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The Mode of Replication Is a Major Factor in Segregational Plasmid Instability in *Lactococcus lactis*

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The effects of the rolling-circle and theta modes of replication on the maintenance of recombinant plasmids in *Lactococcus lactis* were studied. Heterologous *Escherichia coli* or bacteriophage λ DNA fragments of various sizes were inserted into vectors based on either the rolling-circle-type plasmid pWV01 or the theta-type plasmid pAMβ1. All pAMβ1 derivatives were stably maintained. pWV01 derivatives, however, showed size-dependent segregational instability, in particular when large DNA fragments were inserted. All recombinant pWV01 derivatives generated high-molecular-weight plasmid multimers (HMW) in amounts that were positively correlated with plasmid size and inversely correlated with the copy numbers of the monomeric plasmid forms. Formation of HMW or reductions in copy numbers were not observed with pAMβ1 derivatives. The results indicate that HMW formation and/or reduction in plasmid copy numbers is an important factor in the maintenance of pWV01 derivatives. It is concluded that theta-type plasmids are superior to rolling-circle-type plasmids for cloning in lactococci.

Lactococci are important organisms in dairy and other food fermentations. Considerable progress in studies on the molecular genetics of these bacteria has been made since the development of cloning vectors based on the highly related lactococcal plasmids pWV01 (21, 22) and pSH71 (12, 13). Both replicons have an extensive host range, sustaining replication in many gram-positive bacteria, such as *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Propionibacterium*, *Staphylococcus*, *Streptococcus*, *Clostridium*, *Listeria*, and *Bacillus* species, as well as in some strains of the gram-negative bacterium *Escherichia coli* (21, 22, 28, 33).

Analyses of the nucleotide sequences and modes of replication of pWV01 and pSH71 have revealed that both belong to the class of small plasmids that generate single-stranded DNA intermediates during rolling-circle replication (RCR) (16, 26). It is known from studies with *Bacillus subtilis* that RCR-type plasmids are frequently refractory to the cloning of large inserts, and recombinant plasmids often show a high degree of instability. Several studies have indicated that the formation of single-stranded DNA intermediates is an important factor in both structural instability (3, 6, 14, 16) and segregational instability (4–6, 16). The formation of linear high-molecular-weight plasmid multimers (HMW) by RCR plasmids (15, 37) has also been implicated in structural instability (27) and segregational instability (16, 36).

Plasmids that replicate via a theta mechanism also have been used for the construction of cloning vectors in gram-positive bacteria. Theta-type plasmids neither generate single-stranded DNA replication intermediates nor do they form HMW products in wild-type *B. subtilis* strains (15). Several cloning vectors are based on pAMβ1 (19, 32), a broad-host-range *Enterococcus faecalis* plasmid that replicates via a unidirectional theta mechanism (8). Structurally, these plasmids are very stable in *B. subtilis* (19). Some variants, however, are segregationally unstable in this organism (32). The latter property was attributed to the absence of a stability determinant, which was assumed to specify a plasmid resolution function (34).

To date, no systematic studies on the effects of heterologous DNA inserts on plasmid maintenance in lactococci have been reported. This subject is relevant to the development of efficient and stable host-vector systems for these bacteria. The major aim of the present study was to compare the effects of the size of DNA inserts on the maintenance of RCR- and theta-type plasmids in *Lactococcus lactis*. Derivatives of the plasmids pWV01 (RCR type) and pAMβ1 (theta type) were chosen for this comparison. Evidence is presented that the theta-type plasmid is superior to the RCR-type plasmid for cloning in *L. lactis*.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1.

Media. *L. lactis* was grown at 30°C in M17 medium (35) containing 0.5% glucose (GM17 medium). *E. coli* and *B. subtilis* were routinely cultured in TY broth (tryptone, 10 g/liter; yeast extract, 5 g/liter; and NaCl, 10 g/liter [pH 7.4]) at 37°C. When *B. subtilis* was grown to competence, the media and procedures described previously by Bron (2) were used. When required, media were supplemented with antibiotics to the following concentrations: chloramphenicol, 5 μg/ml; erythromycin, 5 μg/ml for *L. lactis* and *B. subtilis* and 100 μg/ml for *E. coli*; ampicillin, 50 μg/ml. For plates, media were supplemented with 1.5% agar.

