in the host chromosome via the pHV60 moiety which was homologous with the chromosome. This can be understood by assuming that integration of pGI401 via the cloned chromosomal region would interrupt an essential unit of transcription and, therefore, would be lethal. Although plasmid pGI406 integrated via the cloned chromosomal insert, the increase in the number of transformants was also small and was comparable to that of pGI401. In this case, insertion of pGI406 might also be lethal, but by fusion with a second chromosome, or part of it, a viable merodiploid structure might have resulted. Cells with such a genetic constitution have been described for *B. subtilis* (1, 2).

In all transformants analyzed, amplification had reached a level of approximately 15 copies per chromosome. Amplification was gradually lost from strain MG404 under nonselective growth conditions. A derivative of this transformant was isolated (MG404-Ia), which contained only one stable copy in the chromosome. The stable maintenance of a single plasmid copy in the chromosome versus the instability of the amplified structure may well relate to differences in size and number of direct repeats present in the structure. In the single-copy situation, the plasmid is flanked by two copies of the DNA insert present in the transforming plasmid (1.3-kb insert), whereas in the amplified structure, several tandem repeats of the whole plasmid are present. These may serve either as a substrate for homologous recombination or for slipped mispairing, which would reduce the copy number of the AU in the chromosome and could account for the minute quantity of free plasmid DNA.

The fact that only one copy of the AU remained in the chromosome under nonselective conditions offers a possible explanation for the discrepancy between the results of the two methods for determining the number of $Cm^r$ cells (Table 3). Since the $Cm^r$ gene is poorly expressed in lactococci (J. M. B. M. van der Vossen, personal communication), we assume that one plasmid copy is insufficient to confer $Cm^r$ on a single cell and therefore would not give rise to a colony in the direct-plating assay. However, since a large number of cells are transferred by the toothpick method, it is conceivable that among the cells transferred a few are present in which amplification had occurred, which thus gave rise to colonies on chloramphenicol-containing plates. If this view is correct, the numbers in Table 3, obtained by direct plating, represent the percentage of cells which possessed an amplified structure in strains MG60, MG404-I, and MG404-Ib. For strain MG404-Ia, the number $2 \times 10^{-4}$ $Cm^r$ colonies after direct plating would represent the frequency at which this strain generates an amplified structure. The loss of amplification in strain MG60 is slower than in strain MG404. This might be correlated with the site of integration or the size of the repeats, although other unknown factors cannot be excluded.

For Campbell-like integration and subsequent amplification, only two additional fragments are expected when the chromosomal DNA is digested with a restriction enzyme, which has a unique restriction site on the integrated plasmid. However, inspection of Fig. 3, lanes 2, 5, and 8, and Fig. 4, lanes 2 to 4, clearly shows that additional hybridizing bands are present. We believe that these extra signals are intimately connected to the presence of the amplified structure, because in the single-copy situation, as in strain MG404-Ia, the extra signals had disappeared. The extra bands may reflect rearrangements in the amplified structure. Alternatively, the plasmid may have integrated at a number of secondary sites in the chromosome. Similar events can be envisaged for strains MG401 and MG406 to explain the presence of additional hybridizing bands. However, the fact that in the single-copy situation only two bordering fragments of the expected size are found argues strongly in favor of the conclusion that the plasmid integrated at a unique site into the chromosome.

Campbell-like integration has been amply documented in the literature for *B. subtilis* (7, 8, 10, 11, 21, 31). In comparison with results obtained for *B. subtilis*, with the same chloramphenicol resistance gene as a selectable marker, both similarities and differences seem to exist. Free plasmid DNA was also observed by Young (32) in *B. subtilis* strains in which an amplified structure was present. In addition, as in the present case, amplification occurred at low levels of selection and was unstable. In contrast, Albertini and Galizzi (1) and Niaudet et al. (21) did not detect extrachromosomal DNA in their *B. subtilis* integrants containing an amplified structure. Jannière et al. (14) and Albertini and Galizzi (1) observed amplification only when the transformants were grown on high levels of the antibiotic. Albertini and Galizzi (1) could not exclude the possibility that amplified copies of the $Cm^r$ gene were lost after growth under nonselective conditions. Jannière et al. (14) reported stable gene amplification when a kanamycin resistance gene was used as a selectable marker. Differences found between data obtained for *B. subtilis* and *L. lactis* subsp. *lactis* may well relate to differences between the two bacterial species or to the antibiotic used for selection.

The data presented in this paper clearly demonstrate the feasibility of using the Campbell-like integration strategy to gain access to the lactococcal chromosome. Amplification appears to be unstable under nonselective conditions in *L.*

*lactis* subsp. *lactis* MG1363, but whether this also applies to amplification with other nonreplicating plasmids remains to be established. Currently, we are investigating the influence of plasmid size and chromosomal insert size on the efficiency of integration and the stability of amplification.

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