Isolation of DNA. Plasmid DNA was isolated by standard alkaline-lysis procedures (2). Total DNA was isolated from *L. lactis* as described previously for *B. subtilis* (2). When HMW were analyzed, shearing of the DNA was avoided by using pipettes with a wide bore.

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TABLE 1. Bacterial strains, plasmids, and DNA inserts

Bacterial strain, plasmid, or insert	Properties	Source or reference
E. coli JM101	*supE thi*Δ(*lac-proAB*) [F'*traD36 proAB lacI*^{q}*lacZ*ΔM15]	30
B. subtilis 8G5	*trpC2 tyrI met his nic purA ura rib*	4
L. lactis		
MG1363	*L. lactis* subsp. *lactis*, plasmid-free; Lac$^-$ Prt$^-$	Lab collection
Wg2	*L. lactis* subsp. *cremoris*, industrial strain	Lab collection
Plasmids		
pWV01	2.2 kb; cryptic plasmid from *L. lactis* Wg2	21
pIL252	Em^r, 4.7 kb; unstable pAMβ1 deletion derivative	32
pMTL23	Ap^r, 2.4 kb; pUC derivative containing *lacZ*α gene	9
pC194	Cm^r, 2.9 kb; *Staphylococcus aureus* plasmid	17
pE194*cop6*	Em^r, 3.7 kb; *Staphylococcus aureus* plasmid	18
pSR11	Em^r, 6.8 kb; stable pIL252 derivative	This work
pAMS100	Em^r Cm^r, 8.4 kb; pAMβ1 or pSR11 derivative	This work
pAMS101 through pAMS171	pAMS100 with various heterologous inserts in the *BclI* site	This work
pKS100	Em^r Cm^r, 5.0 kb; pWV01 derivative	This work
pKS101 through pKS171	pKS100 with various heterologous inserts in the *BclI* site	This work
DNA inserts		
1C	1.2 kb; *E. coli* chromosomal DNA	4
3C	4.2 kb; *E. coli* chromosomal DNA	4
5L	5.6 kb; phage λ DNA; coordinates 22346–27972	31
7L	7.2 kb; phage λ DNA; coordinates 34499–41732	31
8L	8.4 kb; phage λ DNA; coordinates 415–8844	31
10L	10.2 kb; phage λ DNA; coordinates 38814–0–415	31
12L	12.2 kb; phage λ DNA; coordinates 41732–0–5505	31
16L	16.8 kb; phage λ DNA; coordinates 5505–22346	31
112D	8.0 kb; shortened 12L fragment	This work
171	8.4 kb; 1C and 7L fragments	This work

Restriction enzymes, molecular cloning, and gel electrophoresis. DNA-modifying enzymes were used as recommended by the suppliers (Boehringer GmbH, Mannheim, Germany, or New England Biolabs, Beverly, Mass.). General cloning techniques were as described previously (29).

Transformations. *B. subtilis* was grown to competence and transformed as described by Bron (2). *E. coli* was transformed by using the $CaCl_2$ method (29). *L. lactis* was transformed by electroporation as described by Leenhouts et al. (25), using a gene pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.) equipped with a pulse controller unit.

Southern hybridizations. After agarose gel electrophoresis, the DNA was transferred to GeneScreen Plus filters (NEN Research Products, Dreieich, Germany) as described by Chomczynski and Qasba (10). The ECL gene detection system (Amersham International, Amersham, United Kingdom) was used for hybridizations as recommended by the supplier.

Assay of segregational stability. Single colonies of plasmid-carrying strains, which had been purified by two successive platings, were used to inoculate GM17 medium containing chloramphenicol. At an optical density at 660 nm of 0.5, the cultures were diluted 10^6-fold in 100 ml of prewarmed GM17 medium without chloramphenicol. The cells were grown for about 120 generations in successive batch cultures, the optical density of which never exceeded 0.8 (at 660 nm). This prevented the cells from entering the stationary phase of growth. At regular intervals, appropriate dilutions of the cultures were plated on nonselective GM17 agar. At least 200 individual colonies from each sample were transferred to plates containing chloramphenicol. Resistance to the antibiotic was correlated with the presence of a plasmid of the expected size, whereas antibiotic-susceptible colonies were plasmid free (50 colonies of each type were tested for the presence or absence of the plasmid). Restriction analysis of plasmids extracted from cells grown for about 120 generations indicated that deleted variants of the plasmids did not accumulate in these experiments.

Determination of plasmid copy numbers. Plasmid copy numbers were estimated in two different fractions: (i) the circular monomers (covalently closed plus open circular), and (ii) all plasmid forms (circular plus linear HMW). To estimate the copy numbers per chromosome equivalent of the monomeric circular plasmid forms, plasmid-carrying *L. lactis* strains were grown to the late exponential phase in 2-ml portions of GM17 medium supplemented with 5 μg of chloramphenicol per ml and 10 μCi of [*methyl*-^{3}H]thymidine. The addition of chloramphenicol prevented the accumulation of plasmid-free cells. The total DNA of these cultures was extracted, heated for 10 min at 65°C, vortexed at maximum speed for two 30-s intervals, and placed on ice for 5 min. Chromosomal and plasmid DNAs were separated in 0.5% agarose gels, and the ethidium bromide-stained bands were excised and dissolved in a boiling water bath. Fifteen milliliters of Hydroluma (Lumac Systems, Inc., Titusville, Pa.) was added, and the radioactivity in the samples was determined by using a Mark II liquid scintillation counter (Nuclear Chicago Corp., Des Plaines, Ill.). The plasmid copy numbers per chromosome were estimated from the ratios of the radioactivities in the plasmid and chromosomal DNA fractions as described previously (2). The size of the lactococcal chromosome was taken to be 2.446 megabases (24). The densitometric procedure described by Jannière et al. (20) was used to determine the total plasmid copy numbers (including those of HMW). This method involves the com-

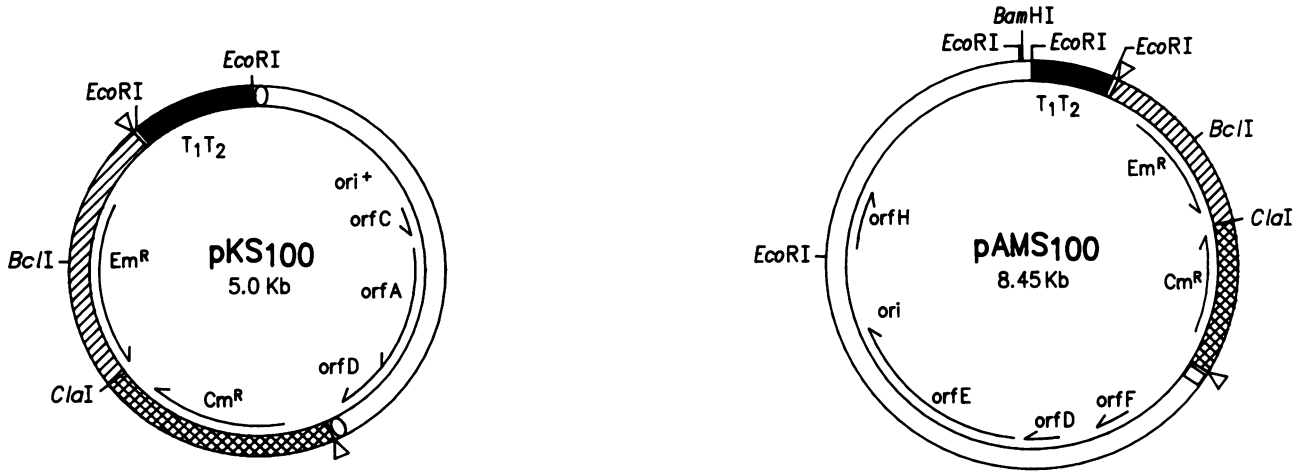

FIG. 1. Plasmids pKS100 and pAMS100. Only sites relevant for the construction and properties of the plasmids are shown. Open reading frames, genes, and the T1T2 transcriptional terminators are indicated. Details of the construction are described in the text. Symbols: ▭, pWV01 or pAMβ1; ▨, pE194; ▩, pC194; ■, T1T2 terminator; ▽, fusion between *Cla*I and *Hpa*II; ○, fusion between *Mbo*I sites; □, fusion between *Nru*I and *Sna*BI.

parison of restriction patterns of the total DNA of cells carrying the plasmid of interest with those of a reference DNA mixture, consisting of a fixed amount of chromosomal DNA to which known amounts of the plasmid DNA are added.

RESULTS

Construction of plasmids. pWV01 was selected as the RCR-type model plasmid for the analysis of the effects of the mode of replication on plasmid maintenance in *L. lactis*. This plasmid has been well characterized and is widely used in lactococci (21, 26). The pAMβ1 derivative pIL252 (32) was chosen as the theta-type model plasmid. An undesirable property of the original pIL252 plasmid was that, because of its construction, it lacks the stability determinant *orfH* (32, 34). The 2.2-kb *orfH*-containing *Eco*RI fragment of a pAMβ1 derivative (pHV1431) was inserted into the *Eco*RI site of pIL252 to avoid instability of this basic construct. The resulting plasmid, pSR11, carried the *Eco*RI fragment in the same orientation as it did in pAMβ1 and, in contrast to pIL252, was stably maintained in *L. lactis*.

pSR11 and pWV01 were subsequently provided with a set of antibiotic resistance markers. First, a cassette was constructed in the *Cla*I site of the multiple cloning site of the *E. coli* plasmid pMTL23 (9). The cassette contained the pC194-derived chloramphenicol resistance (Cm^r) gene (pC194 coordinates 973 to 2008 [17]) and the pE194-derived erythromycin resistance (Em^r) gene (pE194 coordinates 3140 to 1939 [18]). The T1T2 transcriptional terminators from the *E. coli rrnB* rRNA operon (7) were inserted on a 500-bp *Eco*RI fragment into the *Eco*RI site upstream of the Em^r gene to minimize possible read-through transcription from the cassette into plasmid sequences. These terminators are functional in *L. lactis* (35a). The transcription terminator of the Em^r gene was present between the two resistance genes. The cassette was subsequently transferred from pMTL23 to pWV01 and pSR11 as a *Bam*HI-*Bgl*II fragment into the unique *Mbo*I site of pWV01 and as a *Bam*HI-*Nru*I fragment between the *Bam*HI and *Sna*BI sites of pSR11. The resulting plasmids, denoted pKS100 and pAMS100, are shown in Fig. 1. The copy numbers of pKS100 and pAMS100 were comparable (about 15 per chromosome equivalent; see below).

Several heterologous DNA inserts were introduced into the basic plasmids pKS100 and pAMS100. The DNA inserts chosen were the 1C and 3C fragments of *E. coli Bgl*II DNA used previously in plasmid stability studies in *B. subtilis* (4, 5) and several bacteriophage λ DNA fragments which were generated by restriction with *Bam*HI, *Bgl*II, or *Bcl*I. The various fragments were cloned into the unique *Bcl*I site in the Em^r gene of pKS100 and pAMS100, resulting in the pKS and pAMS series of plasmids (Table 1).

Stability of pKS100 and pAMS100 derivatives. The maintenance of the various pKS100 and pAMS100 derivatives in *L. lactis* was studied in the absence of selective antibiotics. The kinetics of appearance of plasmid-free cells were determined with all pKS and pAMS derivatives. Representative examples are shown in Fig. 2. The results show that pKS100 was stably maintained during the entire assay period (140 generations of growth), whereas the pKS derivatives carrying λ DNA fragments of 8 or 12 kb were unstable. Similar results were obtained with several other pKS derivatives (data not shown). In contrast, all pAMS derivatives were stable: no plasmid-free cells were detected within the assay period (Fig. 2). The results of all plasmid stability assays are summarized in Fig. 3, in which the percentages of plasmid-containing cells after 120 generations of growth are plotted as a function of the size of the DNA inserts. Most pKS100 derivatives carrying inserts smaller than about 8 kb were stably maintained. pKS103C (insert, 4.2 kb) and pKS105L (insert, 5 kb), however, were slightly unstable. About 80% (pKS103C) and 90% (pKS105L) of the cells contained the plasmid after 120 generations of growth. All derivatives with inserts larger than 8 kb were poorly maintained, and the instability increased exponentially with the size of the DNA insert. These results suggested that insert size was an important factor in the instability of pKS derivatives. Since plasmid-free cells were not detected with any of the pAMS derivatives, these results indicated that the stability of the theta-type recombinant plasmids was superior to that of the RCR-type recombinants.

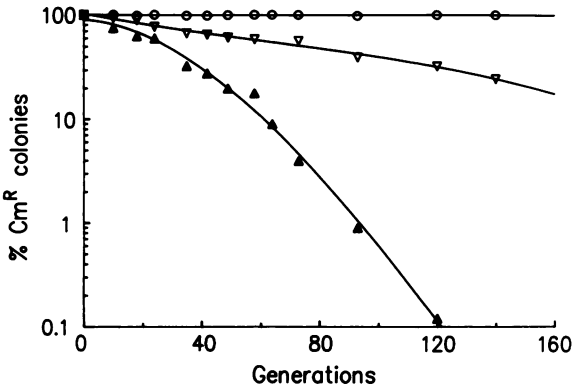

FIG. 2. Kinetics of appearance of plasmid-free cells of representative plasmids in *L. lactis*. Exponentially growing cultures of plasmid-carrying MG1363 strains in medium with chloramphenicol were diluted in antibiotic-free medium and subcultured for approximately 140 generations. After every 10 to 20 generations, samples were plated onto nonselective agar. Colonies were subsequently assayed for resistance to chloramphenicol after transfer to selective plates. Symbols: ○, pKS100 and all pAMS derivatives; ▽, pKS108L; ▲, pKS112L.

Two inserts were altered to assess whether, in addition to size, specific properties of the inserts might also affect the maintenance of pKS derivatives. First, the 7L fragment (7.2 kb), the insertion of which did not cause instability (Fig. 3), was increased in size to 8.4 kb by the addition of the 1.2-kb 1C fragment. The corresponding plasmids were denoted pKS171 and pAMS171. The second fragment, 12L (12.2 kb), which caused high levels of plasmid instability in pKS12L, was shortened to 8.0 kb by deleting two *Bcl*I fragments. The resulting plasmids were denoted pKS112D and pAMS112D. The small fragments (1.6 and 2.6 kb) that were deleted from the 12L fragment were also cloned, separately and together, into the *Bcl*I site of pKS100, resulting in pKS102L, pKS103L, and pKS104L, respectively.

The results of stability assays (Fig. 3) showed that all

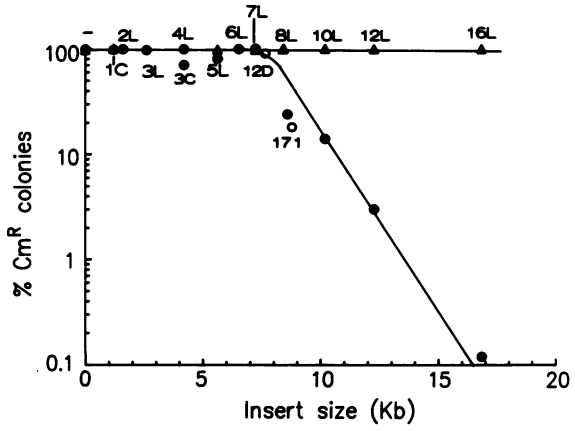

FIG. 3. Relationship between plasmid size and segregational stability in *L. lactis*. The percentage of Cm^r colonies obtained after 120 generations of growth in nonselective media is shown as a function of the size of the inserts. Filled symbols represent plasmids containing the original inserts; open symbols represent plasmids containing the 171 and 112D inserts (see text for explanation). Symbols: ● and ○, pKS derivatives; ▲, pAMS derivatives.

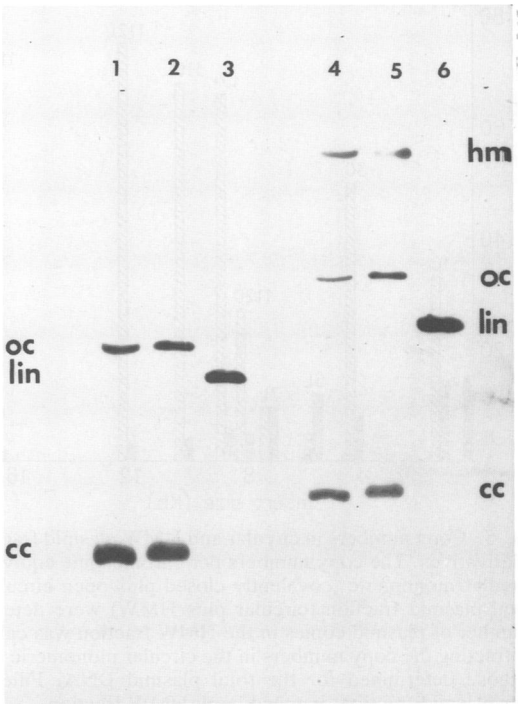

FIG. 4. Analysis of plasmid DNA from *L. lactis*. Total DNA was extracted, separated on agarose gels, blotted, and hybridized using pKS100 or pAMS100 as the probe. Lanes: 1 to 3, pKS100; 4 to 6, pKS101C; 1 and 4, untreated lysates; 2 and 5, total lysates digested with *Bgl*II; 3, and 6, total lysates digested with an enzyme that cleaves each plasmid only once (*Pst*I). cc, covalently closed monomers; oc, nicked monomers; lin, linearized plasmid DNA; hm, high-molecular-weight plasmid forms.

newly constructed pAMS derivatives remained fully stable. However, plasmid pKS171, containing the fusion of the 1C and 7L fragments, was unstable (Fig. 3). Since neither the 1C nor the 7L fragment had an effect on plasmid stability on their own, it is unlikely that a specific sequence on one of these fragments caused the instability of pKS171. Figure 3 also shows that pKS112D (8.0 kb), obtained from the unstable pKS112L (12.2 kb), was almost totally stable during the assay period. Since all small plasmids carrying fragments from pKS112L (pKS102L, -103L, and -104L) were also stable, it seems unlikely that a specific sequence on pKS112 caused its instability. These results indicate that the size of the inserts was the main factor in the instability of pKS derivatives.

Presence of HMW DNA. The cloning of heterologous DNA fragments in RCR-type plasmids often results in the formation of HMW in *B. subtilis* (15, 16) and *E. coli* (11). Experiments were conducted to determine whether the plasmids used in the present study generated HMW DNA in *L. lactis*. Total DNA lysates of cells carrying the various constructs were analyzed by Southern hybridization, using pKS100 or pAMS100 as the probe. HMW DNA was not seen with any of the pAMS derivatives. However, hybridizing DNA migrating at the position of HMW was detected with all pKS chimeras containing either *E. coli* or λ DNA inserts but not with the parental plasmid pKS100. Typical results are shown in Fig. 4. Incubation of the extract containing pKS101C with a restriction enzyme for which no site was present (*Bgl*II) did not affect the migration of the DNA at the

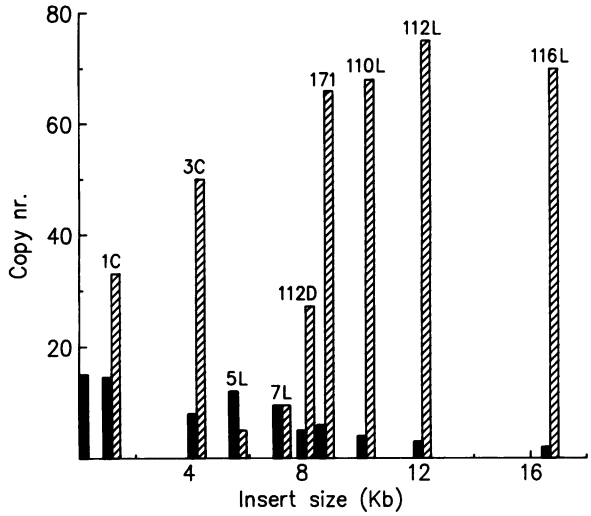

FIG. 5. Copy numbers in circular and HMW plasmid fractions of pKS derivatives. The copy numbers per chromosome equivalent of the circular monomeric (covalently closed plus open circular) and the total plasmid fraction (circular plus HMW) were determined. The number of plasmid copies in the HMW fraction was calculated by subtracting the copy numbers in the circular monomeric fraction from those determined for the total plasmid DNA. Filled bars, circular plasmid fraction; hatched bars, HMW fraction.

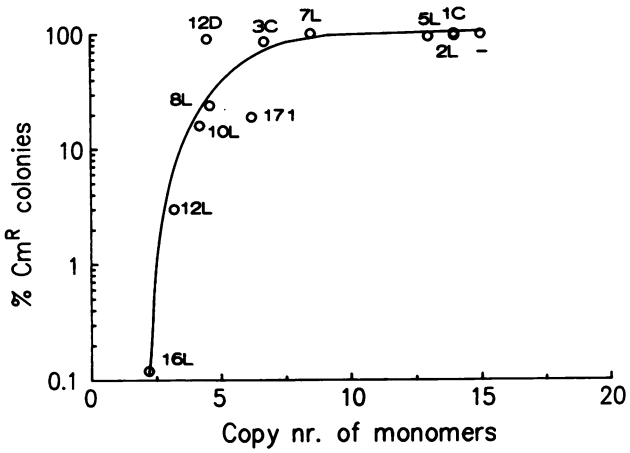

FIG. 6. Relationship between copy numbers of the circular monomers and plasmid stability. The percentages of Cm^r cells correspond to the percentages of plasmid-containing cells after 120 generations of growth in the absence of chloramphenicol. These values were taken from the experiments described in the legends to Fig. 2 and 3.

HMW position. This rules out the possibility that the hybridization signal resulted from nonspecific interaction with chromosomal DNA. When the DNA was pretreated with an enzyme cleaving the plasmids once, one band was detected at the position of the linearized plasmid. This indicates that the pKS-derived HMW consisted of head-to-tail plasmid multimers. We conclude that the appearance of insert-induced HMW in *L. lactis* depended on the mode of replication, since this DNA was only formed with RCR-type plasmids.

Relationships between HMW formation, plasmid copy number, and stability. Possible relationships between the formation of HMW, plasmid copy numbers, and stability were assessed by determining copy numbers in exponentially growing *L. lactis* strains. Two assays were used: one yielding the copy numbers of all possible plasmid forms (circular plus HMW), and the other yielding the copy numbers of the circular plasmid forms. The copy numbers were measured relative to chromosome equivalents. Only monomers in the circular plasmid fraction were considered, since the amounts of dimeric and oligomeric forms were very low.

Each pAMS derivative had a copy number of 15 to 18 in both assays. This indicated that only circular plasmid forms were present, providing further evidence that insert-induced HMW was not formed with pAMS derivatives. However, the copy numbers of pKS derivatives were clearly affected by the presence of heterologous DNA inserts (Fig. 5). With most pKS plasmids, an inverse relationship was observed between the copy numbers of the circular monomers and the insert sizes. For instance, the copy number of pKS100 (with no insert) was 15, whereas that of pKS116L (with an insert of 16.8 kb) was only 2. The copy numbers of the circular plasmid forms were inversely related to the copy numbers in the HMW fraction, which increased with plasmid size. With the largest pKS derivatives, the HMW fraction amounted to about 70 plasmid copies per chromosome equivalent. The relationship between HMW and insert size was not perfect, however. For instance, with the 3C insert (4.2 kb), considerably more plasmid copies were present as HMW than with the larger 5L (5.6 kb) or 7L (7.2 kb) inserts. This means that, in addition to insert size, insert-specific sequences also contributed to HMW formation.

Figure 6 shows the relationship between the maintenance of pKS plasmids and the copy numbers of the circular monomers. Plasmids with copy numbers higher than about 7 were stably maintained. Plasmids with lower copy numbers were, however, unstable, and the instability was inversely related to the copy numbers of the plasmid monomers.

Since the copy numbers determined here are independent of plasmid size, they are not proportional to the mass amounts of the various plasmid forms. Since it is conceivable that the mass amounts of HMW are important for plasmid stability, we also calculated these values for the various pKS derivatives. This was done by multiplying the HMW copy numbers by plasmid size. The results (Fig. 7) revealed an inverse relationship between the mass amounts of HMW and the segregational stabilities of pKS derivatives.

DISCUSSION

The results presented in this article show that in *L. lactis*, the RCR-type plasmid pWV01 suffers from at least one disadvantage that was previously also observed with RCR-type plasmids in *B. subtilis* (4, 5, 6): increased size of DNA inserts results in drastically increased segregational instability. pAMβ1 derivatives with the same inserts were, however, stably maintained, provided that they carried the stability determinant *orfH* (34). Since pAMβ1 replicates via a theta mechanism (8), the difference in replication mechanism is likely to underlie the different segregational stabilities of pWV01 and pAMβ1 derivatives in *L. lactis*. To date, we have not studied the structural stability of recombinant pAMβ1 derivatives in *L. lactis* in detail. We have, however, never observed deleted forms of pAMβ1 derivatives upon gel electrophoresis (data not shown). This suggests that also the structural stability of these recombinant plasmids was

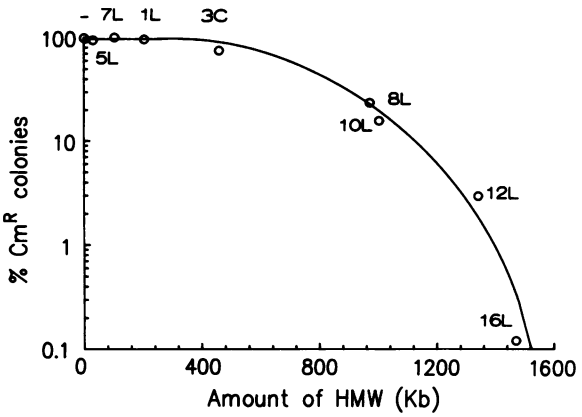

FIG. 7. Relationships between mass of HMW and plasmid stability in *L. lactis*. The mass amounts of HMW, expressed per chromosome equivalent, were calculated as described in the text. Construct names are indicated.

good. High structural stability of pAMβ1 derivatives was previously observed in *B. subtilis* (19).

An additional advantage of pAMβ1 compared with pWV01 is that the copy number of the former does not appear to be reduced by the presence of DNA inserts.

Two observations are relevant to the question of why recombinant pWV01 derivatives show size-dependent segregational instability. First, HMW was induced in mass amounts that were related to insert size; second, increased amounts of HMW were paralleled by decreased copy numbers of the circular plasmid forms. HMW DNA may play a role by interfering with normal cell physiology. Such a mechanism has been suggested by experiments with *B. subtilis* (1, 27, 37). It has also been concluded from studies with *E. coli* that under certain conditions, HMW can cause reduced cell viability (23). Since the maximal mass amount of HMW induced by the largest inserts amounted to about 40% of the total cellular DNA, we consider reduced cellular growth rates as a realistic possibility. The growth disadvantage of HMW-containing cells would increase the rate of plasmid segregation from the population. If this is indeed the role of HMW, it is clear that a certain level of this DNA is required for the induction of instability. This can be concluded from the observation that several derivatives produced (low) amounts of HMW but were nevertheless stably maintained.

The second mechanism by which HMW can be conceived to reduce the maintenance of pWV01 derivatives is interference with the copy control of the circular plasmid forms. Assuming that the circular pWV01 plasmid forms are partitioned randomly to daughter cells during cell division, a reduction in plasmid copy number is expected to result in increased rates of plasmid loss. The present data do not allow us to discriminate between the two possible mechanisms. It is also possible that both mechanisms occur.

The observation that insert-induced HMW formation in *L. lactis* was associated with plasmids using RCR is not unique. Similar observations have been made in wild-type *B. subtilis* (15, 16) and certain *E. coli* strains (11).

The correlation between HMW DNA and insert size indicates that the latter parameter is important for HMW induction. Insert-induced HMW formation is generally believed to result from nontermination of leading-strand displacement during RCR (11, 15, 16). The effect of insert size observed in the present experiments can be explained by assuming that the probability of nontermination is directly related to insert size. Other properties of the inserts also seem to affect HMW formation. This is most obvious with the relatively small 3C *E. coli* DNA insert, which induced considerable amounts of HMW. This may explain why pKS103C is slightly unstable. From studies conducted in *E. coli*, Dabert et al. (11) concluded that the nucleotide composition of the inserts affected HMW formation. We have no indications that this was also the case in the work presented here. Recent studies by Dabert et al. (11a) indicated that the presence of the *chi* sequence on DNA inserts in particular stimulated HMW formation in *E. coli*. We consider it unlikely that similar specific nucleotide sequences were involved in the formation of the HMW described in the present article. This follows from the observation that insert 12L caused high levels of HMW, whereas none of its subfragments was able to do so. Fragment 3C may form an exception: considerable amounts of HMW were formed with this relatively small plasmid.

The present studies, showing that the theta-type plasmid pAMβ1 was more stably maintained in *L. lactis* than the RCR-type plasmid pWV01, make it likely that endogenous lactococcal theta-type plasmids are good candidates for the development of efficient and stable cloning vectors in this organism.

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